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Genetic structure and physiological variation of a widespread European lagoon specialist *Cerastoderma glaucum* (Bivalvia) living in extreme environmental conditions

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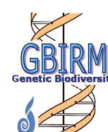

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TO MY PARENTS

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General introduction

1 Introduction

1.1 Goals of the study

The rapid development of molecular methods brought an enormous amount of new data to marine biology studies. Genetic data might be helpful to explain ecological processes. The variety of research tools available to marine biologists nowadays should not be the reason of concurrence, but complementary data obtained with different methods could enrich present knowledge and enable to understand processes which were inexplicable before.

In this study two different types of genetic markers (microsatellites and mitochondrial DNA, cytochrome oxidase subunit I) were applied on *Cerastoderma glaucum* (Bivalvia) populations covering almost the whole distribution area. In order to obtain the most comprehensive view on the influence of different, sometimes extreme environmental conditions, some morphometric and physiological parameters of *C. glaucum*, such as condition, reproductive physiology, respiration rate and biochemical composition were studied as well. This PhD thesis is an attempt to combine physiological and genetic data to support a holistic approach to studies on marine organisms.

Main goals of the project were:

- to investigate population genetic structure of *C. glaucum* using mitochondrial DNA and microsatellites in almost the whole distribution area,
- to form scenarios on the origin, past dispersal, glacial refugia, postglacial colonization and a present gene flow among populations of this species,
- to compare genetic patterns in northern, postglacially colonized regions of Europe and in southern regions, from where the species origins and where it has a longer uninterrupted history,
- to explore the effects of a habitat fragmentation on the population genetic structure and making hypothesis about possible vectors for dispersal,
- to investigate differences in morphometric and physiological parameters among populations living under different often extreme environmental conditions,
- to analyze the strategy of *C. glaucum*, which enables to colonize and persist in extreme lagoon habitats.

1.2 Outline of the thesis

The first part of the thesis contains the goals of the project, explains some characteristics of lagoons and the influence of the specificity of this habitat on the genetic structures of organisms. Some basic information on biology and ecology of *C. glaucum* were given as well. In **Chapter I** morphometric and physiological studies performed on *C. glaucum* were described. **Chapter II** was dedicated to population genetic structure studies. In the first part of it, factors influencing phylogeographic patterns in marine species were presented. Then, the properties of both marker types and the theoretical context of methods, which were applied to analyze the genetic data, were discussed. In the last part of **Chapter II** the results of population genetic structure studies on *C. glaucum* were presented and discussed. All the results were synthesized in **Chapter III**. This chapter also contains suggestions for future studies.

2 Lagoons

2.1 Lagoon habitats

Coastal lagoons are shallow inland marine waters, usually oriented parallel to the coast, separated from the ocean by a barrier, but connected to it by one or more restricted inlets (Kjerfve 1994). They can be created naturally by wave action, storms, land subsidence, sediment redeposition and river channel changes or artificially. Natural as well as anthropogenic processes can also act to destroy lagoons, as they are very delicate (Barnes 1980). Factors used for distinguishing lagoons from estuaries and their classification are the way in which the lagoon is formed, the maintenance of its water chemistry and the exchange between the source water and the lagoon (Barnes 1980; Downie 1996).

Lagoons, especially small ones, are extreme habitats, because they are highly unpredictable and much more subject to short-term variations in temperature, salinity and pH than marine habitats (Bamber *et al*. 1992; Downie 1996). Due to nutrient and organic matter inputs, there are often high levels of primary production in lagoons (Razinkovas *et al.* 2008), leading to occasional anoxia in the benthic environment, which may be the limiting factor for many species (Allen *et al.* 1995). The important feature characterizing

lagoons is their connection with surrounding sea as it has implications for the salinity, the recruitment of species and for the maintenance of the habitats (Barnes 1980).

2.2 Colonization and gene flow in lagoon habitats

Lagoons are interesting sites for studying colonization processes. As they are often isolated and environmental conditions vary among them, colonization does not consist of the gradual expansion of distribution areas, like it is often the case in marine species. It is unclear how lagoon species colonize new habitats. It could take place by rafting on mats of floating algae or by transporting animals attached to birds, which feed in lagoons. A geographic proximity to other lagoons is often a crucial factor, which determines the chance of colonization. Migrants which manage to cross a barrier against dispersal and reach another lagoon are usually few, so a chance plays an important role in the allocation of some species to lagoons. The anthropogenic intervention may also be helpful in colonization processes (Barnes 1988).

Lagoon habitats may be compared to islands (Pearson 2003). Island populations are usually founded by the low number of individuals, so they are subject to genetic loss (Frankham 1997). Moreover, animals with highly specialized habitat requirements naturally occur in small populations (Stacey and Taper 1992). The problem of small populations is an increased likelihood of inbreeding, the loss of heterozygosity and the loss of allelic diversity (Hedrick and Miller 1992). Inbred individuals often suffer from inbreeding depression and a lower fitness compared to non-inbreds (Charlesworth and Charlesworth 1987). It has been proven that inbreeding reduces reproductive success and in a long term may lead to extinction (Frankham 1995). Habitat fragmentation also leads to reduction in local population size, reduced migration, increased population size fluctuations and inbreeding depression. The presence of barriers impeding gene flow among populations may also result in local selection or genetic drift within the populations (Slatkin 1981). However, sometimes either the gene flow from other populations is sufficiently high to counteract the effect of genetic drift or the $N_e s$ is large enough, where N_e is the effective population size and s is the selection coefficient (Tallmon *et al.* 2002). The effective population size (N_e) refers to the subset of individuals in the population that successfully contribute to the next generation. In many marine species high fecundity

together with a very large variance in reproductive success can lead to N_e several orders of magnitude smaller than the total number of individuals N (Hedgecock 1994).

Lagoons may be also considered as marginal habitats. Individuals dispersing to marginal habitats are less likely to raise progeny and will be selected against (Krebs 1994). Therefore, marginal populations at least at the beginning are considered to be demographic sinks, because immigration is needed to maintain the population (Dias 1996). While establishing a new population, natural selection for favorable alleles may increase the genetic loss, unless heterozygote advantage exists (Frankham 1997).

2.3 Need for further studies and conservation of lagoons

Even though coastal lagoons cover 13% of the world coastline (Barnes 1995) and only 5.3% of the European coastline (Kjerfve 1994), they are considered to be the most valuable components of the coastal area (Gonenc and Woflin 2005). It is thought that approximately 10% of lagoon habitats will be lost in the next 20 years (Downie 1996). They have been listed as priority habitats under the European Union Habitats Directive (Council of the European Communities 1992) and are frequently designated as, or lie within Sites of Special Scientific Interest (SSSI), National Nature Reserves, Ramsar sites and Special Protection areas (SPAs). They are of particular conservation importance, because they often support plant and animal species and communities absent or rare in other saline habitats, including a number specialist lagoon species (Barnes 1980; Barnes 1991; Bamber *et al.* 1992). They play a significant role in the regulation of freshwater inputs to the marine environment, being sinks and biogeochemical reactors for nutrients and toxic substances coming from terrestrial sources, as well as nurseries for many marine species and migratory birds (Razinkovas *et al.* 2008). Lagoons as well as estuaries, being transitional ecosystems, differ from freshwater and marine ecosystems in environmental forcing factors, ecological equilibrium dynamics and ecological characteristic scales (McLusky and Elliott 2007).

Lagoons for centuries have had a very important socio-economic role, and have been exploited in many ways even more intensively than adjacent sea areas. Humans have a strong impact on lagoon communities sometimes by deliberate extermination of lagoons' inhabitants or inadvertently through tourism, recreation or commercial exploitation of species. Moreover, pollution, changes in land use or global climate changes have severe

consequences on lagoon species and ecosystems (Spellerberg 1996). Rising sea levels resulting from an increase in the global temperature causing the polar ice caps to melt, may pose a threat to these habitats, because of an increase in storms and the potential for coastal erosion. In the last decades, local factors (e.g. eutrophication, habitat and biodiversity losses) amplified by global processes have resulted in direct and indirect impacts that have considerably reduced the ability of coastal lagoons to meet an ever-increasing demand for their use and development (Crossland *et al.* 2005; Viaroli *et al.* 2007).

To some extent lagoon ecosystems may cope with dynamic fluctuations and perturbations due to complex interactions among benthic vegetation, macrofauna and microbial communities (Nienhuis 1992; Caumette *et al.* 1996). However, above certain pressure thresholds, they react to perturbation in a non-linear way with sudden shifts in community structure and catastrophic changes in biogeochemical processes (De Wit *et al.* 2001). Necessary steps should be undertaken to conserve fragile lagoon habitats. The impact of humankind on ecosystems is increasingly high and thus a responsibility to minimize loss and maximize conservation and survival (Anon 1995).

Scientific knowledge on the lagoon ecology is still insufficient, as compared to both freshwater and marine ecosystems and even estuaries. There is only limited information about the biology, life cycles and reproductive strategies of some lagoon organisms (e.g. Barnes 1994; Sheader 1996; Porter *et al.* 2001; Pearson *et al.* 2002). Investigating lagoons' distribution, their biodiversity and the ecology of associated flora and fauna are essential in managing fragile lagoon resources. There is a link between biodiversity and ecosystem functioning in terms of nutrient cycling efficiency, resilience to disturbances and the removal of toxins. The biodiversity of communities is immense and each community has its unique assemblage of species, so lagoons as well as lagoon species demand individual approach in order to protect them efficiently (Spellerberg 1996).

In the studies on lagoon communities and species various methods may be applied. One of them is investigating genetic structure of species, which is increasingly applied and appreciated, as genetic diversity is considered the prerequisite for all the other kinds of biological diversity (Wilson 1992). Measuring genetic diversity may provide a means to estimate migration in populations, which is a crucial factor in the regional maintenance of species. Understanding genetic diversity of certain key species present in lagoons may be helpful for the conservation of lagoon ecosystems. In order to better predict reactions of lagoon habitats to perturbations, the knowledge of ecophysiological performance and

tolerance of their dwellers is required. Lagoon specialists are interesting objects for studying physiological acclimatization and adaptation.

3 Basic information on *Cerastoderma glaucum*

3.1 Distribution and ecological preferences

C. glaucum (Fig. 3.1) inhabits the Mediterranean Sea lagoons. It is also present in the Atlantic and North Sea region, e.g. in the Netherlands (Eisma 1965), around the British Isles (Bowden and Heppell 1968), in mixohaline bays in Gullmarfjorden in Sweden, near Bergen in Norway (Tulkki 1961), in the Trondheimsfjorden in Norway (Rygg 1970) and in lagoon systems along the Portuguese continental coast (Machado and Costa 1994). Its distribution area reaches also the Aral Lake, the Black Sea, the Caspian Sea and the Baltic Sea (Høpner Petersen and Russell 1973). There is evidence that this species penetrated into the Red Sea (Brock 1987).

C. glaucum inhabits soft, sandy or sandy-muddy bottom (Kingston 1974). It is euryhaline and eurythermic, so various, sometimes even extreme temperatures and salinities do not form a barrier against dispersal. The species is tolerant of salinities ranging from 5 PSU to 84 PSU (Rygg 1970; Labourg and Lasserre 1980) and temperatures from 0°C to over 45°C (Zaouali 1974). The distribution area of *C. glaucum* even if wide is very fragmented. It inhabits usually isolated or semi-isolated, shallow, non-tidal biotopes, like brackish lagoons, estuaries, bays and lakes. It was thought that the main limiting abiotic factor for this cockle is its intolerance to air exposure provoked by tides (Russell 1971, 1972). Nevertheless, there are tidal zones, where *C. glaucum* is found. Apparently, the suitability of the sediment is more important than the direct influence of tides. This species never occurs on bottoms with loose sediment structure. This kind of structure is found in well-sorted sand exposed to currents and waves (Brock 1979). *C. glaucum* often inhabit basins with stagnating water (Russell 1971) and is considered a pioneer species (Zaouali 1979).

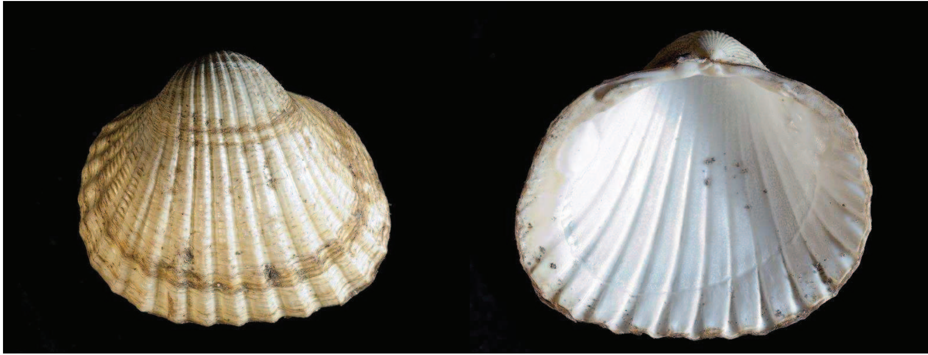

Figure 3.1. *Cerastoderma glaucum* shell (Picton and Morrow 2009).

3.2 Life history

The lagoon cockle usually reaches sexual maturity at the age of two years. Sometimes males reach sexual maturity earlier than females and spawn in their first year (Pearson 2003). Life history and reproductive cycle vary depending on population's location. Northern populations of *C. glaucum* have a monocyclic reproductive cycle with gametogenesis initialization in September/October and spawning between May and July. Spawning starts when water temperature reaches 15-17°C in the whole distribution area, so the more north the population inhabits the later spawning begins (Rygg 1970; Boyden 1971; Kingston 1974; Ivell 1979; Wołowicz 1984; Wołowicz 1991; Brock and Wołowicz 1994). Therefore, Mediterranean populations may spawn up to three times a year (Ivell 1979). Within geographic regions populations from isolated lagoons spawn usually earlier than those from open water basins due to more rapid water temperature increase in spring (Boyden 1971; Barnes 1980).

An interesting aspect is a sex ratio. Boyden (1971) suggested that there is an equal number of males and females in populations of *C. glaucum*. However, Kingston (1974) found significantly more males than females in four populations of *C. glaucum* from the southeast coast of England. Wołowicz (1991) suggested that the sex ratio in *C. glaucum* changes during the year and the number of females in populations decreases after spawning. This could be due to a high cost of gonad maturation and spawning for females, which provokes their increased mortality after spawning, like it was found in *Chlamys islandica* (Brokordt and Guderley 2004) or *Mytilus trossulus* (Wołowicz *et al.* 2006). Presumably, more females are born to compensate for that lost (Wołowicz 1991).

A planktonic larval stage lasts for one week, which is much shorter than in case of the marine common cockle (*Cerastoderma edule*), which has a planktonic larval stage

lasting for 5 weeks (Boyden 1971; Barnes 1980). However, these durations may differ depending on the geographic location of populations. The larvae develop correctly, when the water salinity exceeds 5 PSU, whereas those of *C. edule* cannot develop, when the salinity is lower than 15 PSU (Rygg 1970). The optimal temperature for *C. glaucum* larvae development is from 15 to 31ºC (Kingston 1974).

3.3 Diet

C. glaucum is a filtrator feeding on small suspended macrophyte detrital particles broken down by primary and secondary feeders. Sarà (2007) indicated that in Mediterranean ponds the dominant organic source in the diet of this species is the detritus coming from the seagrass *Cymodocea nodosa*, the macroalgue *Ulva lactuca* and the sand microflora. The studies on stomach contents revealed that the diet of *C. glaucum* consists mostly of diatoms Centricae and Pennatae (Høpner Petersen 1958). Moreover, it has been observed that the sudden removal of nearly all mussels and cockles from an ecosystem led to increased diatoms concentrations (Beukema and Cadée 1996), proving them to be the main food of cockles.

3.4 On taxonomy, divergence and fossil record of C. glaucum

C. glaucum has the following systematic position (Ter Poorten and Gofas 2009):

Kingdom: Animalia

Phylum: Mollusca

Subphylum: Conchifera

Class: Bivalvia

Subclass: Heterodonta

Order: Veneroida

Superfamily: Cardioidea

Family: Cardiidae

Genus: *Cerastoderma*

Synonimized taxa are *Cardium glaucum* (Poiret 1789), *Cardium lamarcki* (Reeve 1845) and *Cerastoderma lamarcki* (Reeve 1845). The original author of *Cardium glaucum* is a serious problem, because Poiret and Bruguière have described this species under the same name at the same time. The genus name *Cardium* was introduced by Linnaeus in 1758, but Tebble (1966) suggested using the name *Cerastoderma* (Poli 1795) as the name of the genus.

The generic and specific names have following origins (Bowden and Heppell 1968):

Cerastoderma (Poli 1795) Testacea utriusque Siciliae, 2, 252, 258.

Cardium edule (Linnaeus 1758) Systema naturae, ed. 10, 1, 681.

Cardium glaucum (Poiret 1789) Voyage en Barbarie 2, 13.

There was a variety of ideas concerning taxonomical divisions within the Ponto-Caspian and Mediterranean populations of *Cardium* (=*Cerastoderma*) sp. which are now, in most of the cases, referred to as *Cerastoderma glaucum* (e.g. Le Renard 2009). Scarlato and Starobogatov (1972) distinguished four species of *Cerastoderma* present in the Black Sea and the Mediterranean Sea differing in depth and habitat preferences: *C. glaucum*, *C. lamarcki*, *C. clodiense* and *C. umbonatum*. Kafanov (1980) proposed the following subdivision: *C. glaucum*, *C. rhomboids*, *C. clodiense* and *C. isthmicum* based mainly on habitat preferences. The two taxa *C. glaucum* and *C. isthmicum* were even indicated from the Caspian Sea (Starobogatov 1994).

The first known fossil record of the genus *Cerastoderma* comes from the Oligocene (33.9-23.0 million years ago) (Keen 1969). Therefore, it is believed that the ancestor of the modern *Cerastoderma* (*Cardium*) existed about 50 million years ago (mya) (Gosling 1994). Fossil records from Libya and Tunisia show that bivalves morphologically indistinguishable from the modern *C. glaucum* thrived in lagoons in the region of the present Mediterranean Sea in the early Pliocene (during Messinian salinity crisis) (Gaillard and Testud 1980; Wołowicz 1991), which suggests that *C. glaucum* diverged from the common ancestor with its closest living relative *C. edule* in the Mediterranean Sea. *Cerastoderma* was probably present in the Mediterranean region at the time of the closure of the connection between the Atlantic Ocean and the Mediterranean Sea around 4 mya in the late Miocene and early Pliocene (Rygg 1970). The combination of tectonic and glacio-eustatic processes which isolated Mediterranean Sea from the open ocean initiated the Messinian salinity crisis. The Mediterranean Sea was split into many basins and lagoons

with varying salinities (Ruggieri 1967; Hsü *et al*. 1977; Krijgsman *et al.* 1999; CIESM 2008). Due to severe environmental conditions at that time, probably the big part of marine Miocene fauna from the Mediterranean Sea (mainly fish) went extinct (CIESM 2008), but it was not the case for *C. glaucum*. It survived in some of the basins with changing salinity, gaining greater euryhalinity, eventually becoming a new species. At the beginning of Pliocene the Strait of Gibraltar opened, provoking the entry of the Atlantic Ocean waters to the Mediterranean Sea (Ruggieri 1967), but *C. glaucum* could not cross with *C. edule*. *C. glaucum* managed to spread north along the Atlantic coast, but *C. edule* did not colonize the Mediterranean Sea (Rygg 1970). This allopatric speciation mechanism seems the most probable for the cockles. Nowadays, there are some locations, where *C. glaucum* and *C. edule* live in sympatry (Brock 1987; Machado and Costa 1994) and have similar spawning periods, but according to electrophoretic investigations on wild populations (Brock 1978; Gosling 1980) and hybridization studies on laboratory populations (Kingston 1973) cross-fertilization does not occur. It is presumably due to a prezygotic reproductive isolation mechanism (Brock 1991), like a gamete mate-recognition system based on gamete surface proteins, which was also found in some echinoderms and gastropods (Palumbi 1992).

Apart from the division into *C. glaucum* and *C. edule*, it has also been discussed whether the Atlantic Ocean and the Mediterranean Sea populations of "*Cerastoderma glaucum* complex" belong to the same species, or there is also an Atlantic species, *C. lamarcki*. The name *C. lamarcki* was first used to name an asymmetric form of *C. glaucum* (Reeve 1845), but finally *C. lamarcki* was stated to be the same species as *C. glaucum* and the latter name was suggested to be the proper one (Bowden and Heppell 1968; Rygg 1970). In the 1970s. the southern and northern populations of *C. glaucum* were considered as conspecific (Høpner Petersen and Russell 1973; Brock 1979). Then, according to immunoelectrophoretical studies (Brock 1987) and the demonstration of the chromosomal DNA differences (Brock and Christiansen 1989) the subdivision into two species has been suggested: the Mediterranean *C. glaucum* and the Atlanto-Baltic *C. lamarcki*. The reason for this separation was claimed to be the adaptation to different environmental conditions in the Mediterranean and in the Atlantic regions since the early Pliocene (4 mya) (Brock and Christiansen 1989; Brock 1991). The findings of Brock (1991) and the study of allozymes variation performed by Hummel *et al.* (1994) supported the division into a rank of subspecies between the Mediterranean *C. glaucum glaucum* and

the Atlanto-Baltic *C. glaucum lamarcki*. Then more detailed studies based on more (16) allozymes (Mariani *et al.* 2002) and on mitochondrial DNA (Nikula and Väinölä 2003) proved that differences between the Mediterranean and Atlanto-Baltic forms of *C. glaucum* are too small to divide them to the rank of subspecies. Moreover, the major divergence within *C. glaucum* is not in the area of the Gibraltar Strait (Nikula and Väinölä 2003; Tarnowska *et al.* 2009).

3.5 Distinguishing between C. glaucum *and* C. edule

The closest relative of the lagoon cockle (*C. glaucum*), the common cockle (*Cerastoderma edule*) differs in some ecological preferences (Hummel *et al.* 1994; Machado and Costa 1994), but due to an extensive overlap in environmental tolerance and preference, there are areas of the sympatric occurrence of these two species in Portugal (Machado and Costa 1994), Finland (Rygg 1970), Germany (Krakau- pers. comm.), Norway, Sweden, Denmark, Netherlands, England and France (Brock 1987). Various morphological traits have been proposed to distinguish between *C. glaucum* and *C. edule* (Table 3.1). However, the cockles display a considerable degree of intraspecific variation in shell morphology, as it depends on environmental factors. For example, soft substrate provokes shell asymmetry and salinity of the water determines the shell size, thickness and the number of ribs (Eisma 1965; Rygg 1970; Barnes 1980).

One of the morphological traits that have been proposed to distinguish between the two species is the visibility (*C. edule*) or invisibility (*C. glaucum*) of the ligament, when cockles are laterally viewed (Boyden and Russell 1972; Brock 1978). Another one is the shape of the ligament, which is short in case of *C. glaucum*, but long, thin and always well visible in case of *C. edule* (Høpner Petersen 1958). Høpner Petersen (1958) chose the relationship between shell width and ligament length as the most striking feature to distinguish between the species. Some equations were suggested to make a clear separation between *C. glaucum* and *C. edule* relaying on this trait (Høpner Petersen 1958; Pohlo 1963; Boyden 1973), but according to Wołowicz (1991) this feature does not enable an unequivocal separation. Other features, which were proposed to distinguish the two species, were the type of calcareous scales (Høpner Petersen and Russell 1973), color differences (Mars 1951), valve profile (Mars 1951; Høpner Petersen 1958; Boyden 1971) and the thickness of periostracum (Høpner Petersen and Russell 1973). Also spermatozoa

of *C. edule* and *C. glaucum* were proven to differ significantly. In *C. glaucum* the spiral-shaped head of spermatozoa has four coils and measures 1/5 of the tail-length, whereas in *C. edule* it has two coils and measures 1/7 of the tail-length (Rygg 1970).

A thorough research concerning morphological criteria for distinguishing *C. glaucum* from *C. edule* from the Portuguese coast was conducted by Machado and Costa (1994) (Table 3.1, Fig. 3.2). The specimens belonging to two cockle species were identified by means of electrophoretic patterns of hepatopancreatic malate dehydrogenase according to Brock's (1978) procedure. The trait introduced by Machado and Costa (1994) for distinguishing both species for which no literature references could be found was a ventral valve junction. According to the authors, the visibility of the ligament cannot be used for distinguishing the specimens from the Portuguese coast, as it appeared not valid for more than 50% of individuals in some samples. The amount of periostracum, the internal posterior brown spot and the shell shape were claimed to be of little or no taxonomic value, which is incongruous with previous findings (Høpner Petersen 1958; Boyden 1971; Van Urk 1973). Apart from morphological traits, other methods for discriminating the two cockles species have also been described based on diagnostic enzyme loci (Brock 1978; Hummel *et al.* 1994), Southern blotting of enzyme digested DNA fragments (Brock and Christiansen 1989), crossed immunoelectrophoresis (Brock 1987), RAPD (André *et al.* 1999) and PCR-RFLP (Freire *et al.* 2005). Microsatellite primers designed for *C. glaucum* did not amplify on *C. edule*, which also proves the separate character of these two species (Pearson 2003).

Table 3.1. The summary of morphological characteristics applied to identify *C. glaucum* and *C. edule* from the Portuguese coast (based on Machado and Costa 1994). Symbols used: "+" useful trait for distinguishing, "+/-" not always correct, a considerable number of exceptions.

Character	***Cerastoderma edule***	***Cerastoderma glaucum***	**Comment**
Valve profile	smooth	sharp	+
Ventral valve junction shape	crenulated	typically straight	+
Type of calcareous scales	laminar type, perpendicularly inserted in valves	typically non-existent or thick, bent to umbonal region	+ (difficult for inexperienced researchers)
Shell color	variable, different from colors typical of *C. glaucum*	typically glaucum-yellow or salt-pit green	+ (difficult for inexperienced researchers)
Posterior valve junction	typically crenulated	typically straight or slightly crenulated	+/-
Visibility of ligament	generally visible or at limit of visibility	typically not visible	+/-

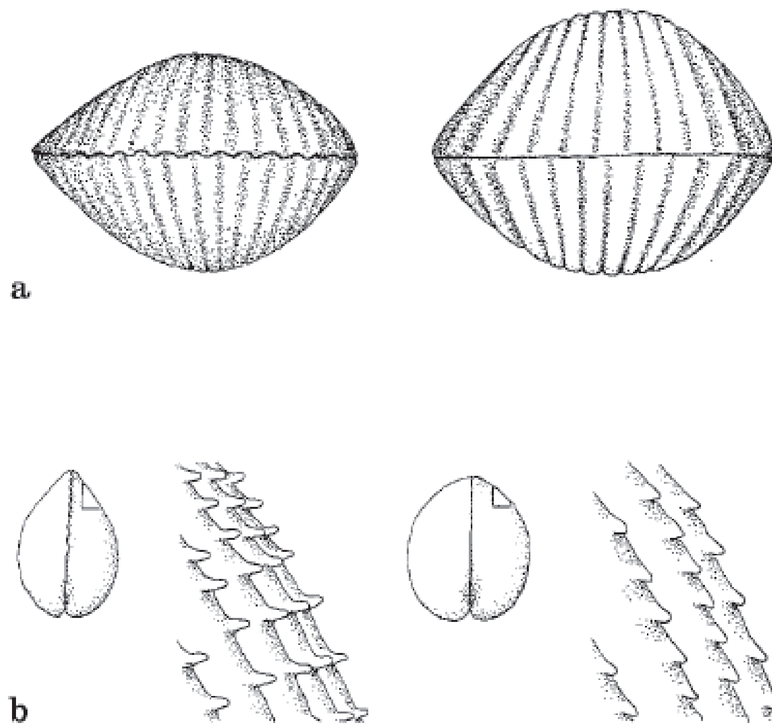

Figure 3.2. *Cerastoderma edule* (on left) and *C. glaucum* (right) (from Machado and Costa 1994): **a)** ventral views showing characteristic valve profile and ventral valve junction in both species, **b)** schematic representation of characteristic shape and type of valve insertion of calcareous scales in both species (front view).

Chapter I: Studies on morphometry and physiology of *Cerastoderma glaucum*

4 Interest in studying physiological responses of *C. glaucum*

C. glaucum is the important component of lagoons it inhabits, as suspension-feeding bivalves are often key species in lagoons, estuaries and coastal habitats (Gili and Coma 1998). If they are found in big densities, the turnover rate of nutrients in lagoon ecosystems is determined mainly by the filtering capacity of benthic filtrators (Nienhuis 1992). They are included in ecological monitoring and impact assessments, because of their trophic position, widespread occurrence and sensitivity (Goldberg *et al*. 1978). They also act as natural controllers of eutrophication processes, as they deposit organic material from the water column onto the bottom sediments. Besides, they accelerate the regeneration of nutrients from the deposited particulate organic matter, thereby enhancing the primary production of phytoplankton (Officer *et al.* 1982). Changes in bivalves growth rate, abundance and distribution may have cascading effects on both benthic and pelagic ecosystems (Norkko *et al*. 2001; Dahlhoff *et al*. 2002; Ellis *et al*. 2002; Newell 2004).

In order to predict bivalves' reactions to environmental changes, like eutrophication (Cloern 2001), pollutants or climate anomalies, the knowledge of preferences and limits towards environmental conditions, such as food availability (quality and quantity), temperature, salinity, pollution level is required. Studying physiological reactions, especially on lagoon specialists, could be informative while predicting consequences of an ongoing global warming, like a shift in species distribution areas (Hughes 2000; Parmesan and Yohe 2003; Root *et al.* 2003). Rising sea levels provoked by a global warming result in the destruction of some current lagoon habitats, but also the possible creation of others. Studying habitats such as lagoons in which organisms are subjected to extremes of temperature and salinity still prosper, can provide information on the kind of organisms that may prosper in more extreme habitats that may be more abundant with the increased global temperatures. Furthermore, effects of climate change on lagoons invertebrates populations could have impacts on fecundity and recruitment, causing problems for over-wintering birds relying on those populations as for the source of food (Lawrence and Soame 2004). The additional threat of climate change in coastal water areas, which are subject to warming, could be related to the temperature dependence of the release of cercariae by the initial molluskan hosts of the trematode parasites (Gates 2006). High temperatures are known to increase the rates of cercariae release, resulting in increased

infection rates, and potentially the mortality of various species that host metacercarial stages of the trematodes.

There is often an intraspecific variation in physiological responses of invertebrates to different environmental conditions. Those differences might be either individual acclimatizations or genetically fixed adaptations (Brock and Wołowicz 1994) and it is important to distinguish between these types of responses (Table 4.1). Also within the same population physiological reactions change during the year, which is associated mainly with thermal and food conditions (Beukema and De Bruin 1977; Bayne and Newell 1983; Iglesias and Navarro 1991; Wołowicz *et al.* 2006). For some bivalves ecophysiological differences were found even for populations from the same region, but from different depths due to different environmental conditions above and below the thermo- and halocline (Wenne and Klusek 1985; Wenne and Styczyńska-Jurewicz 1985; Hummel *et al.* 2000b; Wołowicz *et al.* 2006).

Table. 4.1. Types of responses to the change of the environmental parameter (adapted from Clarke 1991).

Response	Definition
Acute	the adjustment of organism physiology to an immediate change; can include torpor, coma (and death)
Acclimation	the adjustment of organism physiology to a changed environmental parameter in the laboratory
Acclimatization*	the adjustment of organism physiology to changes in environmental parameters; this may be tidal, daily, seasonal or inter-annual
Adaptation	the evolutionary adjustment of organism physiology to environment; this can include adjustment to a seasonal or daily fluctuation in environmental parameter requiring acclimatization

*It should be noted that in laboratory acclimation consists usually of modifying only a single variable (e.g. temperature) while keeping all others constant. An organism undergoing acclimatization in the field is subject to coincident variation in the whole range of environmental variables.

5 Materials and methods

5.1 Sampling and hydrological conditions of sampling sites

Samples serving for physiological analysis were collected from 3 locations (Fig. 5.1):

-the Gulf of Gdańsk (GD), the Baltic Sea, Poland (54°40’ N, 18°30’ E); analysis were performed 5 times: in November 2005, February, May, July and October 2006; samples were collected by dragging at the depth of 5 meters (from the research vessel “Oceanograf II”) (Fig. 5.2),

-the Lake Veere (LV), the North Sea the Netherlands (51°35’ N, 3°38’ E); analysis were performed 4 times: in November 2006, February, May and August 2007; samples were collected by hand at a depth of about 1 meter (Fig. 5.3 and 5.4),

-the Berre Lagoon (BL), the Mediterranean Sea, France (43°24’ N, 5°08’ E); analysis were performed 4 times: in February, May, August and November 2007; samples were collected by hand from the depth of about 1 meter (Fig. 5.5).

At each sampling site each season the water temperature and salinity were measured. As the seasonal differences of these parameters were measured only 4 times a year, data from monitoring databases were taken into consideration as well (Gouze *et al.* 2005; IMGW 2009; RIKZ and RIZA 2009).

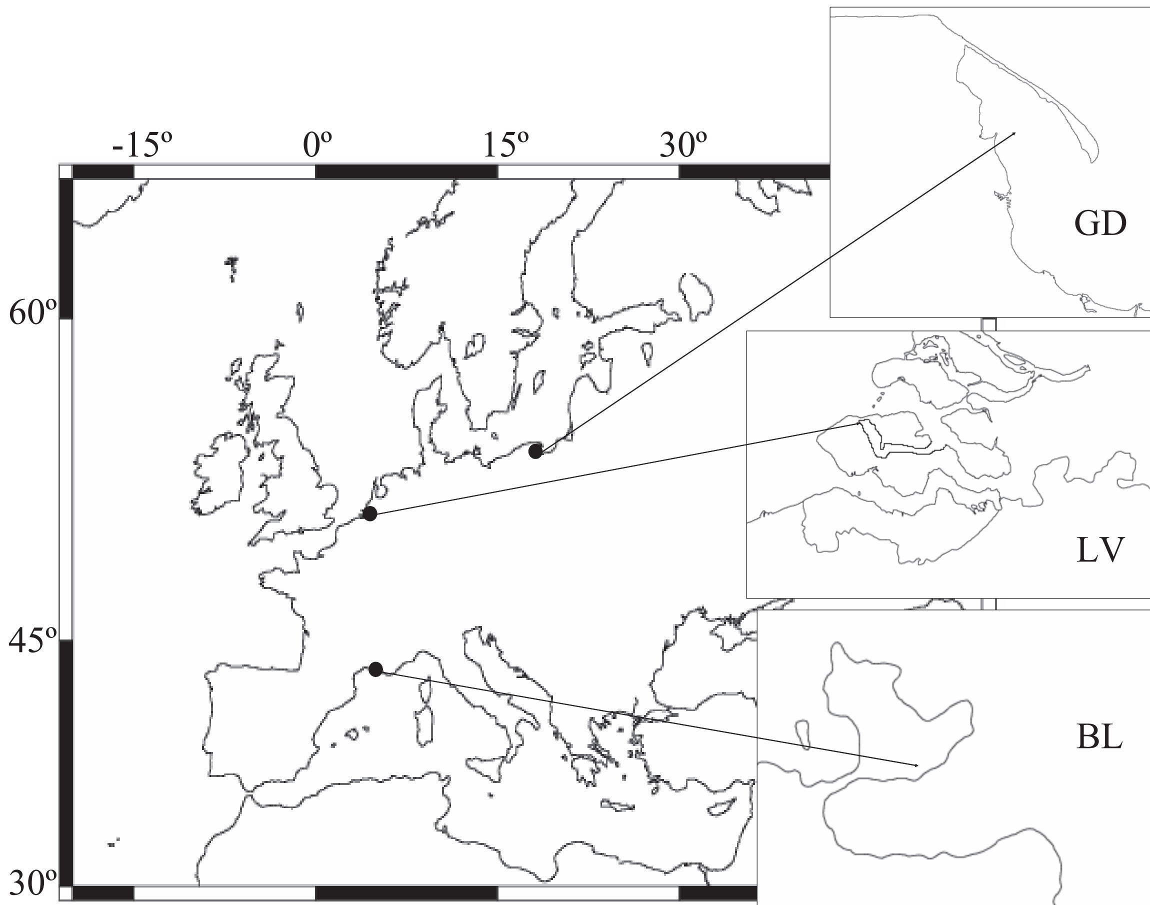

Figure 5.1. Sample collection locations: Gulf of Gdańsk (GD) (54°40' N, 18°30' E), Lake Veere (LV) (51°35' N, 3°38' E), Berre Lagoon (BL) (43°24' N, 5°08' E).

The sampling sites were chosen, so that they differ in environmental conditions. The Baltic Sea (GD) was investigated, because in this area *C. glaucum* is present along the whole coastal area, whereas in the two other sampling sites it is restricted to relatively small and isolated basins. Sampling sites differ in salinity, which is exceptionally low in the Baltic Sea (7 PSU) and relatively high in the Lake Veere and the Berre Lagoon (above 20 PSU). Also thermic conditions differ with two "cold" sampling locations (GD and LV) and one "warm" sampling location (BL) (Gouze *et al.* 2005; IMGW 2009; RIKZ and

RIZA 2009). In order to investigate changes in morphometric and physiological parameters during the year, studies were performed each season.

5.1.1 Hydrological conditions in the Gulf of Gdańsk

The non-tidal Gulf of Gdańsk (Fig. 5.2) is situated in the south of the Baltic Sea, in Poland. It is the first-order estuary of the Vistula River- the second largest river draining into the Baltic Sea, which brings considerable loads of nitrogen and phosphorus (HELCOM 1998). The Gulf of Gdańsk covers an area of 4940 km^2 (1.2% of the entire Baltic Sea surface), has a volume of 291 km^3 and the maximum depth of 118 m (Majewski 1990). The temperature varies during the year from almost 0ºC to over 20ºC. The Gulf of Gdańsk is brackish with a rather uniform salinity of about 7 PSU (IMGW 2009). Bottom waters are occasionally stripped of oxygen and enriched with phosphate. About two-thirds of the bottom is covered by muddy-silty sediments (Uścinowicz and Zachowicz 1994).

An important component of the phytoplankton of the Gulf of Gdańsk are the diatoms (Pliński 1975). Phytoplankton blooms dominated by diatoms and dinoflagellates take place in spring with chlorophyll *a* concentrations reaching around 8-10 µg dm^{-3} (IMGW 2009). There is also a smaller diatom bloom in autumn (Pliński 1995; Wasmund *et al.* 1998). In summer there is a blue algae bloom leading to high chlorophyll *b* concentrations (Latała 1982).

Figure 5.2. Sampling *C. glaucum* by dragging in the Gulf of Gdańsk; Poland. Photo by K. Tarnowska.

5.1.2 Hydrological conditions in the Lake Veere

The Lake Veere (Fig 5.3 and 5.4) is a lagoon in the province of Zeeland, the Netherlands. It was created in 1960-1961 and it is connected with the tidal Oosterschelde estuary with man-operated sluices. It has a surface of between 18 km^2 (during winter) and 21 km^2 (during summer). It is linked to the river Scheldt by the Zandkreekdam lock, which results in a fresh water inflow. The temperature varies from almost 0ºC to above 20ºC and the salinity varies between 22 PSU and 30 PSU (RIKZ and RIZA 2009). Each year in April the salt water from Oosterschelde estuary (the North Sea) is introduced into the lagoon via lock and sluices in the eastern dam. It results in the increase in salinity (of about 3 PSU). Tides are absent and currents are weak. Particles settle easily and consequently the stagnant water of the lagoon, containing the low amount of silt particles is clear, but plankton blooms may color the water mass occasionally (Nienhuis 1992).

The substantial input of nutrient-rich water from agricultural grounds causes a moderate phosphorus load (6 g P m^{-2} yr^{-1}) and a high nitrogen load (34 g N m^{-2} yr^{-1})

resulting in considerable concentrations of ammonium and nitrate at the end of winter, before the phytoplankton spring bloom starts. The phytoplankton and zooplankton communities of the Lake Veere are poor in species. Chlorophyll *a* concentrations derived from phytoplankton, vary considerably between years. In spring a diatom bloom *Skeletonema costatum* produces most of the phytoplankton biomass (Nienhuis 1992) and chlorophyll *a* concentrations reach around 25-55 μg dm^{-3} (RIKZ and RIZA 2009). Large production of phytoplankton biomass may lead to anaerobic conditions in the sediment. The Lake Veere is vulnerable to eutrophication, owing to the long residence time of the water mass and the almost permanent stratification (Nienhuis 1992).

Figure 5.3. Sampling site in the Lake Veere (LV); the Netherlands. Photo by K. Tarnowska.

Figure 5.4. Sampling *C. glaucum* from the shallow water in the Lake Veere (LV); the Netherlands. Photo by K. Pierścieniak.

5.1.3 Hydrological conditions in the Berre Lagoon

The Berre Lagoon (Fig. 5.5) is a water body adjacent to the Mediterranean to the west of Marseille, created by the rise in water levels at the end of the last ice age. Its mean depth is of 6 m and the maximal one is of 9.5 m. It covers the surface of 155 km². It is supplied with fresh water by the rivers Arc, Touloubre and Cadière. In 1966 the canal of the Durance river and the hydroelectric power plant were opened, which caused an excessive fresh water inflow and induced desalination of the Berre Lagoon to the salinity below 10 PSU. The Berre Lagoon was known for being ecologically poor, degradated, strongly influenced by human activities and suffering from the excess of nutrients, heavy metals and hydrocarbons. There were problems of eutrophication, oxygen deficiencies and azote and phosphore accumulation in the sediment. In 1993-1995 the fresh water and slime supplies were limited. Since then the salinity in the Berre Lagoon tended to increase and during the last years it was generally higher than 15 PSU, but there are big seasonal variations. During the drought in August 2005 the salinity reached the value of 30 PSU, which is similar to the values from before 1966, when the hydroelectric power plant was opened (Picon 2002; Gouze *et al.* 2005).

The periods of high chlorophyll concentrations in the Berre Lagoon are not seasonal, which is associated with a salinity increase during the last 10 years. Before 1997 this lagoon used to be hypereutrophicated. Phytoplankton blooms took place in winter and spring and chlorophyll *a* concentrations reached sometimes 300-400 µg dm^{-3}. The chlorophyll concentrations during algal blooms were about ten times higher than medium values in other lagoons. Since then chlorophyll *a* concentrations much decreased (rarely exceeding 20 µg dm^{-3}), there is no spring bloom and phytoplanktonic populations are much more stable, which led to a decrease in the quantity of sestonic matter and diminishing eutrophication. The first level of the food chain has changed rapidly and keeps an acceptable trophic level. The reduction of fresh water inflow provoked the disappearance of the spring bloom and the stabilization of phytoplanktonic populations (Gouze *et al.* 2005). This led to higher water clarity, which enabled the ecosystem to function from the surface to the bottom and to reduce periods of anoxia, which are common in lagoons and often provoke massive summer mortality in benthic communities (Allen *et al*. 1995; Stora *et al*. 1995). In winter there is a big inflow of nutrients into the Berre Lagoon, whereas in summer their quantities are reduced and the nutrients entering the basin are consumed by autotrophic organisms (Gouze *et al*. 2005).

Figure 5.5. Sampling site in the Berre Lagoon (BL); France. Photo by K. Tarnowska.

5.2 Morphometric and physiological analysis

Shell length, dry shell weight, dry tissue weight, condition index, sex, gonad development stage and biochemical composition were asserted on 30 individuals of *C. glaucum* per season per site.

Shell lengths were measured with a slide caliper accurate to 0.01 mm. Soft tissues were freeze-dried until obtaining the constant weight. Shells were dried at 55ºC. Relationships between shell lengths and dry weights of soft tissue and shell were described by power regressions ($y = ax^b$).

Morphometric condition indices were calculated with the following equation (Beukema and De Bruin 1977):

$$CI = \frac{M_c \times 1000}{L^3}$$

where M_c is a dry tissue weight (mg) and L is a shell length (mm).

Sex and the gonad development stage were analyzed under an optical microscope and classified according to the 5-stage scale (Wołowicz 1987).

The gonadic indices (*GI*) were calculated with the following equation:

$$GI = \frac{\sum_{M=0}^{4}(N_M \times M)}{N}$$

where N_M is the number of individuals at each gonad maturity stage, M is the numeric value of the stage and N is the total number of individuals in the sample (Chipperfield 1953) (Table 5.1).

Table 5.1. Gonad development stage (Wołowicz 1987) and the numeric value of each stage to calculate the gonadic index (Chipperfield 1953).

Gonad development stage	**Process**	**Numeric value (*M*) to calculate the gonadic index (*GI*)**
I	initiation of gametogenesis	1
II	development	2
III	ripe stage	3
IV	spawning	4
V	post-spawning	0

The gross biochemical composition was determined on three replicates of each sample. Dry soft tissues were homogenized with the planetary micro mill (Fritsch Pulverisette 7). Dry tissues of all individuals from the same season and location were pooled together and treated as one sample. Protein content was determined according to Lowry *et al.* (1951). Lipids were extracted according to Bligh and Dyer (1959) and lipid content was determined with Marsh and Weinstein (1966) method. Carbohydrate and glycogen contents were determined with Dubois *et al.* (1956) method.

Oxygen consumption was measured every season during 45 to 120 minutes at 4, 10, 17 and 24ºC in temperature-controlled respiration chambers with a volume of 0.3 dm^3 on 5-10 (LV and BL) or about 20 (GD- due to the smaller, average size) individuals per chamber. The individuals were purged overnight in filtered water from the sampling station and at ambient temperature of the site in order to prevent any disturbance in oxygen consumption due to (pseudo)faeces. The decrease in oxygen tension was measured with YSI 5331 oxygen probes (Clarke type polarographic electrodes). The measurements were performed in three replicates (3 respiration chambers) and one control chamber without animals. After the experiment, the animals were frozen in –80ºC and freeze-dried for 72 h to the soft tissue dry weight. Knowing the water salinity and temperature, respiration rates were calculated.

Statistical analyses were performed using STATISTICA v. 8. The χ^2 test was applied to test the significance of differences in the sex ratio. The normality of the data distribution was checked using the Shapiro-Wilk test at the significance level of 5%. Kruskal-Wallis non-parametric ANOVA was applied to verify the statistical significance of differences in morphometric parameters among seasons and sites.

6 Results

Each season the water temperature in BL was higher than in two other sampling sites, while GD differed from other sites, because of a much lower water salinity (Table 6.1).

Table 6.1. Temperatures, T (^{o}C) and salinities, S (PSU), measured at each sampling site each season.

Site	**GD**		**LV**		**BL**	
Parameter	**T**	**S**	**T**	**S**	**T**	**S**
Winter	2.0	7.3	3.0	23.1	15.0	19.5
Spring	12.0	6.0	15.0	27.3	20.0	22.9
Summer	23.9	6.2	19.0	28.5	28.0	25.2
Autumn	14.1	7.0	10.0	28.5	22.0	22.0

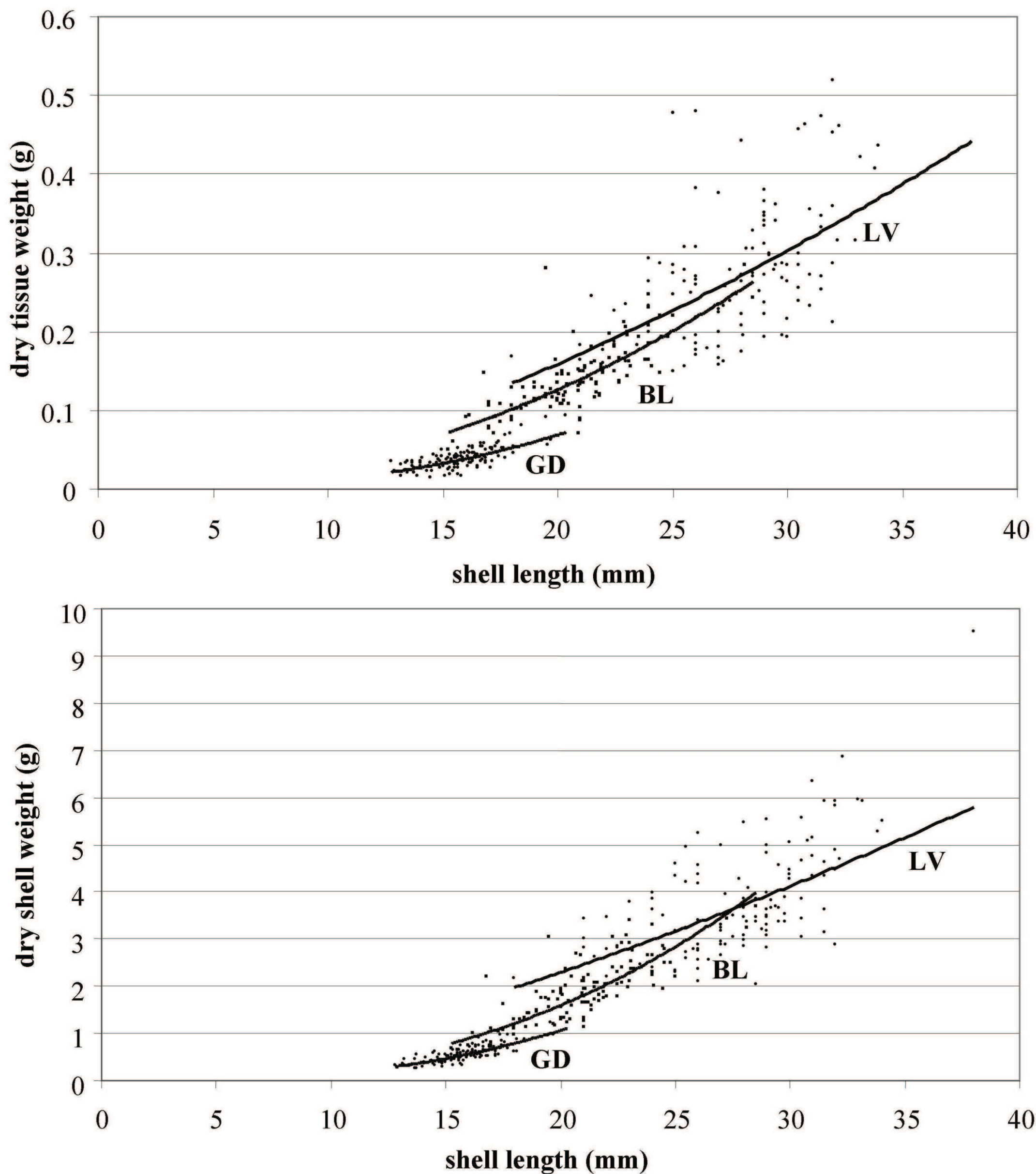

Figure 6.1. Relationship between morphometric parameters of *C. glaucum*:

a) dry tissue weight (g) vs. shell length (mm); GD: $y = 0.00003x^{2.60428}$ ($R^2 = 0.44935$); LV: $y = 0.00134x^{1.59320}$ ($R^2 = 0.35538$), BL: $y = 0.00025x^{2.07862}$ ($R^2 = 0.63470$),

b) dry shell weight (g) vs. shell length (mm); GD: $y = 0.00021x^{2.85317}$ ($R^2 = 0.64458$); LV: $y = 0.03030x^{1.44442}$ ($R^2 = 0.37716$), BL: $y = 0.00069x^{2.58453}$ ($R^2 = 0.67520$).

Individuals from GD were in general much smaller than those from LV and BL. Shell dry weight increased with the increase of shell length the most in BL (Fig. 6.1a-b).

Based on the assumption that the values of morphometric parameters do not change a lot year on year, as our results were similar to those obtained by Wołowicz (1991), the measurements from different seasons were arranged from winter to autumn to facilitate comparisons among populations in the graphs. Most shell length, dry soft tissue weight and dry shell weight data (Appendix 1) did not have normal distributions. Therefore, Kruskal-Wallis non-parametric ANOVA was applied. However, distributions were unimodal, so averages (± SD) instead of medians were calculated. Differences in dry soft tissue weight were significant among sites ($p < 0.001$) and not significant among seasons within sites ($p > 0.01$). Differences in condition index were significant among sites ($p < 0.001$) and among seasons within sites ($p < 0.001$; in case of BL $p < 0.01$) (Fig. 6.2).

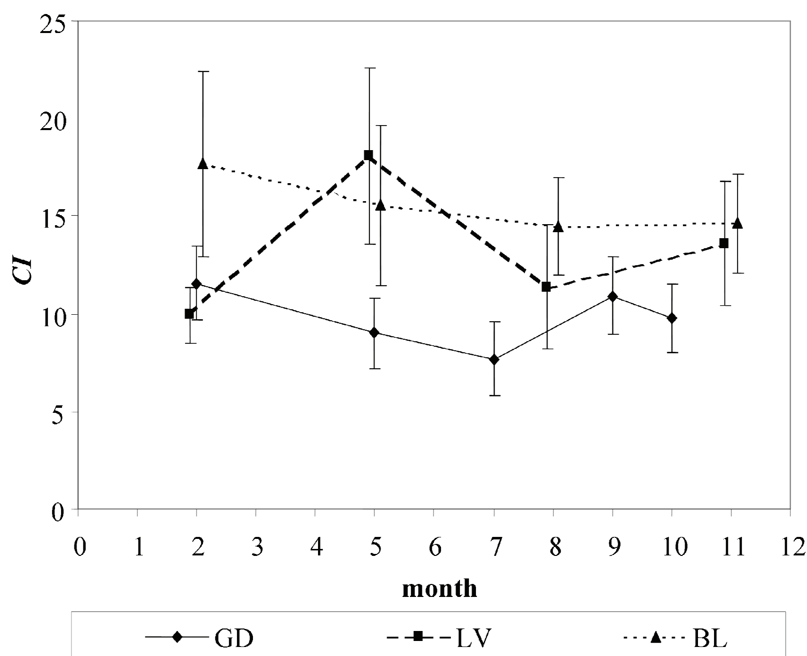

Figure 6.2. Seasonal changes of the condition index *CI*.

The average females to males ratios were 1 : 0.74 for GD, 1 : 1.22 for LV and BL and did not significantly differ from 1 : 1 ($p > 0.05$). Most of the differences in sex ratios among seasons and sites were not significant as well ($p > 0.05$). In GD and LV only one, summer spawning period was observed, whereas in BL differences in gonadic index among seasons were less marked and it was hard to define a spawning season (Fig. 6.3 and 6.4).

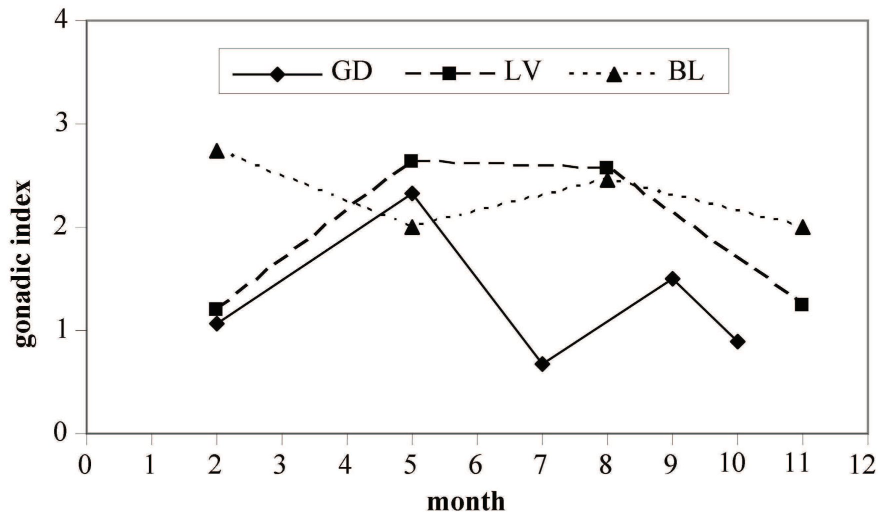

Figure 6.3. Seasonal changes of gonadic index.

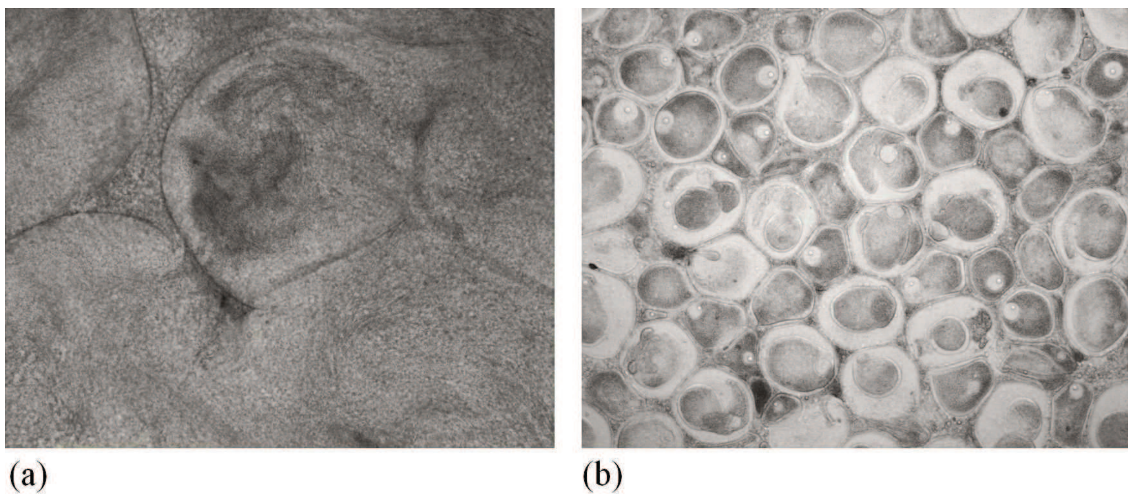

Figure 6.4. Gonads in ripe stage (stage III): **a)** male, **b)** female. Photo by K. Tarnowska.

Protein contents in the dry tissue were between 37.5% and 77.4%, and lipid contents were between 5.7% and 13.6%. For all the populations the protein content was the highest in spring (Fig. 6.5a). In GD and BL lipid content was the highest in spring (Fig. 6.5b). Carbohydrate contents were between 4.1% and 16.1%, glycogen contents were between 2.8% and 13.3%. Glycogen comprised from about half to all of the carbohydrate content. Carbohydrate and glycogen contents in GD and BL were the lowest in spring (Fig. 6.5c-d). In LV differences in lipid, carbohydrate and glycogen contents among seasons were much less pronounced than for other populations (Fig. 6.5b-d). The sum of protein, lipid and carbohydrate contents in the dry soft tissue of *C. glaucum* was between 55.1% and 96.3%.

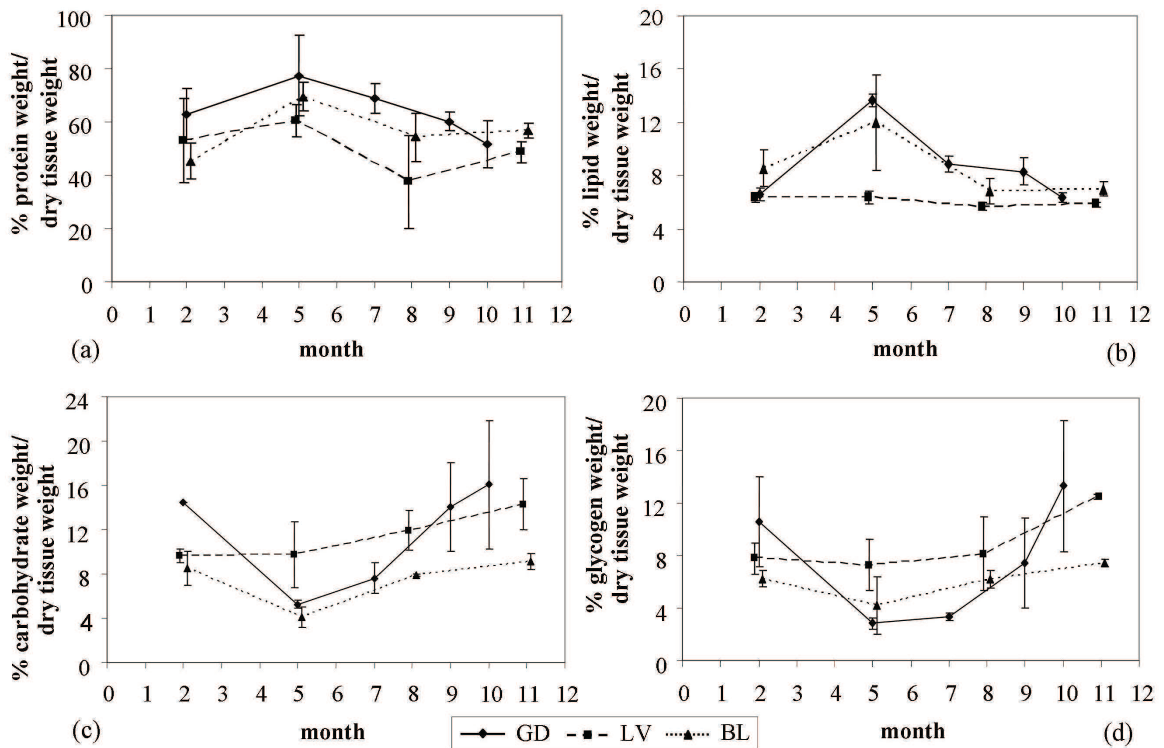

Figure 6.5. Seasonal changes of biochemical components content: **a)** protein content, **b)** lipid content, **c)** carbohydrate content, **d)** glycogen content.

Respiration rates were the lowest at 4°C and increased with a temperature growth. In GD and LV they were the highest in spring and autumn. In GD respiration rates were higher than in other populations reaching even 3.22 mlO_2 gDW^{-1} h^{-1} in spring at 24°C. In BL most of the winter and summer respiration rates were higher than spring and autumn rates for the same temperatures (Fig. 6.6).

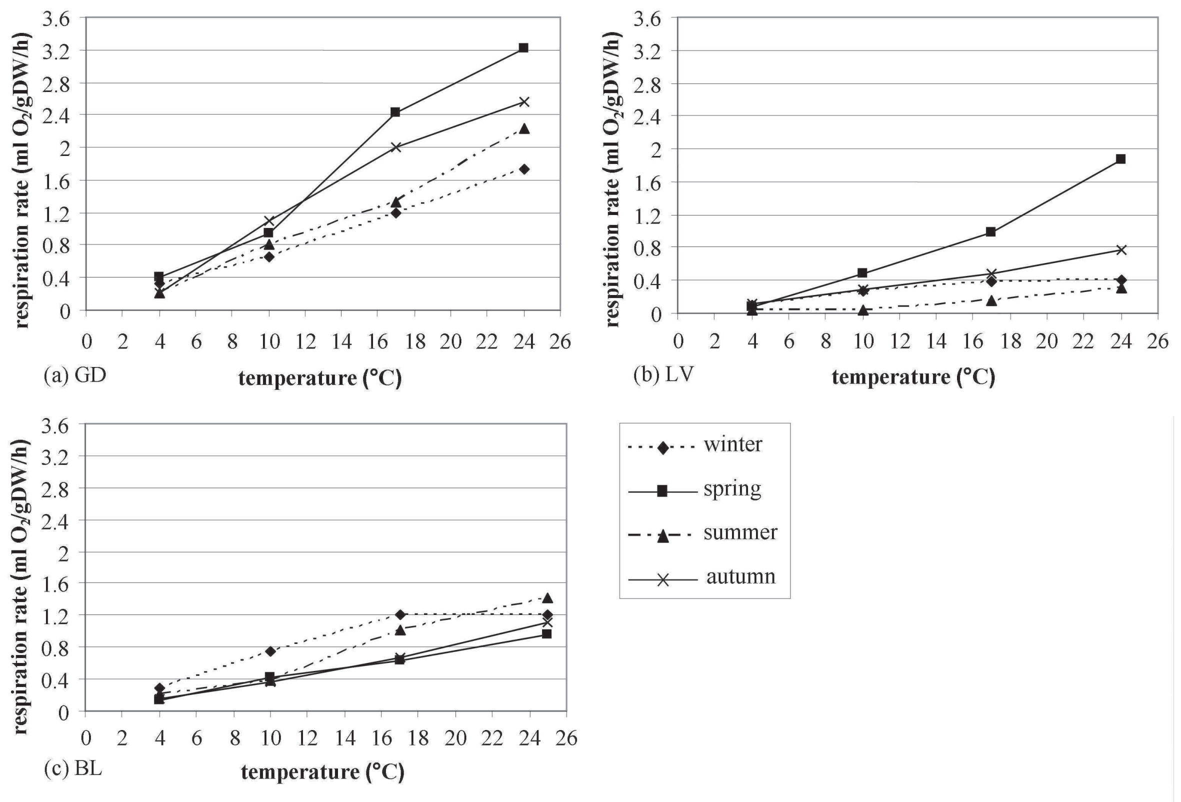

Figure 6.6. Seasonal changes of the respiration rate in different water temperatures. Measurements were averaged among three replicates (standard deviations not showed): **a)** Gulf of Gdańsk (GD), **b)** Lake Veere (LV), **c)** Berre Lagoon (BL).

7 Discussion

7.1 Environmental conditions

In respect of water temperature the northern locations (GD and LV) may be considered similar, whereas in BL there is a high water temperature and intensive evaporation in summer provoking salinity increase (Stora *et al.* 1995). Low salinity of around 5-7 PSU (IMGW 2009) makes GD different from the rest of localities (see: 5.1 Sampling and hydrological conditions of sampling sites).

Food availability is of a crucial importance in promoting individual growth and structuring of benthic communities (Herman *et al.* 1999). As *C. glaucum* does not occur in tidal areas, it is less subject to sudden changes of food supply, than intertidal bivalves, like *C. edule*, which often experiences food shortages due to aerial exposure (Honkoop and Van der Meer 1998). Diatoms, which are considered the main food of *C. glaucum* (Wołowicz 1991; Beukema and Cadée 1996) are an important component of the

phytoplankton in GD (Pliński 1995; Wasmund *et al.* 1998), LV (Nienhuis 1992), and BL (Lelong and Riva 1976). In northern sampling locations spring phytoplankton blooms make trophic conditions favorable (IMGW 2009; RIKZ and RIZA 2009). In BL there is no longer any spring bloom and phytoplanktonic populations are more stable (Gouze *et al.* 2005).

7.2 Morphometric parameters and condition index

Cockles from GD were by far the smallest among sampling sites (Fig. 6.1) due to low average salinity in the Baltic Sea influencing active intracellular transport, feeding rate or nutritive absorption, respiration, excretion and particularly osmoregulation. Organisms under osmotic stress have to expend additional energy to maintain the haemolymph osmolality above that of the environment (Schmidt-Nielsen 1990). Consequently, less energy is expended for growth. In the Baltic Sea, a tendency for shell length to decrease in parallel with a drop in salinity was reported in *Macoma balthica* (Wenne and Klusek 1985). In BL until a certain shell length (around 25 mm) shells were thinner than in LV, but then they became thicker (heavier) (Fig. 6.1). Environmental salinity and temperature influence shell's size and shape (Eisma 1965), as well as its composition (aragonite and calcite proportions) (Kennedy *et al.* 2008).

Condition index depends mainly on bivalves' growth, metabolism or gonad development stage (Lucas and Beninger 1985; Smaal and Stralen 1990; Hummel *et al.* 2000b). For northern populations of European bivalves with a monocyclic reproduction pattern, the highest values of condition index were reported in spring, before spawning (*C. glaucum*, Wołowicz 1991; *M. balthica*, Hummel *et al.* 2000b; *Mytilus trossulus*, Wołowicz *et al.* 2006). The seasonal changes of condition index that were found in the present study in the northern populations (GD, LV) did not fully reflect this pattern. In GD *CI* decreased between spring and summer due to spawning. The following increase between summer and autumn might have been caused by favorable trophic conditions provoked by autumn phytoplankton bloom (Pliński 1995). In LV between winter and spring *CI* increased, because of gonad development and phytoplankton bloom, which surprisingly was not the case in GD. In LV in summer *CI* was low, although according to gonad development stage analysis a spawning stage (stage IV) prevailed, so a high condition index was expected. The reason for that could be the fact that gonad

development stages and condition indices were asserted on different individuals, so the individuals used for *CI* measurements may have spawned already. The winter *CI* for LV was low, which was not the case for GD. Studies on *C. edule*, *Mytilus edulis* and *M. balthica* proved that in the bivalves from temperate latitudes (like GD and LV) body mass (and condition index) declines in winter (Honkoop and Beukema 1997). In BL differences among seasons in condition index were not clearly marked, as the cockles seem to spawn during the whole year. Bivalves' condition may be influenced by parasites, which were not found in this study, because only a small piece of gonad tissue was scanned, but their presence cannot be excluded. *C. glaucum*, as well as *C. edule*, is a host species for metacercariae of parasites from the genus *Meiogymnophallus* (Digenea: Gymnophallidae) (Bowers *et al.* 1996). Condition index was also reported to decrease with increasing age and body size (Wenne and Styczyńska-Jurewicz 1985; Bawazir 2000).

<u>*7.3 Reproduction*</u>

Differences among seasons in sex ratio were mostly not significant. In some mollusks, like *Chlamys islandica* (Brokordt and Guderley 2004) or *M. trossulus* (Wołowicz *et al.* 2006) increased females mortality after spawning was reported due to the high cost of gonad maturation and spawning. In other species, like *Choromytilus meridionalis* (Griffiths 1977) or *Perna perna* (Lasiak and Dye 1989), the male : female sex ratios reportedly did not differ significantly from 1 : 1. The northern populations of *C. glaucum* (GD, LV) had a monocyclic reproductive cycle with gametogenesis initialization in September/October and spawning between May and July (Rygg 1970; Boyden 1971; Kingston 1974; Wołowicz 1987; Wołowicz 1991). LV seemed to reproduce later than GD. It has been reported that even populations experiencing similar thermal regimes (like GD and LV) may exhibit temporal discrepancies in the reproductive cycle as a result of variations in food availability (Newell *et al.* 1982; Navarro *et al.* 1989). In the southern population (BL) it was hard to define one spawning period, because cockles seemed to reproduce all over the year. Higher water temperatures in southern areas provoke prolonged spawning of bivalves, when compared to boreal areas (Pieters *et al.* 1980). Mediterranean populations of *C. glaucum* were previously reported to spawn several times a year (Ivell 1979). Spawning of *C. glaucum* depends on water temperature and it starts at 15-17°C, so the more northerly the locality inhabited by a populations, the later spawning begins (Kingston 1974; Ivell 1979; Wołowicz 1991; Brock and Wołowicz

1994). Temperature and food availability have been proven to be critical for spawning in *M. balthica*, *C. edule*, *M. edulis* (Honkoop and Van der Meer 1998) and also *C. glaucum* (Kingston 1974).

7.4 Gross biochemical composition

Energy input in excess of metabolic requirements can be utilized for somatic growth and/or gamete production. Energy allocation to reproduction differs with age and size, as young bivalves grow rapidly and convert little or no energy into reproduction (Kautsky 1982). Changes in biochemical composition were proven to be correlated with a reproductive cycle in (adult) bivalves, like for example Pelecypoda (Sastry 1979), *M. edulis* (Pazikowska and Szaniawska 1988), *Pecten maximus* (Pazos *et al.* 1997) or *Perna perna* (Bawazir 2000). Bayne (1976) divided bivalves into two groups. In "conservative" species, like *M. trossulus* (Gabbott and Bayne 1973; Bawazir 2000) gametogenesis occurs in autumn-winter at the expense of nutrient stores accumulated during the previous summer, resulting in an inverse relationship between biochemical components level (mainly carbohydrates) and reproductive condition. In "opportunistic" species, like *P. perna* (Bawazir 2000), *Ostrea edulis* (Ruiz *et al.* 1992) or *C. glaucum*, winter is a period of sexual "resting", and gametogenesis starts in spring, when sufficient food to fuel biosynthesis is available. Therefore, gonad development and the accumulation of energy reserves overlap temporally.

Trophic conditions also determine seasonal changes of biochemical components contents. Unfavorable trophic conditions first induce catabolization of carbohydrates, then of lipids and, finally, of structural proteins (Beninger and Lucas 1984). Wołowicz (1991) suggested that in southern populations of *C. glaucum* periods of unfavorable conditions are short, so they result in carbohydrates catabolism, whereas in northern populations long periods of unfavorable environmental conditions lead to lipids decomposition.

The protein and lipid contents in the tissue of *C. glaucum* reported by Wołowicz (1991) were the highest in spring in the northern populations, whereas the differences among seasons in the southern populations were small. These contents increase in female tissues before spawning and then decrease after spawning, since proteins and lipids are the main components of oocytes (Gabbott 1975; Pieters *et al.* 1979; Houlihan 1991). This trend was visible in GD. Proteins are synthesized during gametogenesis, while lipids are

partially moved from somatic tissues to the gonads (Holland 1978; Barber and Blake 1981). In LV, differences among seasons, especially in lipid content, were not distinct. There was no spring increase in lipids content despite gametes development. This may have been due to the pooling of the sexes for the biochemical composition analyses, since no clear relationship between lipid levels and reproduction should be expected in males (Navarro *et al*. 1989). Although there was no clear spawning season in BL, protein and lipid contents decreased between spring and summer, which could have been due to unfavorable trophic conditions, as lipids also serve as an energy reserve during periods of nutritional stress (Beukema and De Bruin 1977; Beninger and Lucas 1984). In many bivalves lipids are stored mainly in the gonads (e.g. *M. edulis*, Zandee *et al.* 1980; *M. balthica*, Wenne and Styczyńska-Jurewicz 1987). Lipids can also be stored in the digestive glands as surplus energy to be transferred to the gonads when needed (*Chlamys opercularis*, Taylor and Venn 1979). High lipid content in *M. balthica*, found not only in reproductive organs, but also in other tissues, was claimed to be related to lipid-rich food source (mainly diatoms) (Wenne and Styczyńska-Jurewicz 1987).

The lowest carbohydrate and glycogen contents were noted in GD in spring, because glycogen is used for gonad development. In females it is converted into lipids in the oocytes (Gabbott 1975). The increase in carbohydrates content in northern populations (GD, LV) between summer and autumn seemed to be correlated with phytoplankton blooms (Wasmund *et al.* 1998). In BL differences among seasons in carbohydrate content were small and they might have been due to changes in trophic conditions (Wołowicz 1991). In LV, the seasonal differences were less marked, which might have also been influenced by the fact that the data from both sexes were pooled and not divided into age groups (Newell and Bayne 1980; Navarro *et al.* 1989; Wołowicz *et al.* 2006).

The protein, lipid and carbohydrate contents did not sum up to 100%, because the other components were: ash, nucleic acids, free amino acids, which comprised around 7-12% of the dry weight (Pieters *et al.* 1980) and the remaining water comprising 2-3% of the dry weight (Beukema and De Bruin 1979). In *P. perna* and *M. trossulus* bivalves, the protein, lipid and carbohydrate contents made up about 60-75% (Bawazir 2000).

7.5 Respiration

Oxygen consumption reflects metabolic activity of an organism and it is proportional to its instantaneous ATP demand. The synthesized ATP is utilized in various processes, including ion pump activity, muscular activity, neural activity, growth, gametogenesis or catabolite excretion (Bayne and Newell 1983; Clarke 1991). Metabolic activity is influenced by ambient temperature (Clarke 1991). Reduced oxygen uptake may indicate stress and reduced feeding activity (Akberali and Trueman 1985; Riisgård *et al.* 2003). Some mollusks are able to use anaerobic pathways as an energy source, when they are exposed to stress (Liu *et al.* 1990; Oeschger 1990), which may lead to wrong conclusions on energy metabolism based on respiration rate measurements. It was found that oxygen consumption increases with bivalve size according to the power law, $R = aW^b$ (where R is oxygen uptake, W is the body weight, a is the intercept, and b is the slope). Metabolic rates rise more slowly than body size, because $b < 1$ (Dame 1996). Therefore, because of significant differences in dry tissue weights among populations ($p < 0.001$), the current results could serve better for comparisons within populations among seasons than among populations.

Apart from GD, where respiration rates were higher than in other populations, oxygen uptake rates were within the same range as those reported for *C. glaucum* (Wilson and Elkaim 1997) and other bivalves (McMahon and Wilson 1981; Wilson and Davis 1984; Wilson 1990; Wilson and Elkaim 1991; Hummel *et al.* 2000b). The exceptionally high respiration rates in GD cockles, which to our knowledge, have not been reported before in the Baltic Sea invertebrates, could be due to elevated metabolic levels, because of the energetic costs of osmotic adjustment to low water salinity (Engel *et al.* 1975; Newell 1979). In spring, the metabolic levels and oxygen consumptions of GD and LV were high, and cockles were sensitive to temperature increase as they needed additional energy for gonad development. The respiration rate can increase by about 30-80% in reproducing cockles (*C. edule*) (Iglesias and Navarro 1991) and by more than 50% in mussels (*M. edulis*) (De Vooys 1976). Metabolic rates decreased in both GD and LV in summer, by which time many individuals had probably already spawned. The autumn metabolic rates were high as well in GD and LV, because of phytoplankton blooms and increased feeding activity (Wołowicz 1991). In winter, the respiration rates were low. The metabolic rate of another cockle species, *C. edule* from England, measured as the rate of oxygen consumption, was the lowest in winter (December to March; Newell and Bayne 1980).

Respiration rates in BL population, which experiences high temperatures during summer, were higher in winter than in summer (except at 24°C). This was also noted for *C. glaucum* from the south-west coast of France (Arcachon) and was interpreted as metabolic down-regulation aimed at saving energy (Wilson and Elkaim 1997). This occurs in some marine ectotherms at high water temperatures during summer (Calow 1975; Jansen *et al.* 2007). On the other hand, the respiration rate in BL at 24°C (which is close to the ambient summer temperature in this lagoon) was the highest in summer, so the current data do not demonstrate metabolic down-regulation in *C. glaucum*.

8 Conclusions

C. glaucum is an eurytopic lagoon specialist adapted to extreme environmental conditions, like the low salinity of the Baltic Sea, or the extremely high summer temperatures in Mediterranean Sea lagoons, like those of the Berre Lagoon. Changing environmental conditions in the Berre Lagoon lead to rapid fluctuations of dominant macrobenthic species and often to their extinctions. However, *C. glaucum* seems to form the stable element of communities in this lagoon (Stora *et al.* 1995; Stora *et al.* 2004), which also demonstrates the resistance of this species to extreme and unstable environmental conditions. There are substantial physiological differences among *C. glaucum* populations (Wołowicz 1987; Wołowicz 1991; Wilson and Elkaim 1997) owing to individual acclimatization or genetically fixed adaptations (Nevo 1978; Brock and Wołowicz 1994). Lagoon isolation impedes gene flow and has been proven to facilitate genetic drifts as well as local adaptations (Porter *et al.* 2001; Pearson 2003). A high level of genetic structuring was found in European populations of *C. glaucum* (Hummel *et al.* 1994; Mariani *et al.* 2002; Nikula and Väinölä 2003), so local adaptations are to be expected.

Chapter II: Phylogeography of *Cerastoderma glaucum*

9 Theoretical background

9.1 Factors influencing genetic structures in marine species

9.1.1 Role of glaciations in genetic structures of marine species

Genetic studies of widely distributed marine species help to build general historical scenarios for regional biotas (Patarnello *et al.* 2007). European seas offer an interesting environment to study the effects of Pleistocene glaciations on the genetic structures of marine species (Quesada *et al.* 1995; Pannacciulli *et al.* 1997; Luttikhuizen *et al.* 2003a). Shallow marine habitats in northern parts of the European coastline are young due to the effect of continental ice and lowering of the sea level during the glacial phase, and were postglacially (re)colonized (Dawson 1992). In contrast, the Mediterranean Sea and southern Atlantic coasts were less severely altered by the cold climatic phases, and potentially served as refugia for species that retreated from the northern areas. So far, European marine phylogeography points to the existence of several Pleistocene glacial refugia (Coyer *et al.* 2003; Jolly *et al.* 2005; Provan *et al.* 2005; Hoarau *et al.* 2007) (Fig. 9.1), and some general postglacial recolonization patterns of the northern European marine regions are emerging (reviewed in Patarnello *et al.* 2007 and Maggs *et al.* 2008).

Figure 9.1. The area covered by ice at any time during four periods of glaciations is shown in white (Encyclopedia Britannica 2009). Areas appearing to have been important Pleistocene refugia (marked with grey; encircled, but exact locations are largely unknown): 1-Hurd Deep in the English Channel, 2- Southwest Irish coast, 3- Iberian Peninsula, 4- Western Mediterranean, 5- Eastern Mediterranean. Persistence of marine species in the Black Sea (6) during the last glacial maximum is controversial (adapted from Nikula 2008).

9.1.2 Dispersal modes and barriers in marine species; the case of C. glaucum

Apart from the past climatic conditions, genetic structures are also shaped by a present gene flow. A present gene flow among benthic organisms with planktonic larval stage strongly depends on the duration of the planktonic larval stage, which differs among species influencing their dispersal capacity. It is of around 5 weeks in *C. edule* and of only around 1 week in *C. glaucum*, which was considered an adaptation aimed at retaining larvae in a natal lagoon (Pearson 2003). However, those durations may differ within the species depending on population's location (Boyden 1971; Barnes 1980). The study of swimming behavior performed on *C. edule* suggests that larvae may actively control their dispersal (Jonsson *et al.* 1991). However, Teske *et al.* (2007) found little effect of planktonic larval performance on connectivity among invertebrate populations.

Understanding pelagic larval transport and settlement variations for benthic species is crucial to the further understanding of migration and gene flow among populations (see Gaines and Bertness 1992; Grosberg and Levitan 1992; Benzie and Williams 1997; Jessopp and McAllen 2007).

Regardless of the duration of larval stage and larval performance, various geographic and hydrological barriers can impede the dispersal. Even species with planktonic dispersal retain often significant spatial population structure and effective long-distance larval dispersal is rare (Barber *et al.* 2002; Gilg and Hilbish 2003; Taylor and Hellberg 2003; Veliz *et al.* 2006; Viard *et al.* 2006; Luttikhuizen *et al.* 2008). Colonizing new areas might also be impeded by other abiotic constraints like temperature, salinity, type of sediment, quantity of light or pH. All these factors might form a barrier, when they are beyond the tolerance limit of the species (Palumbi 1994). Sea currents which are the major means of passive transport for species with planktonic stages, influencing the dispersal direction, may also form a barrier against dispersal, because of physical conditions gradient or simply the force of water movement (Gaylord and Gaines 2000). Distribution of species not only depends on abiotic factors, but also on interactions with other species (Davis *et al.* 1998).

In the case of *C. glaucum*, which often inhabits closed or semi-closed lagoons, ponds or salt water lakes, a long-distance dispersal through planktonic larvae seems very unlikely (Wołowicz 1991). Apparently only in the Baltic Sea *C. glaucum* is present along the whole coast and the rest of the distribution area is discontinuous. The colonization of the northern part the distribution area by planktonic larvae would have been impeded by strong waves and tides on the coast of the Atlantic Ocean and the North Sea. Moreover, the short duration of a larval stage (Barnes 1980) does not facilitate dispersal.

A widely discussed dispersal vector of marine organisms is ships' ballast water, which caused numerous invasions of alien species sometimes completely changing the composition of species and the functioning of ecosystems (Carlton and Geller 1993; Carlton 1996). In the case of *C. glaucum*, which is a benthic species this dispersal mode may be possible only in case of larvae, not adult individuals. However the most typical habitats of *C. glaucum* are often excluded from ships' circulation as they have little connection with an open sea or ocean. Moreover, water has been used as ships' ballast only since around 1900 (Fofonoff *et al*. 2003), so it might be considered as a possible factor influencing a present gene flow. Solid ballasts, which were applied before could play a role in dispersal of benthic species, but not a lagoon cockle, which requires a sandy bottom.

C. glaucum has only a marginal commercial value (Arjonilla *et al.* 1994). Therefore, it is not dispersed by man for aquaculture purposes. However, it may be transported accidentally with aquacultured species or as a result of other anthropogenic activities.

After considering all the above-mentioned dispersal options accessible to *C. glaucum*, the one which should be seriously taken into consideration is a long-distance dispersal by birds. Dispersing eggs, larvae or adult animals by birds is a common phenomenon for many invertebrates. That fact was already noted by Darwin (1859, 1878) who drew attention to mollusks, which can be transported by birds externally on feet or plumage or attached to plants. Propagules of invertebrate animals can also be transported internally, surviving passage through a digestive tube to be defecated even after 26 h in a viable condition (Brown 1933). The dispersal distances through endozoochory may exceed 1000 km (Green and Figuerola 2005), as a duck flies at 60-78 km h^{-1} (Welham 1994). It has been proven that some snails and their eggs can survive passage through the digestive tube of duck, but generally for mollusks the external transport seems to be more common (Wesselingh *et al.* 1999).

Invertebrate eggs are transported mainly attached to birds' feet (Figuerola and Green 2002) rather than on plumage. Adult bivalves can be transported on feet as well (Darwin 1878). There were some cases when *Cerastoderma edule*, a species closely resembling *C. glaucum* morphologically and ecologically, was found attached to birds' feet (sanderling *Calidris alba*, dunlin *Calidris alpine* and curlew sandpiper *Calidris ferruginea*) during the handling of 17000 birds in the Odiel Marshes in Spain (Fig. 9.2). During the same handling about 3% of waders had amputated digits probably as an effect of bivalves attached to feet (JM Sayago, communicated in Green and Figuerola 2005). Populations of *C. glaucum* are often found in ornithological reserves or in their vicinity. Many birds' species perform regular migrations between the southern and the northern parts of Europe. They rest and feed in the lagoons and estuaries like Camargue in the south of France, which is inhabited by *C. glaucum* (Wołowicz 1991).

One of the differences between *C. edule* and *C. glaucum* is that the latter normally inhabits non-tidal areas, which leads to the conclusion, that it cannot survive external transport on birds, because of an aerial exposure. However, past experiments showed that during aerial exposure the LT_{50} (lethal time for 50% of the individuals) was of 220 h in 10°C and of 185 h in 15°C. This species can survive the loss of 22% of water from the

tissues (Boyden 1972). If the cockle is transported together with plants they are moist, which is favorable for the cockle and prevents the temperature growth (Wołowicz 1991).

Also, some man-made basins, inaccessible for larval dispersal, were rapidly colonized by *C. glaucum*, providing circumstantial evidence for effective bird-mediated dispersal (Wołowicz 1991).

Bird-mediated long-distance dispersal and colonization have not been much addressed in the marine phylogeographic literature (but see Muñoz *et al.* 2008), and only begin to be appreciated for freshwater organisms (De Gelas and De Meester 2005; Figuerola *et al.* 2005).

Figure 9.2. Common cockle *Cerastoderma edule* attached to a dunlin *Calidris alpina* mist-netted in the Odiel Marshes, Spain. Photo by J. M. Sayago (from Green and Figuerola 2005).

9.2 Different properties of genetic markers applied

Mitochondrial DNA is a small, circular molecule (14-39 Kb). For the majority of animals it is composed of the following regions which evolve at different rates: control region, 2 genes coding for rRNA, 22 genes coding for tRNA and 13 coding for proteins (subunits I, II and III of the cytochrome oxidase, subunits 6 and 8 of the ATPase,

cytochrome b and 7 subunits NADH reductase system) (Wolstenholme 1992). Mitochondrial genome is haploid and the transmission is uniparental, which provokes the absence of recombination. However, biparental transmission has been proven for some bivalves, like *Mytilus* sp. (Zouros *et al.* 1992, 1994) as well as for mice (Gyllensten *et al.* 1991). The population effective size of mitochondrial genes is four times smaller than this of nuclear loci. Therefore, on average four times more nuclear genomes than mitochondrial genomes are transferred by a migrating individual (Mills and Allendorf 1996). There is not much data concerning the evolution rate of molluskan mtDNA, but in mammals there is about 0.5 to 1% of nucleotidic substitutions in million years, which is 2 to 10 times more than in nuclear genome (Wolstenholme 1992). That is why mtDNA is a very useful marker for phylogenic and phylogeographic studies (Crozier 1990) especially on the intraspecific level (Kolbe *et al*. 2004; Voisin *et al.* 2005). In order to understand phylogeographic relationships among populations or species, mtDNA sequences polymorphism is studied (Fig. 9.3). It is performed by analyzing haplotypes, which are unique combinations of genetic markers present in a chromosome (Hartl and Clark 1997). They differ from one another by one or more nucleotides because of substitutions, insertions or deletions.

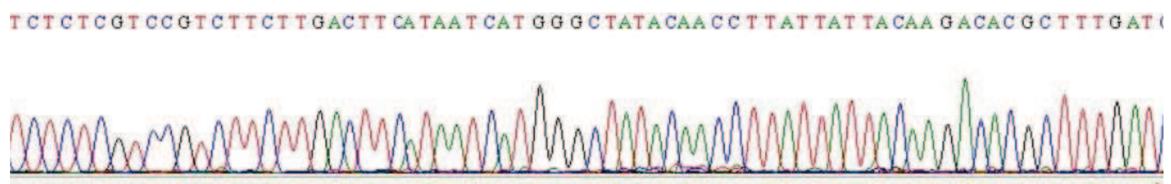

Figure 9.3. The fragment of a mitochondrial DNA sequencing chromatogram (*C. glaucum*- present study).

Microsatellites are nuclear markers composed of repeated nucleotides, each between 1 and 10 base pairs (bp) in length e.g. $(CA)_n$ (Tautz 1989). There are different kinds of motifs: pure motifs (e.g. CACACACACACACACACACA), compound motifs (e.g. CACACACACAGAGAGAGAGA) and interrupted motifs (e.g. CACATTCACACATTCATTCA). The pure motifs are said to evolve more rapidly than other types of motifs (Jarne and Lagoda 1996). Microsatellites are highly polymorphic owing to a high mutation rate (Hancock 1998). The mutation rate is said to vary between 5×10^{-5} and 5×10^{-3} per base per generation for microsatellites while the average mutation rate for the rest of the genome is of 10^{-9} (Estoup and Angers 1998). The main cause of the

hypervariability of microsatellites are mutational events resulting from polymerase slippage while replication, which may increase or diminish the number of repetitions of the base motif (Levinson and Gutman 1987; Schlötterer and Tautz 1992). Microsatellites are biparentally inherited and codominant. It is generally believed that they are neutral, which makes them a good marker for studying genetic structures, the patterns of historical gene flow and demography (Jarne and Lagoda 1996). Generally they are present in non-coding regions (introns), but they have also been found in exons (Hancock 1995). It has been shown that the overall frequency of microsatellites varies widely across genomes (Lagercrantz *et al.* 1993). In bivalves they are proven to be overrepresented in introns (245 loci Mb^{-1}) compared to their frequency in exons (85 loci Mb^{-1}) (Cruz *et al.* 2005). It is not known whether microsatellites have a functional significance (Hancock 1998). It has been suggested that they play a role in gene regulation or recombination (Stallings *et al.* 1991) and they seem to be common in upstream promoter regions of coding sequences giving more evidence for their functional role in coding processes (Kashi and Soller 1998). Most typically phylogeographic studies including microsatellites consist of investigating the differences in the number of repeats among alleles (Fig. 9.4 and 9.5). In this case, mutations of the nucleotidic sequence, which do not result in a sequence length change, are not detected. The main problem which needs to be taken into account while analyzing the length polymorphism of microsatellite alleles is a size homoplasy. It occurs when two alleles have the same length, but do not share a common ancestral sequence. The homoplasy increases as the mutation rate and the time of divergence increase. Microsatellite loci have a finite size, so there could be selection acting to limit allelic repeats length (Estoup and Angers 1998). It results in the reduction of allelic states allowed and at the same time increases the chance of homoplasy (Nauta and Weissing 1996). Another problem is that microsatellite loci must be developed on a species-specific basis (although cross-species amplification is sometimes possible), which requires considerable time and expense (Zane *et al.* 2002).

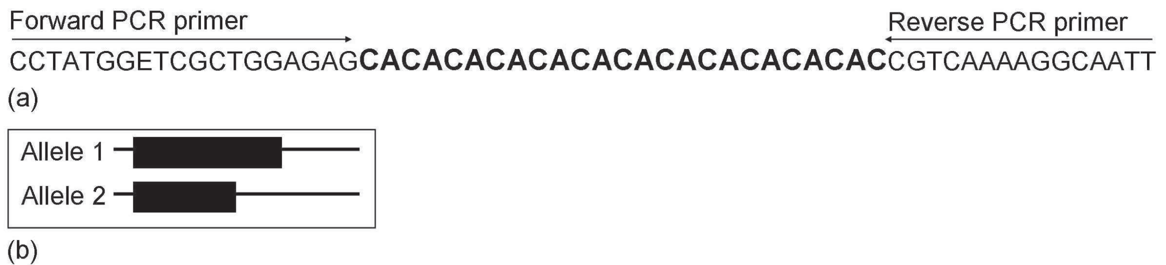

Figure 9.4. Principles of investigating microsatellites: **a)** microsatellite amplification; microsatellite is shown in boldface, **b)** example from a diploid individual that is heterozygote; the number of repeats varies among alleles and among individuals.

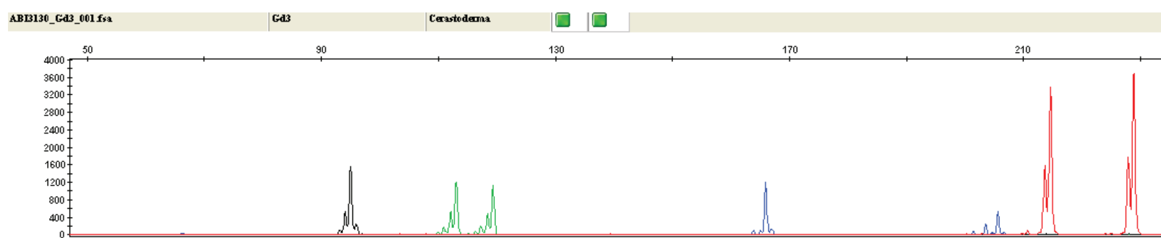

Figure 9.5. Sequencer display of microsatellite alleles of different lengths. Four microsatellites were multiplexed and forward primers were tagged with different dyes (*C. glaucum*- present study).

Demographic events may lead to different effects on mitochondrial and nuclear DNA, because those two genomes are influenced by evolutionary forces in different ways (Féral 2002; Chan and Levin 2005; Chenuil 2006). Combining data from both markers may enable to reveal some phenomena which could not be revealed if only one marker was applied, like differences in migration between both sexes or the processes of introgression on the maternal lineage. The high mutation rate of microsatellites is advantageous for microscale studies of populations, but on the larger scale the results might be influenced by size homoplasy. Studying only hypervariable loci may lead to underestimating a real genetic structure existing among populations (Balloux *et al.* 2000; Estoup *et al.* 2002; Diaz-Almela *et al.* 2004). Therefore, it is worth applying different markers with different mutation rates in one study.

9.3 Theoretical context of methods applied to analyze genetic data

9.3.1 Genetic diversity within populations

Two main types of measurements of genetic diversity within populations can be distinguished: measurements of haplotype polymorphism (number of observed haplotypes, N_H and haplotype diversity, H_d) and measurements of nucleotide polymorphism (number of polymorphic sites, S and nucleotide diversity, Π).

Haplotype diversity (H_d) is calculated with the following equation:

$$H_d = \frac{n}{n-1}\left(1 - \sum_{i=1}^{N_H} p_i^{\,2}\right)$$

where n is number of genes in the sample (in case of a haploid locus equal to the number of individuals) and p_i is a frequency of haplotype i. Haplotype diversity (H_d) defines the probability that two random sequences drew from the sample will be identical (Nei 1987).

Nucleotide diversity (Π) is calculated with the following equation (Tajima 1983):

$$\Pi = \sum_{i=2}^{N_H} \sum_{j<i} p_i p_j \delta_{ij}$$

where p_i and p_j are the respective frequencies of the ith and jth haplotypes, δ_{ij} is the fraction of nucleotide differences between sequence i and j (the number of nucleotide differences between sequence i and j divided by the total number of base pairs of the nucleotide sequence). Therefore, nucleotide diversity measures an average nucleotide divergence among all the pairs of sequences from the sample. It also is the probability that two random sequences of the sample are different at a given nucleotide site.

9.3.2 F-*statistics*

Hardy-Weinberg equilibrium is one of the basic statements of population genetics. A theoretical population of an infinite size remains in Hardy-Weinberg equilibrium, which means that both allele and genotype frequencies remain constant or are in equilibrium from generation to generation, when following conditions are met: panmixia (random mating of individuals), no mutation, no migration, no selection and generations do not overlap (Hartl and Clark 1997). However, those conditions are rarely met and the departures from Hardy-Weinberg equilibrium are often in natural populations. One of the main effects of these departures on genetic diversity is the reduction in observed heterozygosity compared with

expected heterozygosity. The extent of reduction in observed heterozygosity can be used to quantify the level of genetic differentiation between the subpopulations. This quantification has been formalized by Wright (1951) in a series of hierarchical *F*-statistics.

The expected heterozygosity (H_e), also called gene diversity, is the chance that two randomly chosen alleles from the population will be different. This can be expressed by the equation:

$$H_e = 1 - \sum p_i^2$$

where p_i is the frequency of the *i*th allele. Values of H_e range from 0 to 1. H_e is independent of a sample size.

To make the connection between heterozygosity and *F*-statistics, the following terms need to be defined:

H_I- mean observed heterozygosity per individual within subpopulations,

H_S- mean expected heterozygosity within random mating subpopulations,

H_T- expected heterozygosity in random mating total population.

F_{ST}- fixation index- the mean reduction in heterozygosity of a subpopulation relative to the total population; 0 (no differentiation) to 1 (complete differentiation):

$$F_{ST} = \frac{H_T - H_S}{H_T}$$

F_{IS}- inbreeding coefficient- the mean reduction in heterozygosity within a subpopulation; can range from –1 (all individuals are heterozygotes) to +1 (no heterozygotes observed):

$$F_{IS} = \frac{H_S - H_I}{H_S}$$

F_{IT}- overall fixation index- the mean reduction in heterozygosity of an individual relative to the total population:

$$F_{IT} = \frac{H_T - H_I}{H_T}$$

These indices are linked by the following relationship:

$$(1 - F_{IT}) = (1 - F_{IS})(1 - F_{ST})$$

Various authors developed modifications of the way in which F_{ST} can be conceptualized. An example is an ANOVA-like approach to partitioning genetic variance into within- and among-subpopulation components (Weir and Cockerham 1984). Three new indices analogous to F_{ST}, F_{IS}, F_{IT} were defined (after Simon-Bouhet 2006):

θ measuring the amount of differentiation among populations

f estimating the amount of inbreeding within populations

F measuring overall inbreeding in the set of observed populations

It assumes the model with subpopulations of equal sizes, derived from the ancestral population in the Hardy-Weinberg equilibrium and in linkage equilibrium. It is assumed that populations could have derived from one another only as a result of a genetic drift or sampling drift. In case of two alleles locus, θ, f and F estimators are expressed as follows:

$$1-\hat{f}=\frac{b}{b+c}$$

$$1-\hat{F}=\frac{c}{a+b+c}$$

$$\theta=\frac{a}{a+b+c}$$

where a is a component of among subpopulations variance, b is a component of within subpopulations variance and c is a component of within individuals variance. Using θ is advised to summarize the distribution of variation within and among populations when the sampled populations are used to represent the characteristics of a larger set of populations from which they were drawn (random-effect sampling), as it allows unequal sample sizes.

In case of microsatellite data various methods and modifications for estimating genetic structuring have been proposed like Slatkin's R_{ST} (1995), unbiased R_{ST} (UR_{ST}; Goodman 1997) and Wright's F_{ST} (1965) (Valsecchi *et al.* 1997; Gaggiotti *et al.* 1999; Balloux and Lugon-Moulin 2002). The mode of mutation for any given microsatellite locus is generally unknown and as a result using R_{ST} (based on stepwise mutation model) as an estimator may discomfort the level of differentiation between populations (Estoup and Cornuet 1999). Using F_{ST} eliminates this difficulty. It was suggested that F_{ST} performs better as an estimator (than those based on stepwise mutation model) where imperfect microsatellites are included (Carmichael *et al.* 2001) and when population sizes and

number of loci are low (Gaggiotti *et al.* 1999), which is the case of the data analyzed in this study.

Definitions of F_{ST}, which are based on heterozygosity, are not valuable for haploid loci, like mitochondrial DNA sequences. There are a few ways of reconceptualizing F_{ST} for haploid loci like γ_{ST} proposed by Nei (1982), G_{ST} of Takahata and Palumbi (1985) or Φ_{ST} of Excoffier *et al.* (1992). Although each of these statistics for nucleotide data is calculated slightly differently, in reality they are all trying to estimate the same parameter- the proportion of nucleotide diversity (Π) among subpopulations, relative to the total.

A common definition given by Hudson *et al.* (1992) is:

$$F_{ST} = \frac{\prod_{Between} - \prod_{Within}}{\prod_{Between}}$$

where $\Pi_{Between}$ and Π_{Within} represent the average number of pairwise differences between two individuals sampled from different ($\Pi_{Between}$) or the same (Π_{Within}) population. The average pairwise difference within a population can be calculated as the sum of pairwise differences divided by the number of pairs. Using this definition of F_{ST}, the value Π_{Within} should be computed for each population and then averaged. Otherwise, random sampling of pairs within populations put all the weight on the population with the largest sample size.

9.3.3 AMOVA

Analysis of Molecular Variance *(*AMOVA) is a hierarchical analysis, which partitions the total variance in the structure into samples groups. The total covariance component (σ^2) is divided into three components: the component due to intra-populations differences (σ_c^2), due to inter-populations differences within the group (σ_b^2), and inter-groups differences (σ_a^2) (Excoffier *et al*. 1992). These three components are used to define three hierarchical levels of the variation source: intra-population (σ_c^2), inter-population within groups (the function of $\sigma_b^2 + \sigma_c^2$), between groups (the function of $\sigma_a^2 + \sigma_b^2 + \sigma_c^2$). The significance of components associated with different levels of genetic structuring is tested using the procedure of non-parametric permutations.

9.3.4 Multidimensional scaling (MDS)

Multidimensional scaling (MDS) (Kruskal and Wish 1978) serves for analyzing a proximity matrix based on a set of observations in order to represent them as accurately as possible in a limited number of dimensions (usually 2). The disparities are the distances that describe the optimal representation for the observations. This method can be applied to represent distances among populations or pairwise populations F_{ST} without imposing a dichotomic hierarchical clustering scheme of classical phylogenetic trees algorithms (see: 9.3.9 Trees and networks).

Nonmetric MDS is applied when the representation function simply respects the relative order of the observations. Metric MDS is applied when the dissimilarities are transformed into disparities using a specific parametric function.

The difference between the disparities and the distances measured on the representation resulting from the MDS is called the stress: the lower the stress, the better the representation of the observations. The Shepard diagram plotting representation distances against dissimilarities is an empirical, but reliable method, which allows observing some ruptures in the ordination of the values. The more the chart looks linear, the better the representation.

9.3.5 Mantel test

The Mantel test (Mantel 1967; Smouse *et al.* 1986) is aimed at testing the significance of correlation between two matrices. It might be used for example to test the significance of the correlation between the matrices of genetic and geographic distances or between two genetic distances matrices (e.g. distances between the same population pairs obtained using different genetic markers).

When the matrix is symmetrical and there are n objects, the matrix contains $n(n - 1)/2$ distances. Because distances are not independent of each other, since changing the position of one object would change $n - 1$ of these distances, the relationship between the two matrices cannot be assessed by simply evaluating the correlation coefficient between the two sets of distances and testing its statistical significance. The correlation between the two sets of $n(n - 1)/2$ distances is calculated. In order to assess significance of any apparent departure from a zero correlation, the rows and columns of one of the matrices are subjected to random permutations many times, with the correlation being recalculated after

each permutation. The significance of the observed correlation is the proportion of such permutations that lead to a higher correlation coefficient. In addition to overcoming the problems arising from the statistical dependence of elements within each of the two matrices, use of the permutation test means that no reliance is being placed on assumptions about the statistical distributions of elements in the matrices.

9.3.6 Detecting inbreeding

Inbreeding is the result of mating between genetically related individuals. There are two variations. In a very small random mating population it is called panmictic inbreeding. In a large non-random mating population, in which assortative mating occurs it is called partial inbreeding (Charlesworth and Charlesworth 1987). Partial inbreeding can be detected by comparing the proportion of heterozygotes observed and expected under Hardy-Weinberg equilibrium. An inbreeding coefficient F_{IS} can be calculated. It is the probability that two alleles at a locus within a single individual are identical by descent (i.e. correlation of allele within an individual). Observed heterozygote deficiency expressed as positive F_{IS} may be due to many reasons. In case of microsatellites, where the differences in the number of repeats among alleles and individuals are studied, it may be provoked by scoring errors due to large allele drop-out (Wattier *et al*. 1998), due to stuttering (Ewen *et al*. 2000) or due to the presence of null alleles. Null alleles are the alleles that fail to amplify in a PCR, either because the PCR conditions are not ideal or the primer-binding region contains mutations that inhibit binding. As a result, some heterozygotes are genotyped as homozygotes and a few individuals may fail to amplify any alleles (Selkoe and Toonen 2006). The biological factors provoking heterozygote deficiencies are: (1) inbreeding, which causes high proportion of homozygotes across all loci, (2) the Wahlund effect, i.e. the union of two or more populations genetically differentiated in the sample (Castric *et al.* 2002) or (3) selection against heterozygotes.

The method applied in RMES (Robust Multilocus Estimation of Selfing) program (David *et al.* 2007) is based on controlling the distribution of multilocus heterozygosity in population samples and allows specifying the cause of observed heterozygote deficiency. It distinguishes between Hardy-Weinberg disequilibrium due to the presence of null alleles, and the one caused by phenomena supposed to affect all loci similarly, like reproduction among relatives (Castric *et al.* 2002). The selfing rate (s), which in gonochoric species

should be interpreted as inbreeding rate, is deduced from an estimator of the two-locus heterozygosity disequilibrium over all pairs of loci ($\hat{g}_2$) under the assumption of inbreeding and linkage equilibrium. *P*-value for the null hypothesis $s = \hat{g}_2 = 0$ is obtained by resampling genotypes, drawing monolocus genotypes independently.

9.3.7 Neutrality tests

The neutral models provide predictions about the molecular evolution under mutation-drift equilibrium and in the absence of systematic effects, such as natural selection or demographic effects (change of effective size). Tests aimed at detecting natural selection are based on the infinite sites mutation model (Kimura 1969), according to which every mutation is assumed to be independent, as it is in a new nucleotide site each time, so that homoplasy and reverse mutation do not occur.

In population genetics the θ parameter is central. In the neutral model, it is defined as:

$$\theta = 4\mathrm{N}_e\mu$$

where N_e is an effective population size and μ is a mutation rate of a studied locus per generation.

Tajima's (1989) neutrality test compares two estimators of the mutation parameter theta (θ). Tajima's *D* expresses the difference between the estimator of theta (θ) based on the number of polymorphic sites in the sample *E(S)* and the estimator based on the number of nucleotide differences between a pair of sequences *E(Π)*.

$$E(\Pi) = \theta$$

and

$$E(S) = \theta \sum_{i}^{n-1} \frac{1}{i}$$

where *n* is a number of haplotypes in the sample, *S* is a number of polymorphic sites and *Π* is a nucleotide diversity. Two estimators of θ may be obtained from sequence polymorphism:

$$\hat{\theta}_\pi = \hat{\Pi}$$

and

$$\hat{\theta}_S = \frac{S}{\sum_i^{n-1} \frac{1}{i}}$$

In the absence of selection and in a mutation-drift equilibrium $\hat{\theta}_\Pi$ and $\hat{\theta}_S$ are identical. However, those two estimators react differently to selection and the change of population size. Therefore, the equation:

$$\hat{D} = \hat{\theta}_\Pi - \hat{\theta}_S$$

is applied to check whether the population is in mutation-drift equilibrium. $\hat{D} = 0$ where the population is in the mutation-drift equilibrium. $\hat{D} < 0$ suggests that the population is in a demographic expansion or there was a positive selection for a favorable allele. In a demographic expansion mutations acting on studied loci have little chance to be lost and increase rapidly $\hat{\theta}_S$. At the same time the new haplotypes, which are numerous, are close to other haplotypes, so $\hat{\theta}_\Pi$ will increase less rapidly. In the extreme case of positive selection one haplotype may replace all others and the observed variability is only constituted by new haplotypes, deriving from the selected one, and very close to it. $\hat{D} > 0$ suggests a Wahlund effect (a mixture of differentiated populations) or a past demographic reduction. A difference between a selective effect and a change of population size is that the latter has the same effect on $\hat{D}$ on all the loci.

Neutrality test applying D^* and F^* indices of Fu and Li (1993) is based on the assumption that according to the distribution of mutations in the genealogy of a random sample of the gene sequences, ancient mutations in the basic (internal) part of the tree are in general shared by several sequences (=internal mutations) and they differ from recent mutations, situated on the extremities of branches in the recent part (=external mutations). Fu and Li (1993) tests compare the number of internal and external mutations in relation to the one expected in the neutral model. D^* compares the number of singletons η_s and the number of total mutations η. F^* compares the number of singletons η_s with the average number of nucleotide differences between the pair of sequences in a random sample of n sequences from the population. A recent fixation of an advantageous allele in the population will produce an excess of external mutations, because the majority of mutations are supposed to be young. On the other hand, in case of balancing selection, some alleles may be old and there may be an excess of internal mutations and a deficiency of external mutations.

9.3.8 Selection detection- F_{ST}-outlier method

The F_{ST}-outlier method serving for detecting selection is based on the assumption that the level of differentiation experienced at different neutral loci should be the same for all the loci, because of their shared demographic history. Therefore, loci that show unusually low or high levels of genetic differentiation are often subjected to natural selection. For a range of population structures and demographic histories, the distribution of F_{ST} is strongly related to the heterozygosity (polymorphism) at a given locus. Outlying F_{ST} values can be identified in a plot of F_{ST} versus heterozygosity using a null distribution generated by a simple genetic model. The genetic variation at the discrepant locus identified is likely to have been influenced by natural selection, either acting on the locus itself or at a closely linked locus. The F_{ST}-outlier approach is a useful method for eliminating non-neutral outlier loci from data sets before computing most population genetic parameters (e.g. F_{ST}, N_m- the number of migrants, N_e- the effective population size), that require neutral loci. LOSITAN software may be used for detecting selection using the F_{ST}-outlier method (Beaumont and Nichols 1996; Antao *et al.* 2008).

9.3.9 Trees and networks

A typical approach in interspecific phylogeny is constructing trees. Species with similar nucleotide sequences, which could have a common ancestor, are assembled together. However, on the intraspecific level networks should be used instead of trees, because some rules of constructing trees are not valid for the relations among populations of the same species. As intraspecific differentiation is quite recent in the evolutive time scale, ancestral genotypes may coexist with recent genotypes, so multifurcations may appear in trees. Moreover, a given genotype may have several potential origins, which results in creating alternative links and cyclical structures among genotypes (Posada and Crandall 2001).

The construction of neighbor-joining phylogenetic trees (Saitou and Nei 1987) is based on deterministic approach, because they are constructed from matrices of genetic distances among populations. This algorithm is based on the minimum evolution criterion. In practice the topology that results in the shortest total branch length is preferred at each step of the algorithm. However, neighbor-joining may not find the true tree topology with "shortest total branch length" approach, because it is a greedy algorithm that constructs the tree in a step-wise fashion. There are also maximum likelihood phylogenetic trees based on

a probabilistic approach, according to which the most likely tree is the one that maximizes the probability of the given data occurring (Guindon and Gascuel 2003).

The quality of the tree may be estimated with bootstrap method (Efron 1979; Felsenstein 1985). This resampling method samples “columns” (i.e. nucleotide sites) from the alignment and asks whether the sampled data gives the same tree as the one calculated from the full dataset. At the end some columns are not selected, some are selected once, and some are selected several times. The total number of selected columns is as a standard equal to the number of columns in the original dataset. Therefore the bootstrap % on the tree node signifies the % of trees when the sampled data gave the same node as the one calculated from the full dataset.

The analysis of phylogeographic relationships, especially those at the intraspecific level, may result in many different trees based on the same data set. These trees may be represented in the form of one network using the maximum parsimony or median-joining algorithm (Bandelt *et al.* 1999), where various potential evolutive pathways are represented in the form of polytomys.

9.3.10 Structure software

One of the drawbacks of classical *F* or *Φ* statistics is that the populations are defined *a priori*. A set of new statistical methods have been developed to investigate the structure and clustering of individuals relying on molecular data using no *a priori* information on the geographic origin of the individuals included in the simulations (Falush *et al.* 2003).

An individual-based Bayesian approach based on clustering may be taken to address the degree of structuring in the microsatellite multilocus data using STRUCTURE software (Pritchard *et al.* 2000). The STRUCTURE inferences assume that marker loci are unlinked, at linkage equilibrium with one another within clusters and that there is Hardy-Weinberg equilibrium within clusters. The model assumes *K* clusters, each of which is characterized by a set of allele frequencies at each locus. The method attempts to assign individuals to clusters on the basis of their genotypes, while simultaneously estimating clusters allele frequencies (Pritchard *et al.* 2000). Allelic frequencies in the inferred clusters are found with the Markov Chain Monte Carlo (MCMC) method. The number of clusters *K* here is a fixed parameter, so several simulations with different *K* values must be performed. The aim is to choose the best value of *K* by estimating the posterior probability of the data. This posterior probability, marked as *LnP(D)* in STRUCTURE program and as *L(K)* by Evanno *et al.* (2005), is obtained by computing the log likelihood of the data at each step of

Markov Chain Monte Carlo. Then, the average of these values is computed and half their variance is subtracted to the mean, which gives *LnP(D)*. True number of clusters K is often identified using the maximum *LnP(D)* value (Zeisset and Beebee 2001; Vernesi *et al*. 2003). However, Evanno *et al.* (2005) suggested that this approach often leads to overestimating a number of clusters and suggested the approach based on ΔK or the second order rate of change in the log probability of the data *(L(K))* between successive values of K. The statistic ΔK is calculated as the mean (m) of the absolute values of the second order derivative *L"(K)* averaged over several runs with the same K values divided by the standard deviation (s) of *L(K)* over those runs:

$$\Delta K = \frac{m(|L''(K)|)}{s[L(K)]}$$

$$|L''(K)| = |L'(K+1) - L'(K)|$$

$$L'(K) = L(K) - L(K-1)$$

Finally it expands to:

$$\Delta K = \frac{m(|L(K+1) - 2L(K) + L(K-1)|}{s[L(K)]}$$

The peak value of the ΔK has been shown to point the highest hierarchical level of structuring present in a data set (Evanno *et al.* 2005).

9.3.11 Isolation with Migration model

IM software (Nielsen and Wakeley 2001; Hey 2005) applies the Isolation with Migration model using Markov Chain Monte Carlo coalescence simulation method to treat genetic data drawn from a pair of closely related populations or species. The program is designed to jointly estimate likelihoods of a range of demographic parameters for a pair of diverging populations [divergence time, population-wise population mutation parameters ($\theta = 4N_e u$) following and preceding divergence, and directional migration rates] given data on haplotype sequences and frequencies. With six parameters it can capture many of the phenomena that can occur when one population splits into two: the splitting event may

have been long ago or recent, the ancestral and the two descendant populations may differ in size, there may have been gene exchange during the time since population splitting, and this gene exchange may have occurred more in one direction than the other (Fig. 9.6).

The Isolation with Migration model differs sharply from the general family of models in which populations have been exchanging genes for an indefinitely long period of time. The "island model" or the "stepping-stone model" assume that the pattern of variation within and between populations is at equilibrium between the counteracting forces of mutation, genetic drift and gene exchange. In this way the Isolation with Migration model should be more appropriate for the analysis of populations that have recently separated. The IM method assumes that the populations have been constant in size (Nielsen and Wakeley 2001; Hey 2005).

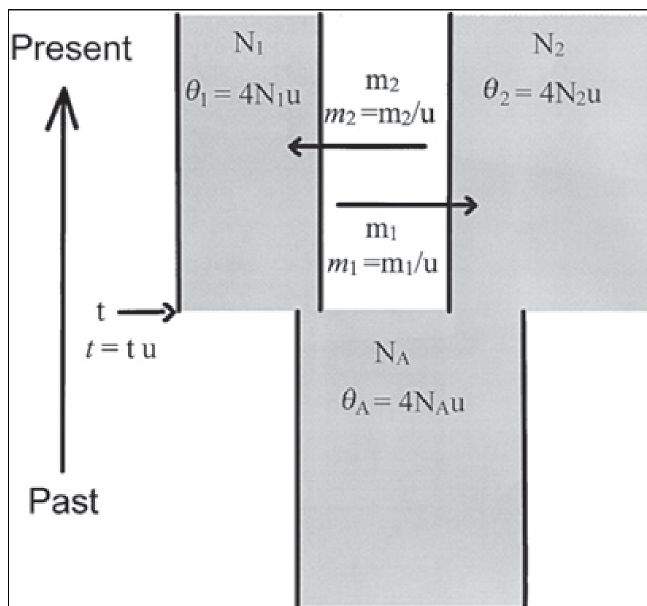

Figure 9.6. The isolation with migration (IM) model is depicted with two parameters sets. The basic demographic parameters are constant effective population sizes (N_1, N_2, and N_A), gene flow rates per gene copy per generation (m_1 and m_2), and the time of population splitting at t generations in the past. The second set of parameters is scaled by the neutral mutation rate μ and used in the model fitting (from Hey and Nielsen 2004).

10 Materials and methods

10.1 Sampling and DNA extractions

Genetic structure of *C. glaucum* was investigated using the combination of nuclear and mitochondrial genetic markers in order to (1) explore the effects a highly fragmented

lagoon habitat on the genetic structure and to (2) compare the genetic patterns in northern, postglacially colonized regions of Europe and in southern regions, where the species has a longer uninterrupted history. Samples of the lagoon cockle were collected from 20 localities from the Baltic Sea, through the Atlantic Ocean and the Mediterranean Sea to the Caspian Sea (Fig. 10.1, Table 10.1). Compared to the previous studies on the genetic structure of this species (Hummel *et al.* 1994; Mariani *et al.* 2002; Nikula and Väinölä 2003), this study uses previously unavailable nuclear markers (microsatellites) and includes previously unsampled regions that proved to be essential in tracing the colonization history.

Samples were stored in 95% ethanol in –80°C. Fragments of a foot tissue (about 1 mm^3) were extracted using the QIAamp DNA Mini Kit according to the manufacturer's (QIAGEN) protocol. The DNA of 22 to 31 individuals per population was extracted, except for the sample from Azerbaijan (BK) which consisted of only 6 individuals. DNA extracts were stored in –20°C. The quality and the quantity of the extracted DNA of each individual were estimated by electrophoresis of 4 µl of each DNA extract on a 2% TAE agarose gel.

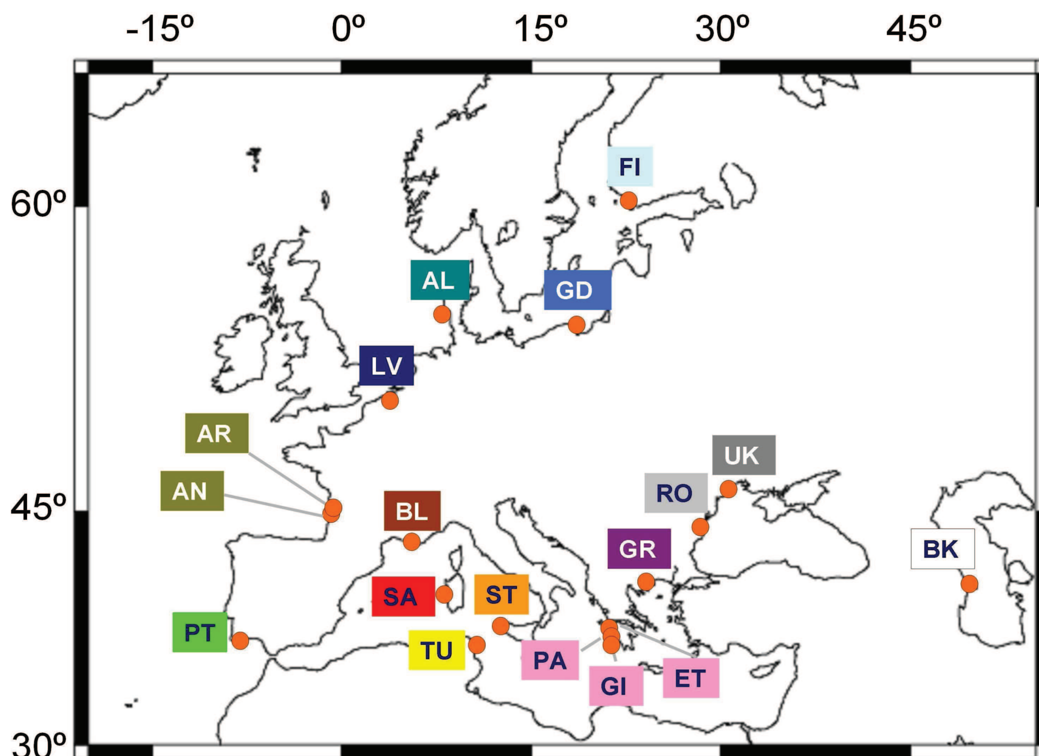

Figure 10.1. Sampling locations for genetic structure analysis of *C. glaucum*. In case of the sample from Sicily (ST) microsatellite analysis were performed on three separate samples (SE, SM, SS). Codes refer to locations as in the Table 10.1.

Table 10.1. Code and geographic coordinates of sampling sites (kindly provided by people who sampled *C. glaucum* or asserted using Google Earth).

Code		Site location	Geographic coordinates	
FI		Tvärminne; Finland	59°50’ N	23°15’ E
GD		Gulf of Gdańsk; Poland	54°40’ N	18°30’ E
AL		Sylt; Germany	55°02’ N	8°24’ E
LV		Lake Veere; the Netherlands	51°35’ N	3°38’ E
AR		Arcachon; site 1; France	44°41’ N	1°03’ W
AN		Arcachon; site 2; France	44°41’ N	1°03’ W
PT		Ria Formosa, Ramalhete; Portugal	37°00’ N	7°58’ W
BL		Berre Lagoon; France	43°24’ N	5°08’ E
SA		Cabras, Sardinia; Italy	39°56’ N	8°31’ E
TU		Tunis Bay; Tunisia	36°47’ N	10°17’ E
SE		Ettore, Sicily; Italy	37°47’ N	12°26’ E
SM	**ST***	Stagnone di Marsala, Sicily; Italy	37°47’ N	12°26’ E
SS		Infersa, Sicily; Italy	37°47' N	12°26’ E
PA		Papas Lagoon; Greece	38°12’ N	21°22’ E
ET		Etolikon; Greece	38°28’ N	21°18’ E
GI		Gialova Lagoon; Greece	36°57’ N	21°40’ E
GR		Porto Lagos Lagoon; Greece	40°59’ N	25°09’ E
RO		Constanta; Romania	44°11’ N	28°39’ E
UK		Seihierovka; Ukraine	46°01’ N	30°24’ E
BK		Baku; Azerbaijan	40°22’ N	49°53’ E

*For mitochondrial DNA analysis samples from Sicily were pooled together (because the problems with amplification and the geographic proximity of sampling sites) and referred to as ST.

10.2 Amplification and sequencing of cytochrome oxidase subunit I (COI) locus

First the universal primers:
HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' and
LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' (Folmer *et al.* 1994)
were tested under various PCR conditions to amplify the cytochrome oxidase subunit I (COI) locus, but the quality of PCR products which were obtained, was not satisfactory. Therefore, specific primers were designed using the 657 bp sequences obtained by amplifying COI mtDNA fragment with universal primers (Folmer *et al.* 1994). Finally, the fragment of 580 bp was amplified with the specific primers:
F: 5'-CTAYCTAGCTTTTTGAGCGGG-3' and
R: 5'-CACCWCCCCCAACTGGATCGA-3'.
Polymerase chain reaction (PCR) was performed in a Bio-Rad DNA Engine Gradient Cycler. It started with the denaturation step at 94°C for 4 min followed by 35 cycles at 94°C for 30 s, 65°C for 30 s and 72° C for 1 min. Each PCR reaction mixture (volume 25 μl) contained 1.5 μl of DNA extract solution, 1 μM of each primer, 265 μM dNTPs, 1×PCR buffer, 2.5 mM $MgCl_2$ and 0.65 units of GoTaq Flexi DNA Polymerase (all reaction chemicals manufactured by Promega). Amplification results were verified by 2% TAE agarose gel electrophoresis. DNA of 311 individuals was sequenced at the Sequencing Platform GENOMER in Roscoff (France) (CNRS, Marine Biology Research Station). Sequences were deposited in GenBank under the accession numbers FJ555376-FJ555465.

10.3 Amplification and genotyping of microsatellites

The microsatellites were isolated and characterized by Pearson (2003) using methods adapted from Kandpal *et al.* (1994). DNA of each individual was amplified using four pairs of microsatellite primers (Pearson 2003) (Table 10.2). PCR program conditions were the following: an initial denaturation step at 94°C for 2 min followed by 25 cycles (30 cycles in case of *Cg9*) consisting of denaturation for 30 s at 94°C, annealing for 30 s (*Cg9* at 45°C, *Cg4* and *Cg11* at 50°C, *Cg7* at 55°C) and elongation for 1 min at 72°C. After all cycles had finished, the additional elongation step of 20 min was performed at 72°C. PCR was performed as for COI, but the reaction volume was 10 μl and fluorescent

forward primers were used. The mixture contained 0.7 µl of DNA extract solution, 1.675 mM (in case of *Cg4* and *Cg7*) or 2 mM (in case of *Cg9* and *Cg11*) of $MgCl_2$, 0.25 units of GoTaq Flexi DNA Polymerase and the other reaction chemicals in the same concentrations as for COI. Microsatellite alleles were visualized on an ABI 3130 automated sequencer using multiplex electrophoresis. Most of the PCR products were diluted 10 times before visualizing. Visualization was performed using 96 well plates. Each well contained 10 µl of deionized formamid (HI-DI, Applied Biosystems), 0.2 µl of size standard (Gene Scan 600-Liz; Applied Biosystems) and 8 µl of 4 PCR products- 2 µl for each microsatellite for a given individual. In each 96-well plate, 5% to 10% individuals had been genotyped on previous runs to allow cross comparisons. After exclusion of the individuals which did not amplify at any locus, the analysis were performed on genotypes from 500 individuals.

Table 10.2. Primers used to amplify 4 microsatellite loci.

	Primers sequences	**Microsatellite sequence**
Cg4	F: GTGTTGGACTCGCCATACC R: GACACAAGTAAAAACAATGTCT	$(GT)_n$
Cg7	F: GATCCAGCCGTTCAAGTCC R: CGAAATAATGCGCGATGC	$(GT)_n$
Cg9	F: CCATATTACCACTGCCCACAC R: TGACCCCCTCCAGTGATTC	$(GACA)_n$
Cg11	F: GGGGCGATTCTGGAGTAGTAG R: GTCAAACCAGGCGCTAAGTC	$(C)_n(CA)_n$

10.4 Cytochrome oxidase subunit I (COI) locus data analysis

COI fragment sequences were aligned using the program BIOEDIT (Hall 1999), Clustal W (Thompson *et al.* 1994). The fragment of 514 bp was chosen for further

analysis. COI sequences from Sicily (SE, SM, SS) were pooled together and referred to as ST. Phylogenetic network of COI haplotypes based on median-joining vectors method was generated with the program NETWORK v.4.5 (Bandelt *et al.* 1999). Additionally, the network of *C. glaucum* haplotypes with one *C. edule* haplotype accessible in the GenBank (accession number: AY226940) was generated. The calculation of average uncorrected percent of differences between the two main haplotype groups and the amino acid translation of the sequences were made using the MEGA 4 program (Tamura *et al.* 2007). The number of segregating sites (*S*), haplotype diversity (H_d) (Nei 1987) and nucleotide diversity (Π) (Nei 1987; Nei and Miller 1990) were computed using the program DNASP v.4.10.9 (Rozas *et al.* 2003), as well as *D* statistics of Tajima (1989) and D^* and F^* of Fu and Li (Fu and Li 1993), aimed at testing the hypothesis of neutrality, which assumes absence of selection and a stable effective size. Pairwise F_{ST} values between populations (Weir and Hill 2002) were computed using ARLEQUIN v.3.1 (Excoffier *et al.* 2005). The metric multidimensional scaling (MDS) of F_{ST} values (XLstat 7.5.2) (Kruskal and Wish 1978) allowed presenting levels of differentiation between populations without assuming tree-like relationships.

The mitochondrial DNA evolutionary model was tested using the online platform (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) based on Posada and Crandall (1998). The maximum likelihood haplotype tree (Guindon and Gascuel 2003) based on the most probable evolutionary model was constructed with PhyML online platform (http://www.atgc-montpellier.fr/phyml) with 100 bootstrap data sets. The tree was rooted with the *C. edule* sequence from the GenBank (AY226940). In order to obtain the sequences of the same length as *C. edule* sequence, the *C. glaucum* sequences were shortened to 506 bp.

A Markov Chain Monte Carlo coalescence simulation method implemented in the program IM (Nielsen and Wakeley 2001; Hey 2005) was used to assess the divergence time parameter (t = divergence time × mutation rate per sequence) among two sets of geographically distant populations within mitochondrial haplogroups I and II. The HKY substitution model was applied (Hasegawa *et al.* 1985), because it is advised in a user manual for mitochondrial DNA sequences. Firstly, the nontrivial ranges of parameters were tested (see Won and Hey 2005). In the final simulations upper ranges of parameters were set according to the posterior distributions from the initial screening runs: for haplogroup I (-l 20000000 –b 2000000 -q1 150 –q2 25 –qA 40 –t 10 – m1 6 –m2 m2 4)

and for haplogroup II (-l 20000000 –b 2000000 -q1 100 –q2 200 –qA 40 –t 5 – m1 2 –m2 m2 2).

10.5 Microsatellite data analysis

Linkage disequilibria between pairs of microsatellite loci were estimated using GENETIX v.4.02 (Belkhir *et al.* 1998), by calculating the correlation coefficients between all possible pairs of loci (Weir 1979). 1000 permutations of monolocus genotypes within each population were performed for testing significance ($\alpha = 0.05$).

Observed and expected heterozygosities (without bias) (H_{obs}, H_{nb}) (Nei 1978) and F_{IS} (Weir and Cockerham 1984) for each population were calculated using GENETIX v.4.02. The significance of observed values was tested by performing 1000 permutations of alleles within each population ($\alpha = 0.05$). The number of private alleles per population and per locus was counted. RMES (Robust Multilocus Estimation of Selfing) program (David *et al.* 2007) was applied to check for the presence of biparental inbreeding in the populations. The selfing rate (s), which in gonochoric species is interpreted as an inbreeding rate, was deduced from an estimator of the two-locus heterozygosity disequilibrium over all pairs of loci ($\hat{g}_2$) under the assumption of inbreeding and linkage equilibrium. P-value for the null hypothesis $s = \hat{g}_2 = 0$ was obtained by resampling genotypes 1000 times, drawing monolocus genotypes independently. The level of significance ($\alpha = 0.05$) was corrected by the method of Benjamini and Hochberg (1995) to control false discovery rate of multiple statistical tests which are independent or positively correlated (Verhoeven *et al.* 2005).

Pairwise F_{ST} values between populations for each locus separately and over all loci were calculated according to Weir and Cockerham (1984) using GENETIX v.4.02. For significance testing, 1000 permutations of individuals among samples while preserving their multilocus genotypes were performed. The significance level was corrected with Benjamini and Hochberg's (1995) method. The metric multidimensional scaling (MDS) (Kruskal and Wish 1978) was employed to illustrate pairwise F_{ST} values between populations. Signals of selection acting on any loci were screened from various subsets of populations with the F_{ST}-outlier approach implemented in LOSITAN software (Beaumont and Nichols 1996; Antao *et al.* 2008). The "neutral mean F_{ST}" and "force mean F_{ST}" options were applied. The stepwise mutation model was chosen and 10000 simulations were performed.

The tree representing relationships among populations was constructed using programs implemented in the PHYLIP 3.6 package (Felsenstein 2005). However, this method has a very limited reliability at an intraspecific level (see: 9.3.9 Trees and networks) and I consider that multidimensional scaling (MDS) of pairwise population F_{ST} better represents among population relationships. The distance matrix based on microsatellite allele frequencies was constructed with Cavalli-Sforza chord distance (Cavalli-Sforza and Edwards 1967). The reconstruction of phylogeny was based on neighbor-joining algorithm (Saitou and Nei 1987) based on the minimum evolution criterion for phylogenetic trees.

An individual-based Bayesian approach was taken to address the structuring in the microsatellite multilocus data. Posterior probability of the data was estimated with the STRUCTURE software (Pritchard *et al.* 2000) under a range of scenarios with the number of panmictic genetic clusters (K) varying from 1 to 20. For each K value tested, 12 iterations were performed. No *a priori* information of the geographic origin of individuals was given (Falush *et al.* 2003). The burn-in length was set at 50000 and the actual run length was 250000 Markov chain repetitions. The admixture model and correlated allele frequencies model were used. The number of clusters was estimated with Evanno's *et al.* (2005) method. Once the number of clusters was inferred, the CLUMPP v.1.1.1 software (Jakobsson and Rosenberg 2007) was used to permute the clusters output by 12 independent runs with the same K value, so that they match up as closely as possible. The "Greedy" algorithm and G' pairwise matrix similarity statistic were applied, testing 1000 random input orders. The DISTRUCT v.1.1 software (Rosenberg 2004) was used to graphically display the results.

10.6 Comparing mitochondrial and nuclear results

The significance of the correlation between the pairwise populations F_{ST} matrices based on mitochondrial DNA data and on multilocus microsatellite data was tested using Mantel test (Mantel 1967; Smouse *et al.* 1986) implemented in ARLEQUIN v.3.1 (Excoffier *et al.* 2005). In order to test for significance 1000 permutations of rows and columns of the matrices were performed. The samples from Sicily (SE, SM, SS in case of microsatellites and ST in case of mitochondrial DNA) were removed from the analysis in order to obtain the matrices of equal sizes.

Phylogeographic structure in mtDNA and microsatellites as well as the accordance between the structures revealed by two marker types were also examined with the analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) using the ARLEQUIN v.3.1 (Excoffier *et al.* 2005). Several geographic groupings were tested to elucidate the existence and location of phylogeographic breaks in *C. glaucum*. The significance was tested with 1000 permutations. The type of permutations is different for each covariance component.

11 Results

Translating COI mtDNA sequences into amino acids revealed that all mutations were synonymous except for one individual singleton. 90 haplotypes resulting from 100 individual mutations at 92 polymorphic sites were observed. The number of haplotypes shared among sites was 11 (Appendix 2, 3 and 4). At microsatellite loci the total number of alleles at each locus was 42 for *Cg4*, 25 for *Cg7*, 63 for *Cg9* and 24 for *Cg11* (Appendix 5 and 6).

11.1 Variability among populations

The haplotype network of COI sequences revealed two main phylogroups assembling populations from the eastern Mediterranean Aegean Sea (GR) and the Ponto-Caspian region (UK, RO, BK) (haplogroup VI) against more westerly samples (haplogroups I-V) with a 6.6% sequence divergence. Nikula and Väinölä (2003) assumed that the divergence between *C. glaucum* and *C. edule* took place during the Messinian salinity crisis, which led to an estimation of the divergence rate within *C. glaucum* of about 5% COI sequence divergence per million years, which translates to the substitution rate of 2.5% per million years. Based on this divergence rate, the estimated divergence between the eastern and the western lineages took place about 1.3 mya. Additionally, the Atlanto-Mediterranean phylogroup was subdivided into four well-defined haplogroups and a group of haplotypes sampled in the Ionian Sea populations, which exhibited high intragroup divergence. Two striking geographic disjunctions were detected in the distribution of haplotype lineages: haplotypes in the Baltic Sea (FI, GD) were similar to the Portuguese (PT) (haplogroup I), and haplotypes from Sardinia (SA) and two haplotypes from the Berre Lagoon (BL) were similar to those from the Atlantic coast of France (AN, AR) and the North Sea (LV, AL) (haplogroup II). Those distant populations belonging to the same haplogroup did not share

any individual haplotypes, but their haplotypes differed usually by one or two mutations (Fig. 11.1, 11.2 and 11.3). The population pairwise F_{ST} for COI sequences (Fig. 11.4a, Table 11.1) revealed the high level of genetic structuring, with significant pairwise F_{ST} even within regions. In the median-joining network *C. edule* haplotype was connected with the haplogroup VI (grouping populations from the Aegean Sea, the Black Sea and the Caspian Sea) (Fig. 11.2).

The most suitable substitution model was found to be the General Time Reversible model (Tavaré 1986). In the maximum likelihood tree of mitochondrial DNA haplotypes based on this model, the divergent character of the haplogroups VI was confirmed (Fig. 11.3). The haplogroup (IV) from Tunisia was also very divergent. The haplotypes from Portugal (PT), Poland (GD) and Finland (FI) (haplogroup I), despite the geographic distance separating the populations, formed a monophyletic group supported by a high bootstrap value. Haplotypes from the haplogroup (II) formed a "fork-like" structure, within which a distinct sub-group contained most of the haplotypes from Sardinia.

The coalescence simulations for haplogroups I and II both yielded the highest posterior density (HPD) for the divergence time of 0.7 substitution units (*t*). Assuming the divergence rate of 5% per million years (Nikula and Väinölä 2003), the divergence time of the distant populations within haplogroup I, as well as within haplogroup II would be around 55 thousand years [or $0.7 \times (2.5 \times 10^{-8} \times 514\text{bp})^{-1}$].

No linkage disequilibrium was detected between microsatellite loci. Microsatellites revealed a main division between the Ionian Sea and all the other regions (Fig. 11.4b, Table 11.2a). The isolation of the Ionian Sea populations (ET, GI, PA) was most strongly supported by locus *Cg11*, at which these populations exhibited a very low genetic diversity (non-biased heterozygosity) when compared to other loci (Appendix 6d). In fact, at *Cg11* locus, positive selection was detected by the F_{ST}-outlier test in the Ionian Sea, when the test was performed on a subset of populations from the Ionian Sea, the Aegean Sea and the Ponto-Caspian region (Fig. 11.5). Therefore, pairwise populations F_{ST} were also calculated omitting *Cg11*, in which case the Ionian populations appeared similar to the Aegean-Ponto-Caspian populations in nuclear data (Fig. 11.4c, Table 11.2b). The F_{ST} revealed the high level of genetic structuring among populations even within regions. F_{ST} between the southern populations were generally lower than F_{ST} between the northern populations (Fig. 11.4b-c, Table 11.2). There was a large variation among loci in pairwise F_{ST} (Fig. 11.6, Table 11.3).

The tree based on microsatellite data confirmed a separate character of the Ionian Sea populations and populations grouping rather consistent with the geographic locations of samples (Fig. 11.7).

The Bayesian analysis performed in STRUCTURE program on microsatellite data identified four panmictic units according to the method of Evanno *et al.* (2005). One unit characterized cockles from the Baltic Sea and the North Sea (FI, GD, AL, LV), another one associated strongly with the cockles from the Atlantic coast of Europe (AR, AN, PT), the third characterized individuals from the Ionian Sea (GI, ET, PA), and the last one- those from the Aegean Sea and the Ponto-Caspian region (GR, RO, UK, BK). The individuals from the Western Mediterranean mainly appeared as a product of admixture. Several groups of geographically close populations strongly matched within the same panmictic units with little geographic discontinuity (Fig. 11.8). The inference of four panmictic units and their associations with the populations was also found when omitting the putatively selection-affected locus *Cg11* from the data set. Therefore, contrary to the MDS representation of pairwise F_{ST}, the STRUCTURE analysis, even without *Cg11,* supported the distinct character of the Ionian Sea populations.

The Mantel test revealed no statistically significant correlation between the matrices of pairwise populations F_{ST} based on mitochondrial DNA data and on microsatellites.

The highest proportion of among-group molecular variation in mitochondrial DNA was revealed with AMOVA, when populations were divided into six arbitrary haplogroups exhibiting geographical discontinuities, like in Fig. 11.2, which was expected, but useful in order to obtain a reference value for comparisons. It was also high, when the Aegean Sea and the Ponto-Caspian region populations were grouped against all the other populations. In contrast, the among-group molecular variation revealed in microsatellites was high when the Ionian Sea populations were grouped against all the other populations or when the populations were divided in accordance to the geographic regions (Table 11.4). Interestingly, the two “best groupings” for mitochondrial DNA corresponded to the two “worst groupings” with microsatellite data, and conversely.

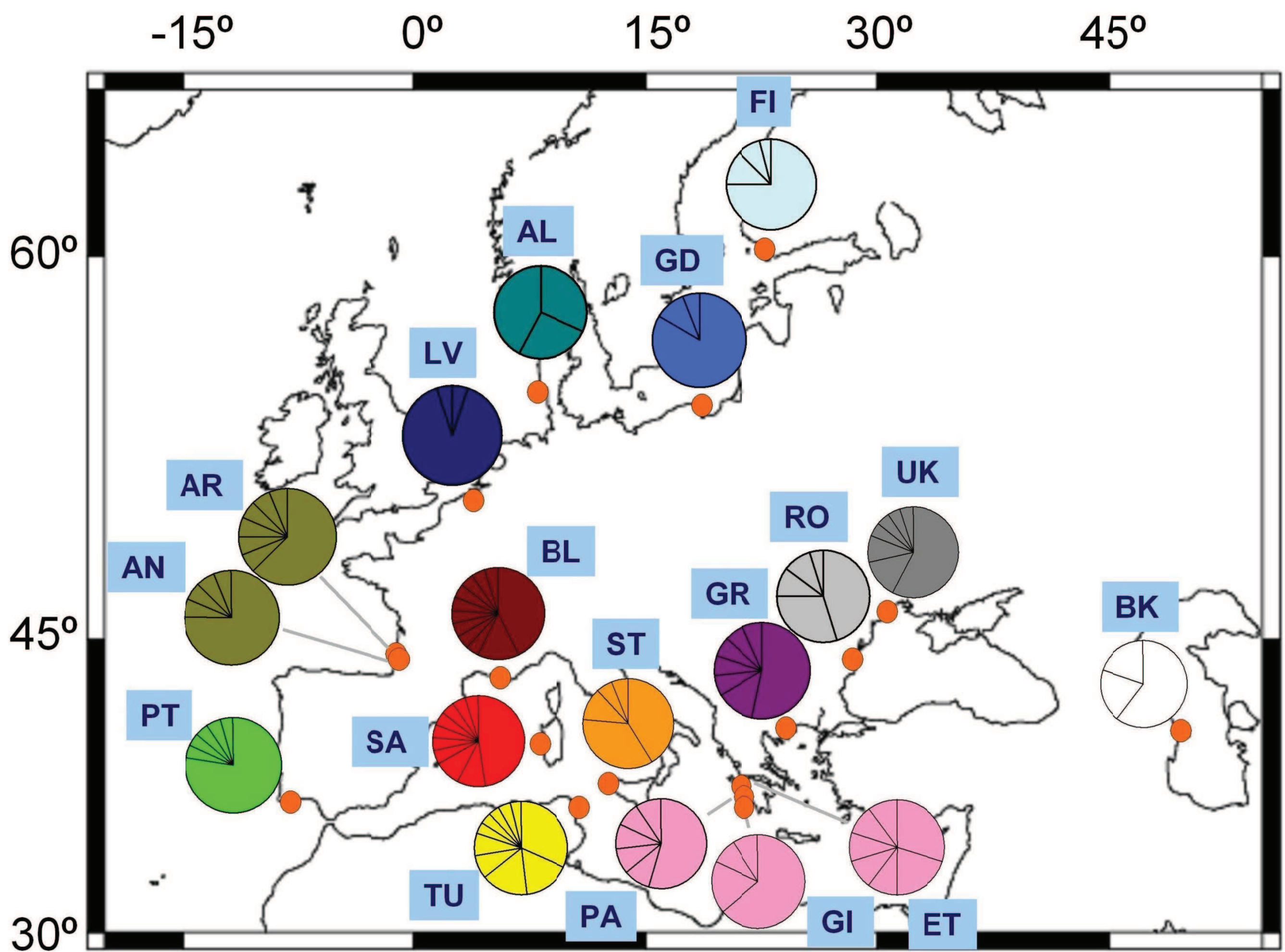

Figure 11.1. COI haplotype frequencies in each population. Distinct haplotypes within populations are visible as slice marks. Color code is the same as in Fig. 11.2. See Table 10.1. for sites abbreviations.

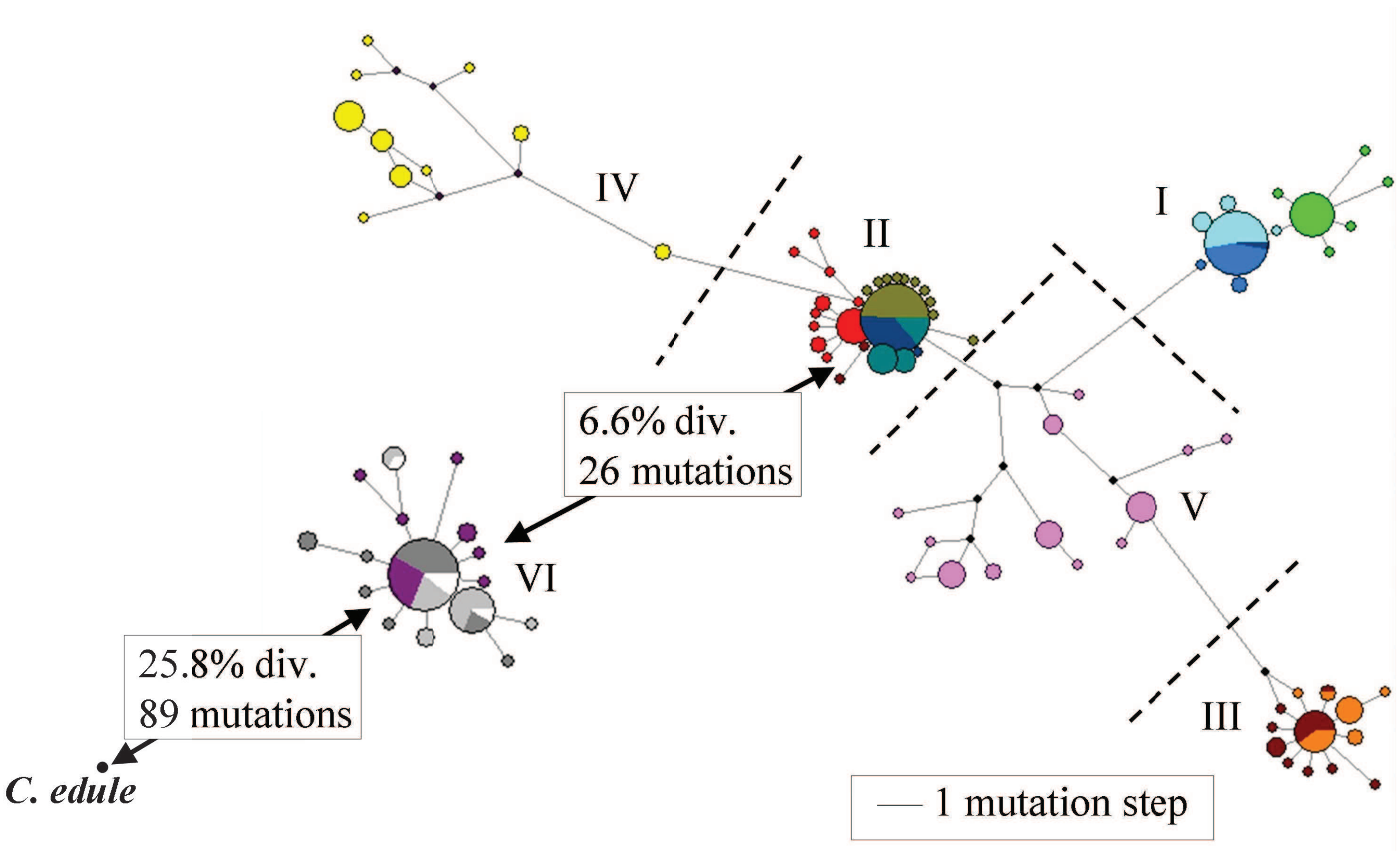

Figure 11.2. COI haplotype network. Circle sizes are proportional to frequencies of each haplotype and haplotypes colors are the same as in Fig. 11.1. The haplogroups are arbitrarily numbered from I to VI to facilitate description. Distances reflecting the number of mutations are marked from the centers of haplotype circles. Lines lengths are proportional to the number of mutations (see the scale bar).

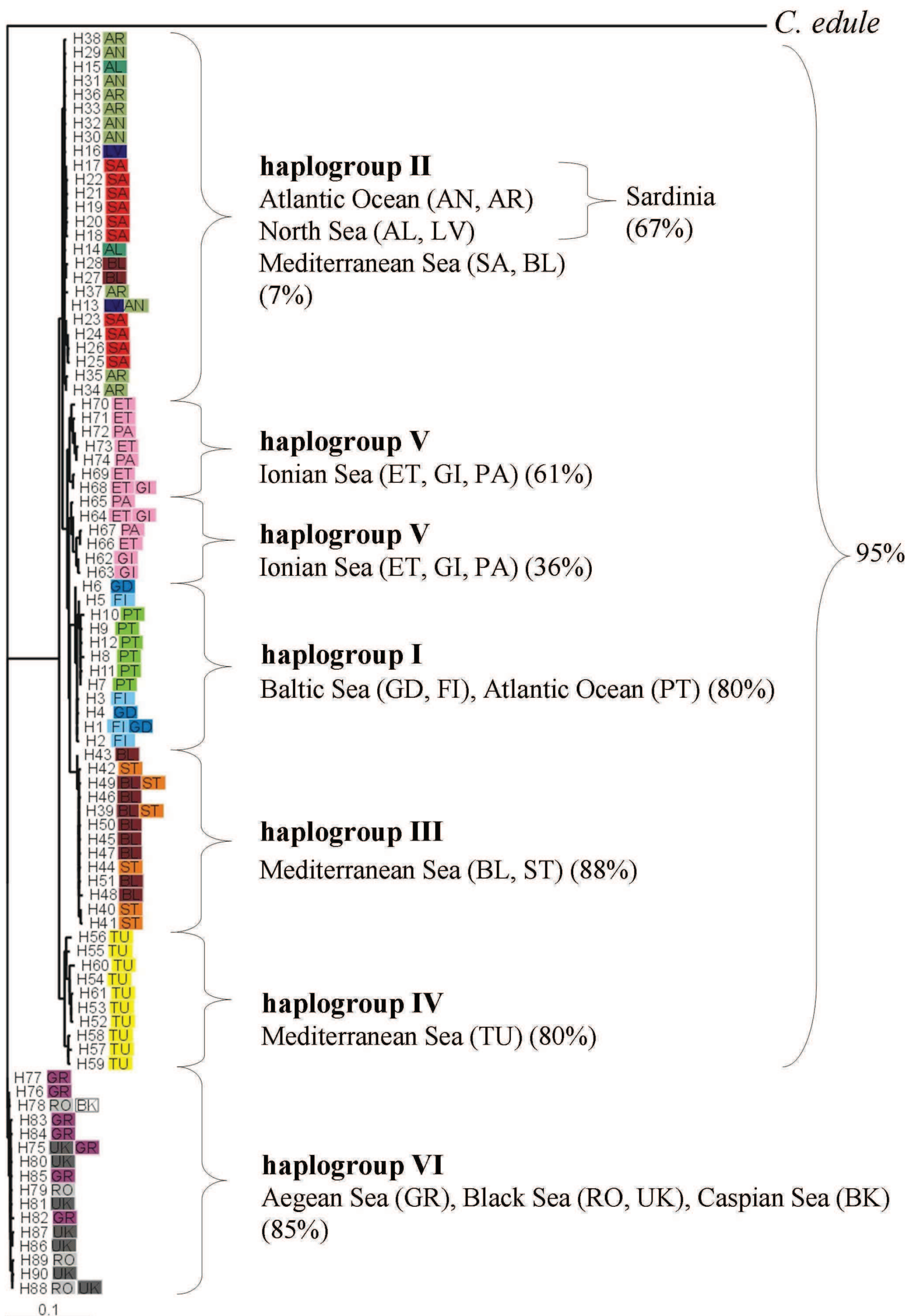

Figure 11.3. Maximum likelihood tree of mitochondrial DNA haplotypes. Bootstrap values are marked in brackets. Haplotype numbers correspond to the numbers in Appendix 3 and 4. See Table 10.1 for sites abbreviations.

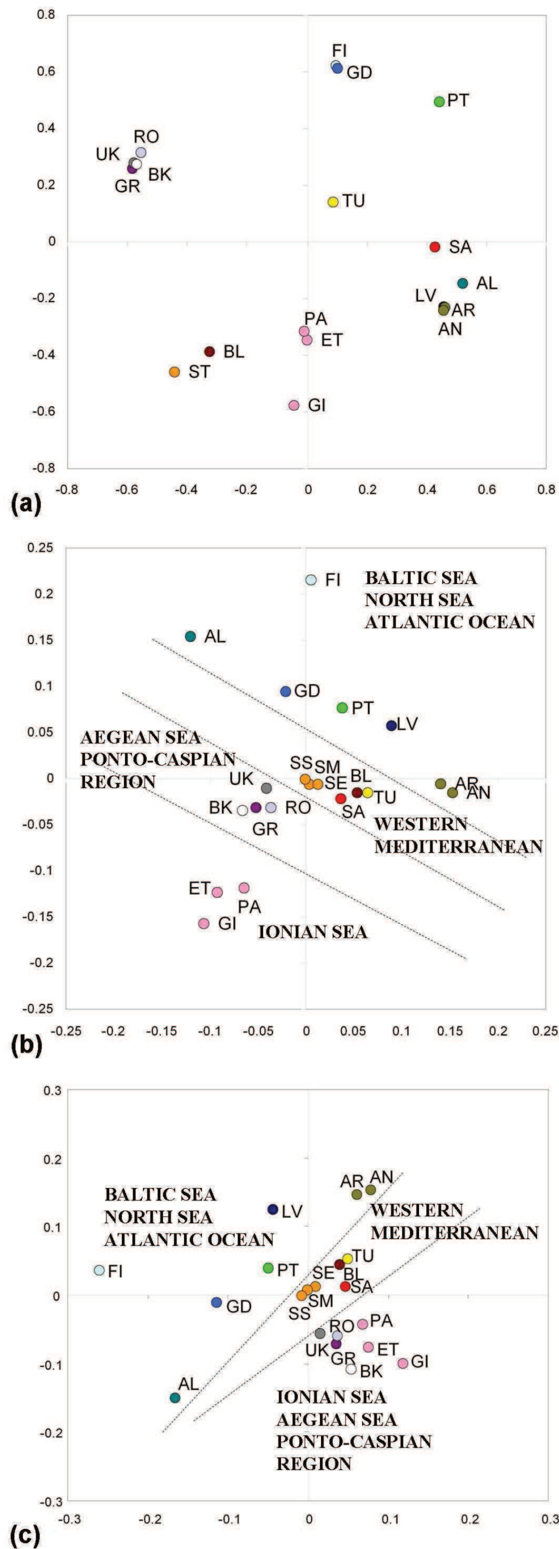

Figure 11.4. Multidimensional scaling (MDS) of population pairwise F_{ST}. Populations are marked with the same colors as in Fig. 11.1. See Table 10.1 for sites abbreviations. Dashed lines show divisions into geographic regions: **a)** mtDNA data (stress = 0.242), **b)** microsatellite data (stress = 0.138), **c)** microsatellite data, locus *Cg11* excluded (stress = 0.135).

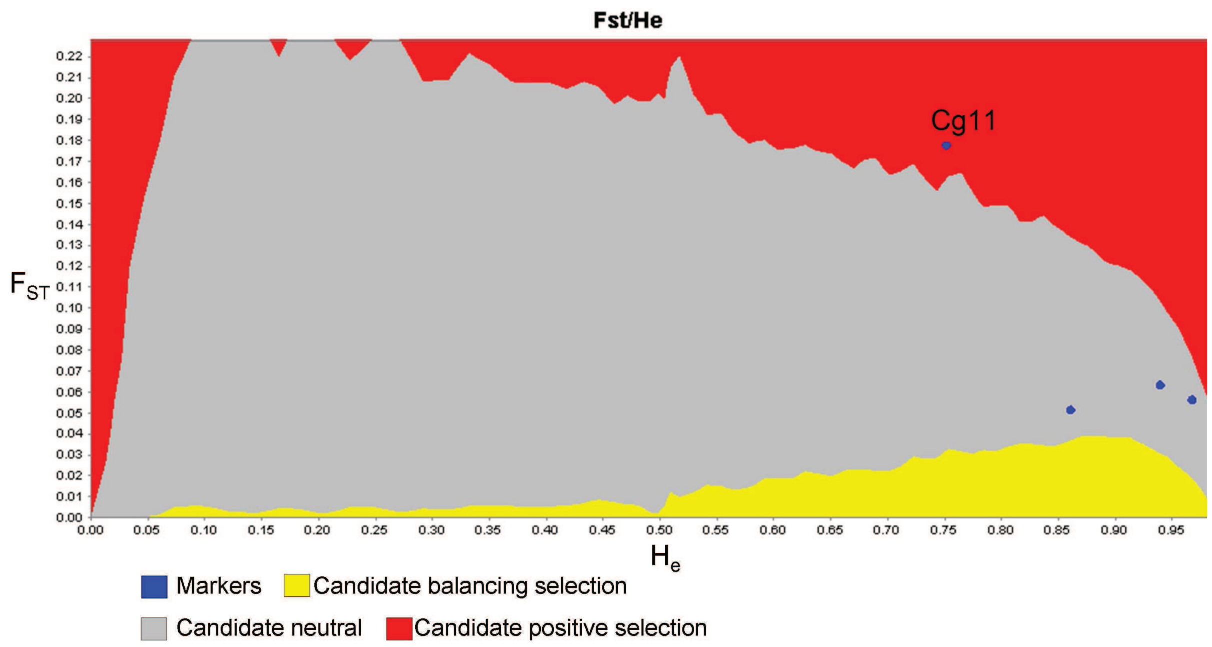

Figure 11.5. F_{ST}-outlier method to detect selection- the output of LOSITAN software (Beaumont and Nichols 1996; Antao *et al.* 2008). *Cg11* is a candidate locus for a positive selection.

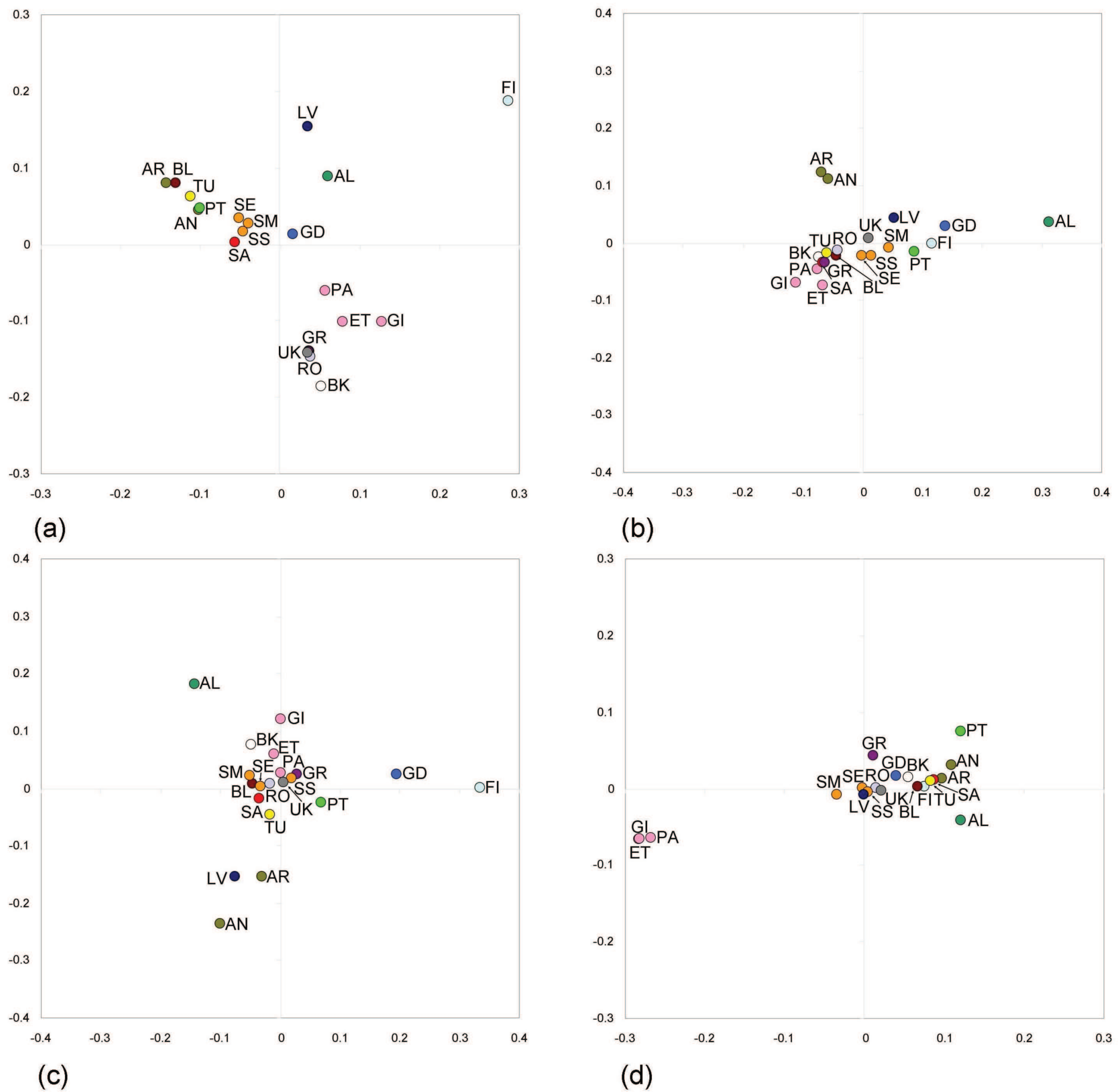

Figure. 11.6. Multidimensional scaling (MDS) of microsatellite pairwise populations F_{ST} for each locus separately. Each population is marked with the same color as in Fig. 11.1 and 11.2. See Table 10.1 for sites abbreviations: **a)** locus *Cg4* (stress = 0.153), **b)** locus *Cg7* (stress = 0.165), **c)** locus *Cg9* (stress = 0.180), **d)** locus *Cg11* (stress = 0.176).

Table 11.1. Pairwise populations F_{ST} (Weir and Hill 2002) based on mtDNA sequences. Not significant values are marked in grey and with italics. See Table 10.1 for sites abbreviations.

	GD	AL	LV	AR	AN	PT	BL	SA	TU	ST	GI	ET	PA	GR	RO	UK	BK
FI	0.05	0.93	0.91	0.93	0.94	0.78	0.83	0.89	0.86	0.93	0.88	0.79	0.81	0.98	0.97	0.98	0.98
GD		0.93	0.92	0.93	0.95	0.80	0.82	0.89	0.85	0.93	0.87	0.77	0.79	0.98	0.97	0.98	0.98
AL			0.19	0.20	0.23	0.92	0.79	0.40	0.73	0.91	0.83	0.70	0.72	0.97	0.97	0.97	0.97
LV				*-0.01*	*-0.01*	0.90	0.78	0.33	0.72	0.90	0.81	0.68	0.70	0.97	0.97	0.97	0.97
AR					*0.01*	0.92	0.78	0.34	0.71	0.90	0.82	0.68	0.70	0.97	0.97	0.97	0.97
AN						0.93	0.80	0.36	0.72	0.92	0.84	0.70	0.72	0.97	0.97	0.97	0.98
PT							0.82	0.88	0.85	0.92	0.86	0.78	0.80	0.97	0.97	0.97	0.98
BL								0.77	0.74	0.09	0.67	0.61	0.66	0.94	0.94	0.94	0.92
SA									0.70	0.87	0.79	0.67	0.69	0.95	0.95	0.96	0.95
TU										0.80	0.76	0.69	0.70	0.92	0.92	0.93	0.90
ST											0.80	0.73	0.77	0.97	0.97	0.97	0.97
GI												0.35	0.47	0.95	0.96	0.96	0.94
ET													*0.08*	0.92	0.93	0.93	0.89
PA														0.92	0.93	0.93	0.90
GR															0.17	0.06	*-0.03*
RO																0.09	*-0.02*
UK																	*-0.02*

Table 11.2. Multilocus pairwise populations F_{ST} (Weir and Cockerman 1984) for microsatellites. Not significant values are marked in grey and with italics. See Table 10.1 for sites abbreviations: **a)** multilocus pairwise populations F_{ST}, **b)** multilocus pairwise populations F_{ST} after locus *Cg11* was excluded.

	GD	**AL**	**LV**	**AR**	**AN**	**PT**	**BL**	**SA**	**TU**	**SE**	**SM**	**SS**	**GI**	**ET**	**PA**	**GR**	**RO**	**UK**	**BK**
FI	0.09	0.24	0.19	0.27	0.32	0.20	0.25	0.24	0.25	0.21	0.24	0.18	0.36	0.32	0.31	0.21	0.24	0.21	0.30
GD		0.15	0.13	0.18	0.21	0.08	0.14	0.14	0.15	0.10	0.10	0.07	0.25	0.21	0.19	0.13	0.13	0.09	0.16
AL			0.19	0.29	0.31	0.21	0.20	0.20	0.23	0.19	0.15	0.17	0.36	0.32	0.32	0.23	0.20	0.18	0.22
LV				0.11	0.11	0.14	0.11	0.12	0.10	0.09	0.08	0.11	0.27	0.23	0.20	0.18	0.14	0.13	0.22
AR					*0.01*	0.13	0.10	0.09	0.07	0.12	0.15	0.13	0.31	0.29	0.24	0.18	0.17	0.16	0.23
AN						0.15	0.12	0.10	0.08	0.14	0.16	0.16	0.33	0.30	0.26	0.19	0.16	0.16	0.22
PT							0.10	0.11	0.10	0.07	0.08	0.08	0.29	0.25	0.23	0.14	0.15	0.12	0.16
BL								0.03	0.02	0.05	0.06	0.06	0.22	0.19	0.17	0.12	0.10	0.11	0.12
SA									*0.01*	0.04	0.06	0.04	0.21	0.19	0.16	0.07	0.05	0.07	0.07
TU										0.05	0.07	0.06	0.24	0.21	0.18	0.11	0.10	0.10	0.13
SE											*0.00*	*0.00*	0.17	0.13	0.10	0.08	0.06	0.07	0.09
SM												*0.01*	0.16	0.12	0.10	0.10	0.07	0.06	0.08
SS													0.16	0.12	0.10	0.08	0.06	0.06	0.08
GI														0.04	0.03	0.14	0.14	0.16	0.16
ET															0.01	0.12	0.11	0.12	0.12
PA																0.11	0.10	0.11	0.13
GR																	0.02	0.03	*0.02*
RO																		*0.00*	*0.00*
UK																			*0.02*

(a)

	GD	AL	LV	AR	AN	PT	BL	SA	TU	SE	SM	SS	GI	ET	PA	GR	RO	UK	BK
FI	0.11	0.32	0.25	0.37	0.41	0.25	0.33	0.32	0.33	0.27	0.28	0.22	0.35	0.31	0.29	0.26	0.30	0.26	0.39
GD		0.18	0.17	0.22	0.26	0.10	0.18	0.18	0.18	0.12	0.13	0.08	0.24	0.18	0.16	0.15	0.17	0.11	0.21
AL			0.23	0.36	0.38	0.24	0.24	0.26	0.30	0.22	0.15	0.19	0.32	0.27	0.28	0.27	0.24	0.23	0.27
LV				0.12	0.11	0.15	0.14	0.13	0.12	0.11	0.10	0.14	0.28	0.24	0.20	0.22	0.18	0.17	0.28
AR					*0.01*	0.14	0.13	0.12	0.09	0.13	0.15	0.15	0.28	0.24	0.18	0.21	0.20	0.19	0.30
AN						0.17	0.15	0.13	0.10	0.15	0.16	0.18	0.28	0.24	0.19	0.22	0.18	0.18	0.29
PT							0.10	0.12	0.11	0.05	0.05	0.05	0.22	0.16	0.14	0.15	0.16	0.12	0.21
BL								0.03	0.02	0.04	0.04	0.06	0.17	0.14	0.11	0.13	0.12	0.13	0.16
SA									*0.02*	0.03	0.04	0.04	0.13	0.10	0.07	0.07	0.06	0.08	0.09
TU										0.03	0.06	0.06	0.18	0.15	0.10	0.12	0.11	0.12	0.17
SE											*0.00*	*0.00*	0.15	0.10	0.07	0.09	0.08	0.08	0.13
SM												*0.01*	0.16	0.11	0.09	0.11	0.08	0.07	0.10
SS													0.15	0.10	0.07	0.08	0.09	0.07	0.11
GI														0.04	0.03	0.09	0.10	0.12	0.10
ET															*0.01*	0.05	0.07	0.07	0.05
PA																0.05	0.06	0.06	0.07
GR																	*0.01*	0.02	*0.01*
RO																		*0.01*	*0.00*
UK																			*0.02*

(b)

Table 11.3. Pairwise populations F_{ST} (Weir and Cockerman 1984) for each microsatellite locus separately. Not significant values are marked in grey and with italics. See Table 10.1 for sites abbreviations: **a)** locus *Cg4*, **b)** locus *Cg7*, **c)** locus *Cg9*, **d)** locus *Cg11*.

	GD	**AL**	**LV**	**AR**	**AN**	**PT**	**BL**	**SA**	**TU**	**SE**	**SM**	**SS**	**GI**	**ET**	**PA**	**GR**	**RO**	**UK**	**BK**
FI	0.22	0.12	0.20	0.51	0.45	0.44	0.49	0.41	0.48	0.38	0.32	0.38	0.38	0.36	0.34	0.36	0.39	0.41	0.49
GD		0.04	0.08	0.14	0.10	0.09	0.13	0.07	0.11	0.05	0.02	0.06	0.12	0.08	0.06	0.12	0.13	0.12	0.16
AL			*0.02*	0.20	0.15	0.15	0.19	0.15	0.18	0.11	0.07	0.13	0.23	0.20	0.17	0.20	0.22	0.22	0.25
LV				0.20	0.18	0.17	0.18	0.18	0.17	0.12	0.09	0.15	0.32	0.29	0.24	0.30	0.31	0.32	0.40
AR					*0.00*	*0.00*	*0.01*	0.10	0.03	0.08	0.07	0.11	0.34	0.30	0.24	0.30	0.32	0.32	0.41
AN						*-0.02*	*0.03*	0.07	0.04	0.06	0.03	0.08	0.26	0.22	0.18	0.22	0.24	0.22	0.28
PT							*0.01*	0.06	*0.02*	0.05	*0.03*	0.07	0.27	0.22	0.18	0.23	0.25	0.23	0.29
BL								0.09	*0.01*	0.06	0.05	0.08	0.32	0.30	0.23	0.30	0.32	0.31	0.40
SA									*0.04*	*0.01*	*0.01*	*0.00*	0.21	0.17	0.13	0.14	0.15	0.14	0.20
TU										0.03	*0.03*	0.04	0.30	0.27	0.21	0.26	0.28	0.27	0.35
SE											*-0.01*	*-0.01*	0.22	0.19	0.14	0.19	0.20	0.19	0.27
SM												*0.00*	0.19	0.15	0.11	0.15	0.16	0.15	0.20
SS													0.20	0.17	0.12	0.16	0.17	0.16	0.22
GI														0.05	0.03	0.13	0.13	0.12	0.14
ET															0.03	0.07	0.07	0.07	0.06
PA																0.10	0.10	0.09	0.12
GR																	*-0.02*	*0.00*	*-0.05*
RO																		*-0.02*	*-0.03*
UK																			*0.01*

(a)

	GD	AL	LV	AR	AN	PT	BL	SA	TU	SE	SM	SS	GI	ET	PA	GR	RO	UK	BK
FI	0.04	0.15	0.07	0.24	0.23	0.03	0.16	0.20	0.20	0.10	0.03	0.07	0.27	0.20	0.22	0.19	0.15	0.06	0.20
GD		0.06	0.05	0.23	0.22	0.06	0.19	0.24	0.22	0.15	0.07	0.10	0.30	0.24	0.24	0.22	0.17	*0.05*	0.22
AL			0.22	0.42	0.42	0.21	0.37	0.42	0.41	0.34	0.22	0.27	0.49	0.43	0.44	0.40	0.36	0.24	0.47
LV				0.13	0.09	0.08	0.10	0.15	0.11	0.08	0.04	0.08	0.23	0.19	0.16	0.16	0.08	-0.01	0.15
AR					*0.00*	0.22	0.16	0.16	0.13	0.15	0.18	0.17	0.22	0.20	0.15	0.15	0.11	0.10	0.18
AN						0.21	0.14	0.16	0.11	0.14	0.16	0.16	0.22	0.20	0.15	0.15	0.10	0.07	0.17
PT							0.13	0.17	0.17	0.04	*0.00*	*0.01*	0.24	0.16	0.17	0.15	0.13	0.06	0.17
BL								*0.00*	*0.00*	0.03	0.06	0.05	0.05	0.06	0.04	0.02	*0.00*	*0.03*	*0.02*
SA									*0.00*	0.05	0.10	0.07	0.03	0.04	*0.02*	*0.00*	*0.00*	0.06	*0.02*
TU										0.05	0.09	0.07	0.05	0.06	*0.02*	0.02	*0.00*	*0.04*	*0.02*
SE											*0.02*	*-0.01*	0.11	0.06	0.05	0.05	0.04	*0.03*	0.08
SM												*0.00*	0.18	0.12	0.12	0.10	0.06	*0.01*	0.10
SS													0.12	0.04	0.06	0.06	0.05	*0.03*	0.08
GI														*0.03*	*0.02*	0.04	0.05	0.13	*0.05*
ET															*0.00*	0.05	0.06	0.11	0.07
PA																*0.01*	*0.02*	0.08	*0.05*
GR																	*-0.01*	0.06	*0.02*
RO																		*0.01*	*0.01*
UK																			*0.02*

(b)

	GD	AL	LV	AR	AN	PT	BL	SA	TU	SE	SM	SS	GI	ET	PA	GR	RO	UK	BK
FI	*0.04*	0.55	0.48	0.38	0.57	0.24	0.36	0.36	0.31	0.36	0.49	0.22	0.41	0.36	0.31	0.23	0.38	0.30	0.50
GD		0.36	0.35	0.28	0.46	0.15	0.21	0.22	0.20	0.17	0.28	0.08	0.28	0.23	0.18	0.11	0.22	0.16	0.26
AL			0.37	0.42	0.51	0.31	0.16	0.17	0.27	0.20	0.16	0.16	0.25	0.20	0.22	0.21	0.15	0.23	*0.09*
LV				*0.02*	*0.03*	0.21	0.14	0.07	0.08	0.15	0.17	0.19	0.30	0.24	0.18	0.21	0.12	0.18	0.30
AR					*0.03*	0.14	0.16	0.09	0.06	0.13	0.19	0.17	0.28	0.22	0.16	0.18	0.14	0.14	0.31
AN						0.27	0.24	0.16	0.14	0.26	0.28	0.29	0.36	0.31	0.26	0.30	0.22	0.26	0.44
PT							0.12	0.11	0.09	0.07	0.12	0.06	0.17	0.11	0.07	0.06	0.11	0.06	0.16
BL								*0.02*	0.06	*0.03*	*0.02*	0.05	0.13	0.07	0.06	0.06	0.03	0.06	*0.05*
SA									0.01	*0.01*	*0.00*	0.04	0.14	0.08	0.05	0.07	*0.01*	0.04	*0.06*
TU										*0.03*	*0.03*	0.05	0.16	0.10	0.06	0.07	0.05	0.05	0.13
SE											*0.00*	*0.00*	0.11	0.04	*0.02*	*0.03*	*-0.02*	*0.02*	*0.03*
SM												*0.04*	0.12	0.05	0.05	0.07	*0.02*	*0.04*	*-0.01*
SS													0.12	0.06	0.04	*0.01*	0.04	*0.03*	*0.04*
GI														0.04	0.05	0.10	0.12	0.10	0.10
ET															*0.00*	0.05	0.06	0.04	*0.03*
PA																0.02	0.04	0.02	0.05
GR																	0.05	*0.00*	*0.05*
RO																		0.04	*0.04*
UK																			*0.04*

(c)

	GD	AL	LV	AR	AN	PT	BL	SA	TU	SE	SM	SS	GI	ET	PA	GR	RO	UK	BK
FI	0.04	0.03	0.04	*0.01*	0.05	0.08	*0.00*	*0.01*	*0.07*	0.07	0.11	0.07	0.37	0.37	0.36	0.07	0.06	0.06	*0.03*
GD		0.10	*0.02*	0.06	0.06	*0.03*	0.05	0.03	0.05	*0.03*	*0.03*	0.05	0.32	0.32	0.30	0.07	0.03	0.04	*-0.02*
AL			0.10	0.08	0.12	0.14	0.07	0.04	0.05	0.12	0.17	0.12	0.45	0.45	0.43	0.12	0.08	0.05	0.10
LV				0.08	0.11	0.12	0.03	0.06	0.06	*0.02*	*0.02*	*0.02*	0.22	0.21	0.20	0.06	*0.02*	0.03	*0.02*
AR					*0.00*	0.11	0.03	*0.01*	*0.02*	0.09	0.14	0.07	0.42	0.42	0.40	0.08	0.08	0.09	*0.02*
AN						0.08	0.06	*0.02*	*0.03*	0.10	0.14	0.08	0.45	0.45	0.43	0.11	0.09	0.11	*0.00*
PT							0.08	0.07	0.07	0.12	0.14	0.15	0.49	0.50	0.48	0.12	0.10	0.12	*0.02*
BL								*0.02*	*0.00*	0.07	0.11	0.07	0.35	0.34	0.34	0.07	0.05	0.06	*0.01*
SA									*0.01*	0.07	0.12	0.06	0.43	0.42	0.41	0.08	0.05	0.06	*0.00*
TU										0.08	0.12	0.06	0.41	0.40	0.39	0.09	0.05	0.06	*-0.01*
SE											*0.01*	*0.00*	0.23	0.24	0.21	0.04	*0.00*	*0.02*	*0.00*
SM												*0.02*	0.17	0.17	0.15	0.08	*0.02*	*0.03*	*0.01*
SS													0.23	0.23	0.20	0.07	*0.00*	*0.00*	*-0.05*
GI														*0.01*	*-0.01*	0.30	0.26	0.32	0.42
ET															*0.01*	0.31	0.27	0.33	0.43
PA																0.29	0.24	0.30	0.39
GR																	0.03	0.05	*0.04*
RO																		*-0.02*	*-0.03*
UK																			*0.01*

(d)

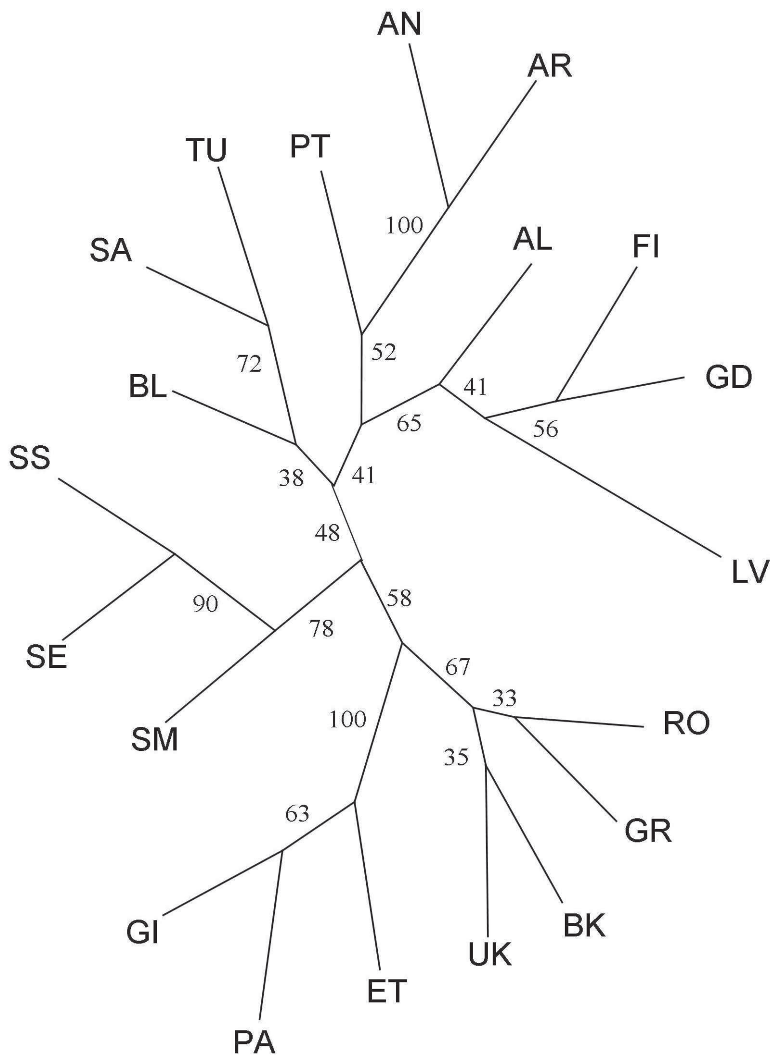

Figure 11.7. Neighbor-joining tree constructed using microsatellite data. Bootstrap values (in %) are marked on the branches. See Table 10.1 for sites abbreviations.

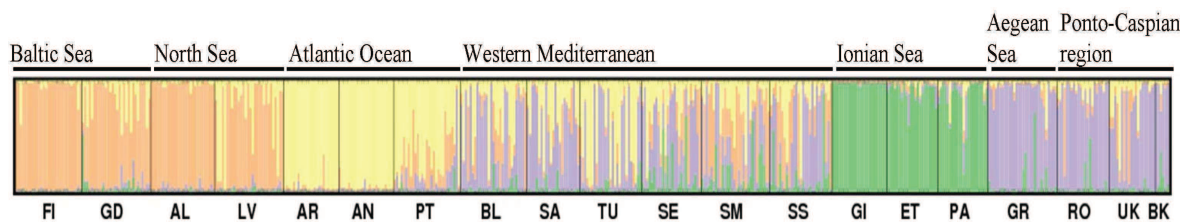

Figure 11.8. Panmictic units showing Hardy-Weinberg equilibrium inferred by STRUCTURE software. Each individual is represented by a single vertical line, which is partitioned into colored segments that represent that individual's estimated membership fraction in each of the *K* inferred clusters.

Table 11.4. The proportions of among-group molecular variation in the various population groups tested with AMOVA.

Population groups tested	% of among-group molecular variation	
	Mitochondrial DNA	**Microsatellites**
1. Aegean Sea and Ponto-Caspian 2. All the other populations	78.04	3.05
1. Ionian Sea 2. All the other populations	9.05	13.67
6 groups consistent with mtDNA haplogroups (see Fig. 11.2)	87.46	8.45
5 geographic groups: 1. Baltic and North Sea 2. Atlantic Ocean 3. Western Mediterranean 4. Ionian Sea 5. Aegean Sea and Ponto-Caspian region	64.54	10.85

11.2 Variability within populations

The number of COI haplotypes per population varied between 3 and 11 (Fig. 11.1). Nucleotide diversity (*Π*) diminished with the latitude (Fig. 11.9). The results of neutrality tests were significant for three Atlantic coast populations (AR, AN, PT) and one North Sea population (LV) (Table 11.5).

For microsatellites, the observed heterozygosity (H_{obs}) was lower than the non-biased expected heterozygosity (H_{nb}) under the Hardy-Weinberg equilibrium hypothesis, for all the populations except for BK, GI and LV (multilocus analysis) (Table 11.6). Eleven populations showed significant heterozygote deficiencies with significant F_{IS} varying from 0.09 to 0.38, but the results differed among loci. Five populations were inferred to exhibit Hardy-Weinberg disequilibrium due to biological reasons (according to results obtained with the RMES program) (Table 11.7).

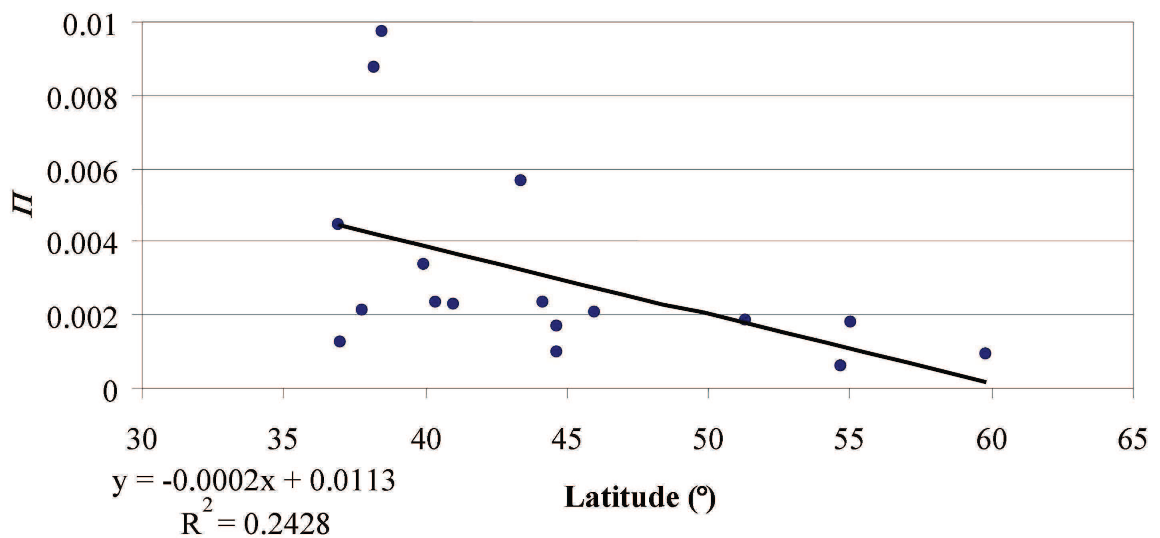

Figure 11.9. The relationship between nucleotide diversity (*Π*) and latitude.

Table 11.5. Polymorphism of COI mtDNA and neutrality tests; *N*- number of individuals, N_H- number of haplotypes, *S*- number of polymorphic sites, H_d- haplotype diversity, *Π*- nucleotide diversity, *D* Taijma, Fu and Li's *D** and *F**, n.s.- not significant. Populations were arranged from the Baltic Sea, through the Atlantic Ocean and the Mediterranean Sea to the Black and the Caspian Sea. Table 10.1 for sites abbreviations.

Site	*N*	N_H	*S*	H_d	$\Pi \times 10^2$	*D* Tajima	Fu and Li's *D**	Fu and Li's *F**
FI	24	4	3	0.431	0.092	n.s.	n.s.	n.s.
GD	18	3	2	0.307	0.062	n.s.	n.s.	n.s.
AL	19	3	2	0.690	0.180	n.s.	n.s.	n.s.
LV	19	3	9	0.205	0.184	-2.20	-3.19	-3.37
AR	16	7	6	0.625	0.169	-2.08	-2.78	-2.97
AN	16	5	4	0.450	0.097	-1.83	-2.41	-2.58
PT	22	6	7	0.411	0.124	-2.14	-3.21	-3.36
BL	21	11	19	0.814	0.565	n.s.	n.s.	n.s.
SA	21	10	10	0.776	0.339	n.s.	n.s.	n.s.
TU	25	10	15	0.860	0.724	n.s.	n.s.	n.s.
ST	17	6	5	0.772	0.212	n.s.	n.s.	n.s.
GI	11	4	8	0.600	0.446	n.s.	n.s.	n.s.
ET	10	7	15	0.911	0.973	n.s.	n.s.	n.s.
PA	11	6	15	0.727	0.877	n.s.	n.s.	n.s.
GR	15	7	7	0.724	0.226	n.s.	n.s.	n.s.
RO	20	5	5	0.721	0.232	n.s.	n.s.	n.s.
UK	21	7	6	0.667	0.204	n.s.	n.s.	n.s.
BK	5	3	3	0.700	0.233	n.s.	n.s.	n.s.
Average	17	6	8	0.633	0.330			
Total	311	90	92					

Table 11.6. Genetic diversity of *C. glaucum* in microsatellites; *N*- number of individuals, H_{nb}- expected heterozygosity without bias, H_{obs}- observed heterozygosity, mean number of alleles per locus, number of private alleles at each locus. Populations were arranged from the Baltic Sea, through the Atlantic Ocean and the Mediterranean Sea to the Black and the Caspian Sea. See Table 10.1 for sites abbreviations.

Site	*N*	H_{nb}	H_{obs}	Mean no. of alleles/locus	Number of private alleles *Cg4*/*Cg7*/*Cg9*/*Cg11*
FI	29	0.56	0.54	6.25	1/1/0/1
GD	30	0.71	0.57	10.25	5/0/1/0
AL	28	0.58	0.57	5.25	0/0/0/0
LV	30	0.65	0.70	5.50	0/0/0/0
AR	24	0.65	0.63	7.50	1/0/0/0
AN	24	0.63	0.60	7.50	0/0/0/0
PT	29	0.72	0.53	7.25	0/0/0/0
BL	29	0.75	0.72	10.75	0/0/0/1
SA	23	0.80	0.68	10.25	2/0/2/1
TU	27	0.74	0.59	9.75	0/2/0/0
SE	26	0.82	0.78	8.75	0/0/0/0
SM	27	0.78	0.49	7.25	0/0/0/0
SS	27	0.81	0.60	10.00	0/0/0/0
GI	24	0.70	0.72	8.50	3/2/3/0
ET	22	0.75	0.64	15.75	0/1/9/0
PA	22	0.75	0.69	13.00	1/0/6/0
GR	30	0.87	0.67	13.25	0/0/0/0
RO	23	0.86	0.74	10.50	0/0/0/0
UK	20	0.86	0.72	9.00	0/1/1/0
BK	6	0.85	0.88	5.75	0/0/0/0
Average	25	0.74	0.65	9.10	

Table 11.7. F_{IS} for each microsatellite locus separately and multilocus; *s*- inbreeding evaluated with RMES (Robust Multilocus Estimation of Selfing) program. Populations were arranged from the Baltic Sea, through the Atlantic Ocean and the Mediterranean Sea to the Black and the Caspian Sea. See Table 10.1 for sites abbreviations.

Site	F_{IS}					$s(\hat{g}_2)$
	Cg4	***Cg7***	***Cg9***	***Cg11***	**Multilocus**	
FI	-0.27	0.01	**0.63**	-0.02	0.03	0.20
GD	-0.06	0.02	**0.74**	**0.25**	**0.20**	0.00
AL	0.17	-0.18	0.07	-0.08	0.02	0.00
LV	-0.34	0.18	-0.24	-0.08	-0.09	0.25
AR	-0.24	-0.16	0.29	**0.18**	0.03	0.03
AN	-0.02	-0.10	0.18	**0.19**	0.05	0.14
PT	**0.20**	**0.24**	**0.31**	**0.29**	**0.26**	0.44
BL	-0.17	-0.05	0.10	**0.18**	0.04	0.00
SA	0.18	0.00	**0.21**	**0.23**	**0.15**	0.00
TU	0.05	0.10	**0.39**	**0.25**	**0.21**	**0.37**
SE	-0.18	0.01	0.26	0.05	0.05	**0.46**
SM	0.15	0.12	**0.72**	**0.50**	**0.38**	**0.59**
SS	-0.12	**0.16**	**0.34**	**0.61**	**0.26**	0.11
GI	-0.02	0.03	-0.07	-0.10	-0.03	0.05
ET	**0.15**	0.13	**0.22**	-0.09	**0.15**	0.21
PA	0.13	-0.03	**0.20**	-0.09	**0.09**	0.03
GR	0.12	**0.33**	**0.26**	**0.24**	**0.24**	0.17
RO	0.10	**0.28**	0.08	0.09	**0.14**	**0.49**
UK	-0.07	**0.53**	**0.38**	-0.19	**0.16**	**0.77**
BK	-0.25	0.15	0.09	-0.11	-0.03	0.25

12 Discussion

12.1 Local genotypic structure in C. glaucum *and other lagoon species*

The causes and consequences of heterozygote deficiencies are the problems that have been largely debated (reviewed in Zouros and Foltz 1984; David 1998). I detected heterozygote deficiencies (significant F_{IS}) in most studied populations. The presence of null alleles was not a sufficient explanation for heterozygote deficiency in three populations from the Western Mediterranean (SE, SM, TU) and two from the Black Sea (RO, UK) (Table 11.7). Biological causes of heterozygote deficiency include: inbreeding, the Wahlund effect and selection against heterozygotes.

According to the results of the STRUCTURE analyses the Mediterranean Sea populations (SE, SM, TU) were composed of a mixture of genotypes of different origins (Fig. 11.8), therefore significant results revealed by RMES may be, at least partly, due to the Wahlund effect. For the populations from the Black Sea (UK, RO), however, it seems necessary to invoke inbreeding. Detection of inbreeding in *C. glaucum* is not surprising as this species often inhabits small isolated lagoons (Pearson 2003). Populations of lagoon specialists are subjected to marked temporal variation in population size, potentially leading to bottlenecks and founder events (e.g. Mason 1986; Reise 2003; Kevrekidis *et al.* 2005) provoking low effective population sizes. Small isolated wild populations often experience high levels of inbreeding (Frankham 1995; Madsen *et al.* 1996; Lacy 1997). Strong heterozygote deficiencies and lower intra-population variability compared to *C. edule* were also revealed in *C. glaucum* by isoenzyme electrophoresis (Hummel *et al.* 1994). Heterozygote deficiencies provoking inbreeding depression in isolated populations may lead to lower reproductive output and viability (Madsen *et al.* 1996).

12.2 Genetic patterns of C. glaucum *in southern areas*

It has been suggested that C. *glaucum* diverged from the common ancestor with its closest living relative *C. edule* during late Miocene- early Pliocene about 5.5 mya, when the Atlantic Ocean became isolated from the Mediterranean Sea (Rygg 1970) (see: 3.4 On taxonomy, divergence and fossil record of *C. glaucu*m) The Mediterranean Sea can be therefore regarded as a historical core distribution area of *C. glaucum*, from where it spread to other European seas and brackish-water basins.

The estimated divergence rate within *C. glaucum* was of 5% divergence per million year (Nikula and Väinölä 2003). There seems to be a lot of variation in COI divergence rates among invertebrates, see e.g. Marko 2002. The divergence rate found for *Macoma balthica* COIII based on the timing of trans-Arctic interchange overlaps with the 5% per million years (see Nikula *et al.* 2007). A divergence rate estimated for *Mytilus edulis* COI by Wares and Cunningham (2001) was even higher.

Both mitochondrial DNA and microsatellites confirmed the previous suggestions (Mariani *et al.* 2002; Nikula and Väinölä 2003) that the subdivision within *C. glaucum* into the Atlantic and the Mediterranean species or subspecies (Brock 1987; Brock and Christiansen 1989; Hummel *et al.* 1994) should be definitely abandoned. The Strait of Gibraltar does not represent a major barrier to gene flow for many marine species (Borsa *et al.* 1997; Patarnello *et al.* 2007) and the faunas of Mediterranean Sea and the adjacent Atlantic Ocean are similar enough for the regions to be recognized as a single Temperate Northern Atlantic province (Spalding *et al.* 2007).

Deep subdivisions were found between mitochondrial lineages of *C. glaucum*. Marked preglacial phylogeographic structures were found in other European marine species and explained by allopatric divergence in glacial refugia (Coyer *et al.* 2003; Luttikhuizen *et al.* 2003a; Papadopoulos *et al.* 2005; Provan *et al.* 2005; Pérez-Losada *et al.* 2007), which could also be the case for *C. glaucum*. A main phylogeographic break revealed with mtDNA, grouping the Aegean Sea, the Black Sea and the Caspian Sea populations against more western populations, was consistent with the observations of Nikula and Väinölä (2003) with the same marker. It indicated that populations from these regions were effectively isolated for a long time. At the end of the Pliocene (about 1.8 mya), when the connection between the Aegean Sea and the Black Sea was established, the Mediterranean fauna entered the Black Sea (Bacescu 1985). This was the time when *C. glaucum* might have colonized the Black Sea for the first time, which is in a rough agreement with our estimation of the divergence time among the major lineages (1.3 mya). The fact that the Black Sea has probably played a major role in the evolution of the lagoon cockle is especially interesting. Most of the marine taxa are believed to have gone extinct in the Black Sea due to a severe salinity drop during the last glacial period (Nikula and Väinölä 2003; Peijnenburg *et al.* 2004). It was not the case for C. *glaucum*, which tolerates low salinities and seems to have survived inside the Black Sea isolated from conspecific populations in the Mediterranean Sea.

The level of divergence between the two main *C. glaucum* mtDNA lineages has often been found between species-level taxa, like clams (mtDNA COI and rDNA 16S) (Peek *et al*. 1998), sea urchins (mtDNA COI) (McCartney *et al.* 2000) or shrimps (mtDNA COI and allozymes) (Gusmão *et al.* 2000). However, taxonomic decisions should not be based on mtDNA only, because of the possibility of introgressions (Currat *et al.* 2008).

In the haplotype network where *C. edule* sequence was included, the Ponto-Caspian populations of *C. glaucum* were found to be the least diverged from *C. edule*, which was unexpected and may suggest that a haplotype structure in this region is quite conserved and has changed relatively little since the separation of species lineages. However, the "starlike" character of this haplogroup (haplogroup VI) does not confirm its ancestral character. It was argued that phylogenetic signals of the mtDNA are often unreliable and may deviate from the actual phylogeny, because of the retention of ancestral polymorphism, male-biased gene flow, selection on any part of the mitochondrial genome or introgression (Ballard and Whitlock 2004; Moritz and Cicero 2004; Monaghan *et al.* 2006).

A major geographic break was also revealed by microsatellites. It singled out the Ionian Sea populations as clearly distinct from the rest of the sample populations. The distinctiveness of the Ionian Sea populations largely resulted from the locus *Cg11*. The F_{ST}-outlier test indicated positive selection acting on *Cg11* in the Ionian Sea, suggesting that selective pressures could have contributed to the differentiation of the Ionian Sea populations from the nearby Aegean Sea and Ponto-Caspian region populations. However, the low number of loci investigated diminish the credibility of the selection detection test (Beaumont and Nichols 1996). A similarly deviant locus, *Mpi*, was present in the allozyme data set of Nikula and Väinölä (2003) as well. Pairwise F_{ST} between populations without locus *Cg11* rendered the Ionian samples fairly similar to the Aegean Sea and Ponto-Caspian region samples, and suggested that the nuclear gene pool of the Ionian Sea populations has an "Aegean-Ponto-Caspian" origin. This affinity was also found at many allozyme loci (Nikula and Väinölä 2003). However, the exclusion of *Cg11* from the STRUCTURE analysis did not change the clustering pattern and confirmed the separate character of the Ionian Sea populations. The Ionian Sea populations were found to possess mtDNA haplotypes that group with the western lineage, and this study thus confirmed (with another type of a nuclear marker) the mixed cytonuclear character of the Ionian Sea *C. glaucum*, which has been previously explained by an old introgression of Atlanto-

Mediterranean mitochondria, into a dominantly Ponto-Caspian nuclear background (Nikula and Väinölä 2003). It is striking that these Ionian Sea populations shared a particular group of mitochondrial haplotypes (haplogroup V) found nowhere else. They displayed by far the highest nucleotide diversity of all haplogroups (except for Tunisia), no traces of bottlenecks and demographic expansions, and had a central position in a global network. This suggests that this region hosts the most direct descendants of the ancestral *C. glaucum* mitochondrial genomes. Tunisian haplotypes also displayed a very high nucleotide diversity, and a private mitochondrial haplogroup, though they were not central in the network. The lagoon cockle probably survived in the regions of Tunisia and the Ionian Sea through several Pleistocene climatic cycles. Many terrestrial species survived glacial periods in refugia on the Balkan Peninsula (reviewed in Hewitt 2000) and in the region of Tunisia (Magri *et al.* 2007).

Nikula and Väinölä (2003) explained the presence of the main phylogeographic break in *C. glaucum* in the region of the Ionian Sea by hydrological conditions. There could be a gene flow through planktonic larvae from the Black Sea to the Mediterranean Sea, as there is a cold, surface current of low-salinity Black Sea water, but not in the opposite direction, as there is a sub-surface current of warmer, high-salinity water from the Mediterranean Sea, which is soon depleted of oxygen on its way to the Black Sea (Vergnaud-Grazzini 1985). The Ponto-Caspian haplotypes established in the Aegean Sea could not colonize the rest of the Mediterranean Sea, because the northern basin of the Aegean Sea is influenced by low-salinity water circulating cyclonically. Moreover, geological conditions between these two basins differ as in the southern part of the Aegean Sea, contrary to the northern part, the continental shelf is very limited (Moraitou-Apostolopoulou 1985). Similar genetic structures with the main barrier against gene flow in the Aegean Sea were found for some other species (see Domingues *et al.* 2005 and references therein). However, a long-distance dispersal by planktonic larvae between isolated lagoon habitats of *C. glaucum* does not seem very probable, except for the areas like in the Baltic Sea, where this species forms continuous populations along the vast areas of the coastal zone. Therefore, hydrological conditions in the open sea area of the Aegean Sea is probably not the most important factor influencing gene flow in this region.

12.3 Genetic structure in postglacially recolonized northern areas

Elaborating the scenario of colonization of the northern parts of the distribution area by *C. glaucum* and defining glacial refugia for this species is not straightforward, since the structures revealed by mtDNA and the four microsatellite loci were not congruent, which was confirmed by the Mantel test and the analysis of molecular variance (AMOVA). Firstly, I will base the colonization scenario on mitochondrial DNA, since it provides genealogical relationships among alleles (haplotypes) unlike microsatellites. Then I will try to reconcile data from both marker types considering potential factors that may explain the discrepancies.

The genetic structures of species with the distribution area reaching the north of Europe, like in case of a lagoon cockle, are influenced by Pleistocene glaciations. Due to a lack of suitable habitats during ice ages, northern populations are assumed to be the most recently established. Therefore, southern populations are usually expected and found to bear higher genetic diversity than their northern counterparts (Bernatchez and Wilson 1996; Gysels *et al.* 2004; Hewitt 2004; Maggs *et al.* 2008). This is the case for *C. glaucum*, which exhibited lower intrapopulation diversity in the northern compared to the southern regions (Fig. 11.9). Southern populations were more divergent from each other, forming monophyletic haplogroups. Haplotypes from the haplogroup II, where the populations from the North Sea and the Atlantic Ocean belong, formed a "fork-like" structure in a haplotype tree, so they seemed to have diverged more recently (Fig. 11.3). This was supported by the significance of the neutrality tests for three populations from the Atlantic Ocean (PT, AR, AN) and one from the North Sea (LV) (Table 11.5) and indicated that northern populations experienced bottlenecking and/or founder events associated with postglacial colonization (Hartl and Clark 1997).

However, microsatellite allele frequencies revealed that populations from postglacially colonized areas from the northern Europe were more divergent from each other than the southern, presumably older populations (Fig. 11.4b-c). Similar patterns of northern heterogeneity versus southern homogeneity have been reported in European terrestrial fauna, and were attributed to increased effects of genetic drift in stressful northern conditions, where census population sizes tend to fluctuate (e.g. Johansson *et al.* 2006).

Sometimes there are exceptions to the expected northwards decrease in the genetic diversity of fauna and flora. Surprisingly, in the marine cockle *C. edule* the genetic diversity studied with mitochondrial DNA sequences was found to increase toward north (Krakau 2008). This structure was explained by the possible glacial refugia in the north, but their existence is still under debate. Some studies lend credence to northern refugia (Stewart and Lister 2001; Maggs *et al.* 2008), other do not (Brochmann *et al.* 2003). The pattern of genetic diversity increasing towards the north is uncommon, but has been encountered before, for example in the subtidal bivalve *Arctica islandica* (Dahlgren *et al*. 2000) and was assumed to be caused by extinctions of this cold-adapted species in the south. High genetic diversity in the temperate North Sea area was also detected for the seagrasses *Zostera noltii and Z. marina* (Coyer *et al*. 2004; Olsen *et al.* 2004).

In mtDNA structure of *C. glaucum* two striking geographic discontinuities were detected: haplotypes in the Baltic Sea (GD, FI) were similar to the Portuguese (PT) (haplogroup I) and haplotypes from Sardinia (SA) and two haplotypes from the Berre Lagoon (BL) grouped together with those from the Atlantic coast of France (AN, AR) and the North Sea (LV, AL) (haplogroup II). The haplogroup I formed a monophyletic haplogroup supported by a high bootstrap value (Fig. 11.3). The areas of Sardinia and Portugal could have remained populated by *C. glaucum* through the Pleistocene glaciations and thereafter served as source populations for its postglacial (re)colonization of the northern European coasts. Coalescence simulations performed using IM framework favored the hypothesis that the separation of the northern and southern mitochondrial populations in the haplogroups I and II took place in the early postglacial times, as opposed to the alternative that the disjunct distributions of the haplogroups are due to human-mediated dispersal in modern times. To be precise, the best estimate of the divergence time fell in the middle of the previous glacial epoch, but given the recently accumulated evidence for fast short term substitution rates (e.g. Audzijonytė and Väinölä 2006; Waters *et al.* 2007; Peterson and Masel 2009), the IM-estimates could be considered compatible with the early postglacial hypothesis. Therefore, a non-anthropogenic dispersal event should be evoked. However IM estimates must be understood as very imprecise, given (1) the low number of substitution units estimated, (2) the high stochasticity of the coalescence process, and (3) the possibility of model violations such as for instance varying mutation rates, effective sizes etc.

If the postglacial colonization sources revealed by mitochondrial DNA genetic structure are true, then migrating birds seem a possible vector for gene flow between such disjunct areas as Sardinia and the Atlantic Ocean and the North Sea or Portugal and the Baltic Sea (see: 9.1.2 Dispersal modes and barriers in marine species; the case of *C. glaucum*). Some waders (Charadriiformes) that undergo particularly long migratory flights use the East Atlantic Flyway, which is a major route for long-distance transport between the Iberian Peninsula and the Baltic Sea (Sánchez *et al.* 2006; Sánchez *et al.* 2007). The Ria Formosa lagoon in Portugal, where the sample PT was collected, is the basin which is commonly used by waders. Various duck and wader species migrate also between the region of Sardinia and the North Sea or the Atlantic coast of France (Andy Green pers. comm.). The transportation of spat by migrating waterfowl has been considered an important means of dispersal for *C. glaucum* around the British Isles (Boyden and Russell 1972). It has also been suggested that immigration of *C. glaucum* from the Black Sea to the Caspian Sea could have occurred via waterfowls as their major migration route passes between these basins, and there were no waterways between them at the time when first *C. glaucum* fossils appear in the Caspian Sea (Nikula 2008). Bird-mediated long-distance dispersal into postglacially emerged northern European areas could to some extent explain the lack of a geographic continuity revealed in mtDNA of *C. glaucum*. On the other hand, it is also possible that the haplotypes present in the Baltic Sea and the North Sea were present in a few allopatric refugia, so the present populations do not come from the region of Portugal and Sardinia respectively, but from some other unsampled refugia (Jaramillo-Correa *et al.* 2004).

The microsatellite identities of the northern European populations were not in conflict with the geographically disjunct mtDNA affinities, as the microsatellite similarity of the northern populations with any of the southern populations is generally very low (Fig. 11.4b-c), presumably due to a founder effect and a genetic drift, as explained earlier. Therefore, postglacial colonization pathways cannot be tracked down from the microsatellite data. There was some similarity between the Portuguese (PT) and the Baltic Sea population (GD) in microsatellites confirming the results obtained with mtDNA (Fig 11.4b-c). However, the visual characterization of the populations obtained with STRUCTURE analysis (Fig. 11.8) did not entirely fit the colonization scenario based on mtDNA. In the STRUCTURE graph Baltic Sea and North Sea populations were found to be fairly similar in composition, whereas their mtDNAs belonged to different haplogroups.

Moreover, the Bay of Biscay populations according to STRUCTURE analysis most resembled the Portuguese population, but they belonged to distinct mtDNA haplogroups. These patterns seemed to imply a certain degree of geographic connectivity in microsatellites, but not in mtDNA. Judging from the overall high level of inferred admixture in individual cockles, however, the geographic patterns implied by the STRUCTURE assignments are not very well grounded, and may be biased due to the small number of studied loci. Moreover, STRUCTURE method assumes Hardy-Weinberg equilibrium within populations (Pritchard *et al.* 2000), which is not the case in all the population studied, but this method has been applied here anyway, as an exploratory tool. The patterns obtained were very clear and seem unlikely to have arisen by chance alone. Should the observed patterns be real, gene flow among populations within the North Sea and the Baltic Sea region and among the Atlantic coast populations could be hypothesized to occur *via* birds. The bird dispersal mode, even on the short distances, is more probable than the dispersal of planktonic larvae among isolated lagoons. I suggest that the role of migrating birds on the genetic structures of coastal marine organisms and on the past colonization and present gene flow has been seriously underestimated until now.

Another prominent feature of the STRUCTURE assignment graph was the high variability in assignment proportions between the Western Mediterranean individuals, and their generally highly mixed assignments to the four inferred panmictic units. The mixed assignments seemed to suggest admixture mainly of the Atlantic, the Aegean and the Ponto-Caspian gene pools in the Western Mediterranean *C. glaucum,* but there is no evidence for such admixture in mtDNA data. Rather, the inference of high admixture in the Western Mediterranean may have reflected the inapplicability of this type of analysis (or its usual interpretations) to a situation where genetically simpler, young offshoot populations exist along with older, genetically much richer populations, and where local populations are not in Hardy-Weinberg equilibrium. Moreover, the considerable amount of missing data in the samples from Sicily could have also influenced the STRUCTURE analysis results for this region.

12.4 Reconciling mtDNA and nuclear genetic structures

In general, the mitochondrial DNA structure of *C. glaucum* exhibited much more abrupt geographic breaks than the nuclear structure inferred from microsatellites.

Furthermore, the different marker types were in conflict at several geographic populations, because the populations did not possess the mitochondrial lineage of the population they appear most closely related with based on microsatellite data.

Previous studies on another nuclear marker type, allozymes, also revealed the high level of genetic structuring among *C. glaucum* populations with no or little geographic discontinuities and the separate character of the Ionian Sea, confirming the results obtained with microsatellites. Hummel *et al.* (1994) claimed that the populations from the Mediterranean Sea belong to the different subspecies (*C. glaucum glaucum*) than those from the Atlantic (*C. glaucum lamarcki*). However, that study was based on only 7 allozymes and only one Mediterranean Sea population was included. Mariani *et al.* (2002) studied 20 populations from the Mediterranean Sea and one from the Baltic Sea with 16 allozymes. Spatial structuring found among the populations fitted a stepping-stone model at a wide scale, with gene flow inversely related to geographic distance, as 4 major groups in the Mediterranean Sea were distinguished: Tyrrhenian, Adriatic, Aegean and Ionian. At small scale, genetic relationships among samples could not be interpreted as simply the effect of physical distance among populations. In the study of Nikula *et al.* (2003) 16 allozymes were tested on 7 European populations, which grouped according to geographical regions and the Ionian Sea population was quite distinct from the neighboring Aegean Sea populations. Thus, the microsatellite data were in agreement with the previous allozyme data, and in contrast with the mitochondrial sequences.

Nuclear and cytoplasmic markers revealed different and apparently conflicting patterns in many studies on marine organisms (Arnaud-Haond *et al.* 2003a; Hoarau *et al.* 2004; Peijnenburg *et al.* 2006; Gruenthal *et al.* 2007; Gérard *et al.* 2008). Those differences in patterns revealed by different marker types have been interpreted as the result of different factors, such as natural selection (e.g. Pogson *et al.* 1995), founder effect (e.g. Poteaux *et al.* 2001), or introgression (e.g. Krafsur 2002). Quantitative, but not qualitative differences, were explained by either male-biased effective sex ratio or cytoplasmic effective population size differing from the nuclear one (Karl *et al.* 1992; Palumbi and Baker 1994; Fitzsimmons *et al.* 1997; Lyrholm *et al.* 1999; Arnaud-Haond *et al.* 2003b).

The two genomes are potentially influenced in different ways by evolutionary forces, such as population demography and natural selection (Féral 2002; Ballard and Whitlock 2004; Chan and Levin 2005; Chenuil 2006). Due to the differences in ploidy and

inheritance (see: 9.2 Different properties of genetic markers applied), the mitochondrial effective population size ($N_{e(mt)}$) is four times smaller than for nuclear loci (Birky *et al.* 1989), and thus more susceptible to the effects of genetic drift. Consequently, estimates of population differentiation are expected to be higher for mitochondrial markers (see discussion in Crochet 2000, but also Birky *et al*. 1989 for possible exceptions). The discrepancy in the strength of the patterns could be explained by the different effects of random genetic drift and migration on the nuclear and mitochondrial gene pools (Ballard and Whitlock 2004) in fairly isolated local populations. Dispersal between local populations is more efficient in keeping the nuclear than mitochondrial gene pools homogeneous, as on average four times more nuclear genomes than mitochondrial genomes are transferred by a migrating individual (Mills and Allendorf 1996). Moreover, the values of F_{ST} tend to decrease together with the markers' polymorphism increase and in case of highly variable markers, such as microsatellites, pairwise population F_{ST} should be interpreted with caution (Charlesworth 1998). However, in the present data, not only the strength, but the patterns themselves are different between genomes, which was confirmed by the lack of correlation between pairwise population F_{ST} matrices based on mitochondrial and microsatellite data and by the analysis of molecular variance (AMOVA). Thus further explanations are required. The reciprocally monophyletic status of mitochondrial haplotypes that was observed among many regional groups of *C. glaucum* may have been promoted by selective sweeps that have been shown to affect the nonrecombining mitochondrial genome more readily than the nuclear genome (Gillespie 2001; Ballard and Whitlock 2004; Bazin *et al.* 2006). Even in the absence of selection, introgression is more efficient in organelle genes compared to nuclear genes (Currat *et al.* 2008). Introgression events may have occurred repeatedly in *C. glaucum* populations at the stage of their initial establishment, and could have entailed differential introgression of nuclear and mitochondrial genomes.

The fact that mitochondrial lineages are nearly monophyletic and clearly separated even among geographically close populations may have resulted from a high historical mtDNA polymorphism that has undergone a differential lineage sorting (stochastic loss of lineages) in relatively recently isolated local populations (Avise *et al.* 1984, 1987). However, it is not very likely that *C. glaucum* has ever had large continuous (panmictic) populations along the European coasts, which would have produced the high historical and

geographically unsegregated mtDNA polymorphism required to make this explanation credible.

Another possible phenomenon that might have facilitated the high mitochondrial differentiation and the less marked nuclear structuring is a gender-biased dispersal (Avise *et al.* 1987), which has been found in several species, mainly mammals and birds (Pusey 1987). Male-biased migration or female phylopatry (homing) have indeed been suspected in several instances (Fitzsimmons *et al.* 1997; Baker *et al.* 1998; Lyrholm *et al.* 1999), but such arguments are not applicable to sessile species. In case of *C. glaucum* if males, but not females, can effectively migrate and transfer their genes among local populations, it could lead to a nuclear homogenization among local populations, while mitochondrial lineages would differentiate. A gender-biased dispersal could hypothetically be caused by gender differences in survival during the aerial exposure and the starvation that are involved with the passive long-distance dispersal mechanisms available to *C. glaucum* (transport by migrating birds or transport by human activities). In other bivalve species the differences in the strength of mitochondrial and nuclear genetic patterns were explained by an unbalanced sex ratio or a sex-biased differential reproductive success between males and females (Arnaud-Haond *et al.* 2003b; Diaz-Almela *et al.* 2004). The variance in a reproductive success is known to be high in bivalves (Hedgecock 1994; Li and Hedgecock 1998) and supposedly higher in females than in males (Boudry *et al.* 2002).

The above mechanisms may lie behind the different nuclear and cytoplasmic population structures in *C. glaucum*, but they cannot account for the disjunct distributions of haplogroups I and II (Figs. 11.1, 11.2 and 11.3). Long-distance dispersal events of some sort have to be evoked to explain them. Bird-mediated early postglacial long-distance dispersal from southern European populations is the most plausible scenario. The microsatellite data are compatible with this explanation, if it is assumed that the vast differentiation among the populations north of Portugal (Fig. 11.4c) stems from an extreme stochastic drift associated with the postglacial population expansions that have erased most of the resemblance with the southern source populations.

12.5 Comparison of the level of genetic structuring with other bivalves

Like in previous studies (Hummel *et al.* 1994; Mariani *et al.* 2002; Nikula and Väinölä 2003; Pearson 2003) the high level of genetic structuring among populations of

C. glaucum was found compared to many other bivalve species (Grant *et al.* 1992; Sarver *et al.* 1992; Saavedra *et al.* 1993; Borsa *et al.* 1994). The genetic studies of marine bivalve mollusks have revealed the range of population structures from a high gene flow and limited genetic differentiation among populations over large geographic scales (e.g. Levinton and Suchanek 1978; Benzie and Williams 1992; Murray-Jones and Ayre 1997; Vadopalas *et al.* 2004) to a significant genetic differentiation and large F_{ST} values among populations over regional to small geographic scales (e.g. Luttikhuizen *et al.* 2003b; Kenchington *et al.* 2006; Lind *et al.* 2007). Despite planktonic larval stage, dispersal is often impeded by the influence of hydrographic features including ocean currents, fronts and mechanisms of larval retention that may operate at the range of geographic scales (e.g. Kenchington *et al.* 2006; Lind *et al.* 2007).

Other marine European bivalves with a similar distribution range to the one of *C. glaucum* revealed in general the lower level of genetic structuring and some interesting patterns of genetic diversity. The main factor shaping the present genetic structure of *C. glaucum* is lagoons' isolation, as the divergence among European populations of its marine counterpart, the common cockle *C. edule* is much less marked (Hummel *et al.* 1994; Krakau 2008). A barrier to gene flow in *C. edule* was found around the English Channel. However, there was no deep divergence between the populations on the both sides of the English Channel.

Within *Mytilus edulis*, no clear division was found among European populations which may be attributed to aquaculture based mixing (Riginos and Henzel 2008). *M. trossulus* has a disjunct distribution, occurring in the Baltic Sea as well as on the eastern Pacific and the eastern Canadian coasts, and a barrier against dispersal was found between the North Sea and the Baltic Sea (McDonald *et al.* 1991; Väinölä and Hvilsom 1991). In *Macoma balthica*, the Baltic Sea populations represent an admixture of two strongly diverged clades, the Pacific one and the Atlantic one (Luttikhuizen *et al.* 2003a; Strelkov *et al.* 2007; Nikula *et al.* 2008). Those and many other studies on European marine species proved that there is a barrier to gene flow between the Baltic and the North Sea and the extreme environment of the Baltic Sea induces extensive adaptive genetic changes (reviewed in Johannesson and André 2006). The findings on *C. glaucum* also revealed a barrier against gene flow between the North Sea and the Baltic Sea in mtDNA, but it was not confirmed by microsatellites studies. *C. edule* did not colonize the Baltic Sea presumably due to its low salinity.

The microsatellites studies on European populations of *Ostrea edulis* revealed a mild, but significant isolation-by-distance profile, providing support for the relative independence of local stocks instead of the existence of a single large panmictic population for this larvae-broadcasting species (Launey *et al.* 2002). European populations of another bivalve, *Mya arenaria* indicated a very limited genetic variation among populations (Lasota *et al.* 2004; Strasser and Barber 2009).

Chapter III: General conclusions and perspectives

14 Physiology and genetic structure of lagoon specialists

14.1 Strategies of lagoon species for efficient colonization and persistence in extreme habitats; the case of C. glaucum

Two main contrasting, but not mutually exclusive mechanisms may enable the species to establish in a new habitat (Parker *et al.* 2003): the presence of general purpose genotypes with high plasticity (e.g. Bamber and Henderson 1988; Pearson *et al.* 2002; Lasota 2009) or the capacity of a rapid adaptation.

The general purpose genotypes (Baker 1965) enable a phenotypic (physiological) tolerance and plasticity, which is a solution to changing environments. As a result, the genetic structure among populations is independent of environmental conditions, because genotypes are not adapted to specific local conditions, but to a wide range of them. The genetic structure is more influenced by other factors, like climatic past of the region. It has been argued that clonal reproduction is favored in species with general purpose genotypes, because then selection acts on the entire composite genotype of an asexual maintaining the advantageous and "plastic" combinations (Vrijenhoek 1998; Pearson 2003). Broadly adapted parthenogens are considered to produce broadly adapted offspring, whereas broadly adapted sexuals produce fewer broadly adapted offspring, because of recombination (Lynch 1984).

A distinct strategy of the efficient colonization of habitats differing in environmental conditions is the capacity of rapid adaptation. The species, which genetically adapt to environmental conditions in a newly colonized habitat, need to have high population sizes and high genetic diversity within loci, because of the genetic load associated with selection. These species usually reproduce sexually, which enables the recombination, giving a possibility to create new genetic combinations adapted to local conditions. In this case, there is a relationship between genotypes and environmental conditions (distinct genotypes adapted to distinct conditions).

I consider that one of the above strategies should prevail in *C. glaucum*. If this species had general purpose genotypes, then the physiological differences found among populations living in different environmental conditions are acclimatizations developed during lifetime. If instead, *C. glaucum* has the capacity of a rapid adaptation to newly

colonized habitats, then the physiological differences among populations are genetically determined.

There are some arguments in favor of physiological plasticity in the cockle**.** Lagoon specialists are often eurythermic and euryhaline (Bamber *et al.* 1992). It has been proven that semi-isolated and physically variable environments, such as estuaries and coastal brackish lagoons, act to select for generalist genotypes (Bamber and Henderson 1988). Environmental changes in lagoons are very rapid (not only seasonal), so in case of genetic adaptations the selective load would be too high. A good example is the Berre Lagoon, where the dominating zoobenthic species change often, due to a wide range of changes in environmental conditions (Stora *et al.* 1995; Stora *et al.* 2004). *C. glaucum* forms a stable element of this lagoon, which proves its phenotypic plasticity. Moreover, the genetic diversity found within this population and other southern European populations is high. Harsh environmental conditions with extremely high water temperatures in summer in southern locations and the periods of anoxia do not seem to reduce genetic diversity on neutral loci. It could be considered as an indication, that there is the same situation for the rest of the genome. Therefore, directional selection does not seem to have reduced the genetic diversity, which confirms the presence of general purpose genotypes. If there was selection on adaptive traits acting within southern populations, like from the Berre Lagoon, then the genetic variability within populations, reduced by the rapid change of environmental conditions, would need to be reconstructed by gene flow from other populations, which in case of the isolated lagoon habitats does not seem possible.

Moreover, low intrapopulation haplotype and nucleotide diversities within northern populations may be explained by postglacial colonization, which provoked bottlenecks and founder effects. Therefore, there is no need to invoke adaptation.

Previous comparative studies on respiration of the lagoon *C. glaucum* and marine *C. edule* revealed that *C. glaucum* appeared to be adapted to a wider range of environmental conditions and seemed to have a greater ability to regulate its metabolism than its marine counterpart, showing its phenotypic plasticity. It is able to reduce energy expenditure at high temperatures, which has been suggested to be a selective advantage for *C. glaucum* over *C. edule* in the lagoon environment, where animals may be subjected to prolonged periods of high temperatures in summer (Wilson and Elkaim 1997). In the present study marked physiological differences were found among populations of *C. glaucum*, as well as within populations among seasons.

On the other hand, the present study revealed a high level of genetic structuring among *C. glaucum* populations. The observation of a high genetic differentiation (at neutral loci) proves that local adaptation is possible since gene flow can be limited.

As for the reduced variability within northern populations, the adaptation to local environmental conditions is also possible. Johannesson and André (2006) claimed that the genetic variability of the Baltic Sea populations of various species is reduced, not only due to founder effect and bottlenecks, by mainly due to the adaptive selection to this extreme habitat. If the northern populations of *C. glaucum* are adapted to local conditions (they are not general purpose genotypes), then low levels of genetic diversity reduce the populations' ability to respond to environmental changes and increase the probability of extinction (Caro and Laurenson 1994). Therefore, in case of an environmental conditions change due to climate change or some local perturbations, this low genetic diversity (if resulting from local adaptations) can make northern populations more susceptible and threatened with extinction than southern ones.

To conclude, there is probably a considerable degree of adaptation to different environmental conditions among isolated populations of *C. glaucum*, but the capacity of coping with rapid changes of environmental conditions within habitats is possible mainly due to a high level of genetic plasticity associated with general purpose genotypes.

Another interesting aspect linking physiology and genetics of *C. glaucum* is that southern populations spawn several times a year and the selective pressures are distinct according to spawning time. Therefore, if natural selection is responsible for adaptation to this changing environment, southern populations should remain genetically more diverse than northern ones (on the selected loci), since at a given time individuals are not sensible to the same factors, because they are in the different stage of a life cycle.

14.2 Conclusions for the future studies on lagoons

There are few studies on the genetic population structure of lagoon specialists, but the majority has shown a high level of genetic divergence among populations (Johnson and Black 1998; Darling *et al.* 2004; Astolfi *et al.* 2005). In many cases no correlation between genetic and geographic distances was found (Pearson *et al*. 2002; Jolly *et al.* 2003; González-Wangüement *et al.* 2006). However, sometimes a significant correlation between genetic differentiation and geographic distance indicating some migration between

locations was proven (e.g. Congiu *et al.* 2002; Astolfi *et al.* 2005). In isolated populations forces such as genetic drift (Städler and Jarne 1997) and selection will exert a strong influence on the genome resulting in lineage generation and divergence from source populations located in the adjacent marine environment. Therefore, lagoons are potentially exciting environments to explore mechanisms of evolution and speciation in marine organisms. Unlike many other "extreme" environments, lagoons are easy to access and may be subject to long-term studies analyzing changes in the size and genetic composition of populations over time. Many lagoon species are also tolerant of long-term study in laboratory aquaria allowing controlled manipulation of the physical conditions in which they live.

Those extreme and fascinating habitats are also delicate and ephemeral. Small, isolated populations are subject to extreme variations in physical environmental factors that may also result in marked temporal changes in population size, making those populations vulnerable to bottlenecks or even extinction. The most probable causes of reduced variation and bottlenecks are inbreeding and genetic drift combined with insufficient time since the reduction in effective population size (associated with initial founding event) for either mutation or/and selection to act. The effects of bottleneck on the genetic structure will depend on population size, the size of the bottleneck and the recovery rate from the bottleneck (Chakraborty and Nei 1977).

There is not sufficient information to provide relevant strategies for management and conservation of lagoons and its species. It is important to note that lagoons are dynamic systems, differing from one another in respect of physico-chemical regimes. Therefore, it is not possible to use the data from one lagoon for the conservation of a given species in all lagoons (Gates 2006). At the same time the species inhabiting lagoons first need an individual approach and many of them should be studied to enable to find some general patterns applicable in lagoon species conservation. Lagoon management strategies should involve a continued monitoring of environmental conditions, such as water temperature and salinity. An important consideration for the conservation of any habitat is protection from the anthropogenic influences. The enclosed nature of lagoons and many terrestrial influences places them at a higher risk from anthropogenic activities than most marine habitats. For example Mar Menor lagoon in Spain is subject to metal pollution after heavy rainfall as a result of run-off from nearby mining facilities (Marin-Guirao *et al.* 2005). There is a reduced possibility if dispersal of polluting agents in lagoons, in comparison to

estuaries or the open sea, so the lagoons organisms are subject to higher concentrations of pollutants for longer periods of time than their marine counterparts, in similar circumstances. Such events can cause devastating effects on lagoon communities.

15 Summary of the most important results and conclusions

- The subdivision into Atlantic and Mediterranean species or subspecies should definitely be abandoned as the main subdivision within *C. glaucum* was found in the region of the Ionian Sea.
- The lineages in the mitochondrial DNA were very deeply divergent, which suggests preglacial divergence, dating to about 1.3 mya in case of the most divergent Aegean Sea and Ponto-Caspian region haplogroup.
- The genetic structure revealed by two marker types was not congruent, which cannot be totally explained by the different properties of the two markers only.
- Populations from the Ionian Sea possessed mitochondrial DNA of an Atlanto-Mediterranean origin and a nuclear DNA of a “Ponto-Caspian-Aegean” origin.
- A possible selection event was detected on one of the microsatellite loci (*Cg11*) in the Ionian Sea.
- Genetic structures found in the Ionian Sea and in Tunisia suggest that these regions remained unaffected by glacial events, and the Ionian Sea may be a core distribution area for *C. glaucum*, from where it spread into other European seas.
- Geographic discontinuities in the population genetic structure were found with mtDNA.
- Intrapopulation variability in the northern populations was lower than in the southern populations, which was probably due to recent, postglacial colonization of these regions.
- Inbreeding has been proven in some populations, which may be connected with the fragmented nature of the habitat.
- The habitats isolation makes the dispersal by planktonic larval stage not very probable.

- Dispersal via migrating birds could play an important role in gene flow in *C. glaucum* and other marine species at different geographic scales in the past and nowadays.
- *C. glaucum* revealed strong interpopulation variablitity in respect of morphometric and physiological parameters among populations.
- The northern populations have a monocyclic reproduction cycle, whereas the southern one spawn a few times a year.
- Respiration rates in the Gulf of Gdańsk (GD) were the highest among populations, which could be due to increased metabolic level provoked by osmotic stress.
- There is probably a considerable degree of adaptation to different environmental conditions among isolated populations of *C. glaucum*, but also a high level of genetic plasticity to cope with rapid changes of environmental conditions.
- Further studies are indispensable to better understand a molecular background of adaptations to different, often extreme, lagoon habitats in *C. glaucum*.

16 Not answered questions- axes for the future studies

This study leaves many questions unanswered and encourages future studies, which could go in several directions.

16.1 Detailed study on the physiological differences among populations of C. glaucum

The physiological analysis performed in this study should be developed to better understand the functioning on *C. glaucum* in different environmental conditions. The question whether the differences among populations are the result physiological acclimatization or genetically fixed adaptation could be addressed by translocation experiments aimed at verifying how the cockle copes with the changes in water temperature and salinity, as well as in other factors, like food concentration, seasonal cycles and possible pollutants in the water. The experiment could be organized similarly to the one performed by Hummel *et al.* (2000a), where the *Macoma balthica* was translocated (wrapped in dry cloths on a top of melting ice in a cooling box) from several stations in the Netherlands to several stations in the south-west of France (at least 1000 specimens per location). At intervals of 3 months some translocated specimens were collected and the

condition index, the biochemical composition and the respiration rate were measured (see Hummel *et al.* 2000a for details). This kind of experiment performed on *C. glaucum* could be helpful to understand whether the wide tolerance of the cockle towards environmental conditions results from the presence of general purpose genotype or the capacity of rapid adaptation.

A factor worth studying is a Scope For Growth (SFG) which describes the energy available to be utilized in growth and reproduction and is considered as an indicator of stress, so might be helpful to understand both the intrinsic and the extrinsic stressors. Even lagoon specialists well adapted to seasonal changes of environmental conditions may experience great stress in case of unseasonal variation of e.g. temperature or salinity as it was proven for *Gammarus insensibilis* (Gates 2006).

16.2 Exploring other accessible genetic markers

As the genetic structures of *C. glaucum* revealed by nuclear and mitochondrial markers differed a lot, it would be advisable to perform the analysis using the additional marker, to better understand the differentiation within *C. glaucum* and the time and modes of colonization of the present distribution area.

The number of independent loci studied has a crucial importance to understand a genetic structure of the organism (Beaumont and Nichols 1996). Isolating more microsatellite loci would be advisable for future studies. The problem associated with microsatellite primers is the fact that they are often species specific, which increases the time and the cost of the research and limits microsatellites applicability (Zane *et al.* 2002). The microsatellite from this study failed to amplify loci of *C. edule*- the close relative of *C. glaucum*.

The microsatellite data are imprecise as variants are characterized by fragment length (and not sequenced), which makes them prone to size homoplasy. The value and reliability of the nuclear data results would increase, if nuclear marker studied with sequence polymorphism analysis was included. The easy and time-saving way of doing it could be studying EPIC (Exon Primed Intron Crossing) loci. Those are universal nuclear markers. Around 50 introns were found for which 93 primer pairs were designed. The PCR tests performed on a few ascidians, echinoderms and cnidarians species as well as two bivalve species (*Macoma balthica*, *C. edule*) gave promising results and tests on other species are

planned (Chenuil *et al.*- in preparation). Some tests performed on *C. glaucum* allowed to isolate promising EPIC loci. Introns have many advantages when compared to microsatellites. EPICs are much less prone to null alleles, since primers are positioned in exons. They are also much less susceptible to homoplasy and after sequencing the variants, evolutionary relationships among alleles can be much more accurately reconstructed. Finally, if the intron loci are universal, there is no need to develop new marker for each species, which enables to save time and money (Zhang and Hewitt 2003).

16.3 Studies on the possible dispersal of marine organisms by birds

The knowledge of the lagoon colonization and the gene flow among lagoons is crucial for establishing lagoons management strategies. In this study I suggested the role of migrating birds in the dispersal of *C. glaucum* as well as other lagoon and marine species. Although there are some previous studies stressing the importance of this phenomenon, direct proofs are missing. The joined effort of migrating birds' specialists and molecular biologists is needed to approach these problems. First of all, it needs to be checked if *C. glaucum* (not just *C. edule*) is found attached to bird's feet. If such specimens were found, it should be verified, whether they come from distant regions (by sequencing the DNA fragment or genotyping a specimen). Some experiments could be performed as well, like the test of survival time of *C. glaucum* during the aerial exposure.

16.4 Comparing C. glaucum *with its closest marine counterpart* C. edule

In order to determine how lagoon specialists differ from their freshwater, estuarine or marine congeners, comparative studies are necessary. Detailed comparisons of individuals from a range of similar species (preferably congeners) from within lagoons, just outside lagoons and further away may provide insights into the adaptations that allow an organism to specialize in the lagoon habitat. This is especially the case, where closely related marine and lagoon sister species can be directly compared with respect to changes in the genome and proteome leading to alterations in phenotypes. Genomic approaches to study the bases for differences in tolerance of variation in physical conditions between *C. glaucum* and the sister species *C. edule* may be instructive in understanding the evolution of lagoon species. It could also enable to better understand whether adaptations favoring generalist genotypes or niche specialization are the features of invasion of marginal habitats.

16.5 More detailed combined studies on physiology and genetics of the cockle

The results obtained in the present study could also be instructive for the choice of populations for the future research of the similar type. First of all, the physiological parameters should be investigated monthly, not only seasonally, and more populations from different regions should be included. The future physiological studies should be performed on genetically divergent populations. The Ionian Sea populations seem particularly interesting for studying physiology, because of their distinct genetic character (nuclear and mitochondrial DNA of different origins) and a possible positive selection, which has been detected. It would also be interesting to perform physiological studies on genetically close populations living under different environmental conditions (like from the Baltic Sea and Portugal according to mtDNA).

Summary in Polish / Streszczenie

Struktura genetyczna oraz fizjologiczne zróżnicowanie europejskich populacji sercówki *Cerastoderma glaucum* (Bivalvia) występującej w ekstremalnych warunkach środowiska

1 Wstęp

1.1 Cele pracy

Struktura genetyczna 20 populacji sercówki (*Cerastoderma glaucum*) została przeanalizowana w oparciu o fragment mitochondrialnego DNA (podjednostki 1 oksydazy cytochromowej, COI) oraz 4 loci mikrosatelitarne, w celu:

- określenia akwenu pochodzenia tego gatunku, refugiów lodowcowych oraz dróg kolonizacji polodowcowej,
- porównania zróżnicowania genetycznego populacji z akwenów północnej i południowej Europy,
- określenia wpływu izolacji geograficznej populacji na strukturę genetyczną,
- przedstawienia hipotezy dotyczącej wektorów rozprzestrzeniania się sercówki.

Ponadto, przeanalizowano różnice parametrów morfometrycznych i fizjologicznych populacji zamieszkujących zbiorniki wodne o bardzo zróżnicowanych warunkach środowiska. Uzyskane dane genetyczne i fizjologiczne pozwoliły na charakterystykę strategii adaptacyjnych umożliwiających sercówce kolonizację i przetrwanie w ekstremalnych warunkach środowiskowych.

1.2 Laguny jako specyficzne środowisko życia

Laguny przybrzeżne to płytkie akweny częściowo lub całkowicie oddzielone od morza, utworzone w wyniku naturalnych procesów lub w wyniku działalności człowieka (Kjerfve 1994). Charakteryzują się one dużą zmiennością fizycznych i chemicznych parametrów środowiskowych (Bamber i in. 1992; Downie 1996). Współczesna kolonizacja lagun przez organizmy zachodzi między innymi w wyniku działalności antropogenicznej lub poprzez transport organizmów na przykład przez ptaki migrujące (Barnes 1988). Populacje zamieszkujące laguny najczęściej są zakładane przez niewielką liczbę

osobników, a z powodu izolacji geograficznej przepływ genów między sąsiadującymi populacjami jest ograniczony. Sprzyja to procesom lokalnej selekcji, a także dryfu genetycznego oraz inbredu (Slatkin 1981; Frankham 1995).

Laguny to unikalne ekosystemy, w których występują zespoły gatunków nieobecne w innych biotopach (Barnes 1980; Barnes 1991; Bamber i in. 1992). Są one obszarami żerowania, rozrodu i odpoczynku wielu gatunków migrujących ptaków wodnych (Razinkovas i in. 2008) oraz są ważne z socjo-ekonomicznego punktu widzenia. Działalność antropogeniczna (Spellerberg 1996), a także zmiany warunków klimatycznych (Viaroli i in. 2007) mają istotny wpływ na funkcjonowanie tych ekosystemów.

1.3 Charakterystyka gatunku

Sercówka (Rys. 3.1) występuje w Morzu Bałtyckim, w Morzu Północnym, na wybrzeżu atlantyckim zachodniej Europy, w Morzu Śródziemnym, w Morzu Czarnym, w Morzu Kaspijskim i w Jeziorze Aralskim (Høpner Petersen i Russell 1973). Gatunek ten toleruje temperatury od 0°C do ponad 45°C (Zaouali 1974) i zasolenia między 5 a 84 PSU (Rygg 1970; Labourg i Lasserre 1980). Najczęściej występuje on w izolowanych lagunach, estuariach, zatokach i jeziorach (Kingston 1974). *C. glaucum* rzadko zamieszkuje strefy pływów, ponieważ nie toleruje dobrze wysortowanego, gruboziarnistego piasku, który jest osadem typowym dla akwenów o dużej dynamice wód (Brock 1979).

Sercówka jest gatunkiem gonochorycznym. Temperatura wody odgrywa ważną rolę w procesie gametogenezy (Rygg 1970). W cyklu życiowym *C. glaucum* występują planktonowe stadia larwalne, których okres życia trwa około tygodnia lub nieco dłużej i zależy od temperatury wody. Stadia larwalne blisko spokrewnionego gatunku, sercówki jadalnej *Cerastoderma edule*, pozostają w toni wodnej nawet do pięciu tygodni (Barnes 1980).

Cerastoderma glaucum jest filtratorem. Odżywia się mikroflorą bentosową (głównie okrzemkami), a także detrytusem pochodzącym z morskich roślin naczyniowych, oraz makroglonów (Høpner Petersen 1958; Beukema i Cadée 1996; Sarà 2007).

Pierwsze znane materiały fosylne rodzaju *Cerastoderma* pochodzą z oligocenu (33,9-23,0 milionów lat temu) z obszaru Morza Tetydy (Keen 1969). Linie ewolucyjne *C. glaucum* i *C. edule* rozdzieliły się, gdy doszło do izolacji Morza Śródziemnego od Atlantyku między miocenem, a pliocenem (około 4 miliony lat temu) (Rygg 1970).

Potwierdzają to pochodzące z pliocenu materiały fosylne z Libii i Tunezji (Gaillard i Testud 1980; Wołowicz 1991). W wyniku izolacji Morza Śródziemnego powstało wiele lagun o zmiennych, często bardzo zróżnicowanych warunkach termicznych i zasoleniowych (kryzys messyński) (CIESM 2008). Poddana silnej presji zmiennego środowiska sercówka przetrwała w tych ekstremalnych habitatach, a gdy na początku pliocenu połączenie między Morzem Śródziemnym, a Atlantykiem otworzyło się ponownie (Ruggieri 1967), nie krzyżowała się już z *C. edule*.

2 Morfometria i fizjologia populacji *Cerastoderma glaucum*

2.1 Wstęp

Małże są często gatunkami kluczowymi w lagunach, estuariach i strefie płytkowodnej (Gili i Coma 1998). Ze względu na ich liczebność i stałość populacji, pozycję w sieci troficznej, duży zasięg występowania i wrażliwość na zmiany warunków środowiska, wykorzystywane są często do badań monitoringowych (Goldberg i in. 1978). Zmiany, które mogą zachodzić w populacji (m.in. w wielkości populacji, tempie wzrostu osobników, zasięgu występowania), mogą w istotny sposób oddziaływać na funkcjonowanie tych ekosystemów (Norkko i in. 2001; Dahlhoff i in. 2002; Ellis i in. 2002; Newell 2004).

2.2 Materiały i metody

Analizy morfometryczne i fizjologiczne sercówki (Tabela A) przeprowadzono sezonowo na 30 osobnikach z trzech europejskich populacji (Rys. 5.1-5.5):

-Zatoka Gdańska (Morze Bałtyckie, Polska) (GD), analizy przeprowadzono w listopadzie 2005, lutym, maju, lipcu oraz październiku 2006,

-Jezioro Veere (Morze Północne, Holandia) (LV), analizy przeprowadzono w listopadzie 2006, lutym, maju oraz sierpniu 2007,

- Laguna Berre (Morze Śródziemne, Francja) (BL), analizy przeprowadzono w lutym, maju, sierpniu oraz listopadzie 2007.

Analizy statystyczne wykonywano za pomocą programu STATISTICA v. 8. Istotność różnic statystycznych w proporcji płci przetestowano testem χ^2. Normalność danych została sprawdzona za pomocą testu Shapiro-Wilka na poziomie istotności 5%. Test Kruskala-Wallisa, który jest nieparametrycznym odpowiednikiem ANOVA, zastosowano w celu sprawdzenia istotności statystycznej różnic suchej masy tkanki, długości muszli oraz wskaźnika kondycji.

Tabela A. Zestawienie przeprowadzonych analiz środowiskowych, morfometrycznych i fizjologicznych.

Parametry środowiskowe	• temperatura i zasolenie w każdym z punktów poboru, w każdym sezonie
Parametry morfometryczne	• długość muszli • sucha masa tkanki miękkiej (po zliofilizowaniu) • sucha masa muszli (po wysuszeniu w 55°C) • zależność między suchą masą muszli/tkanki, a długością muszli dla każdej populacji; $W = a \times L^b$, W- sucha masa muszli/tkanki (g), L- długość muszli (mm), a, b- parametry równania
Wskaźnik kondycji	• $CI = (M_c \times 1000)/L^3$, M_c- sucha masa tkanki (mg); L- długość muszli (mm) (Beukema i De Bruin 1977)
Biologia rozrodu	• stadium rozwoju gonad- skala 5-stopniowa (Wołowicz 1979; Wołowicz 1987) • indeks gonad GI = [∑ (liczba osobników w danym stadium rozwoju gonad × wartość numeryczna tego stadium)/całkowita liczba osobników w próbie)] (Tabela 5.1) (Chipperfield 1953)
Skład biochemiczny	• zawartość białek (Lowry i in. 1951), tłuszczów (ekstrakcja- Bligh i Dyer 1959, pomiar- De Marsh i Weinstein 1966), cukrów i glikogenu (Dubois i in. 1956) w suchej masie tkanki miękkiej
Pomiar tempa respiracji (na 5-10 osobnikach dla LV i BL, oraz na około 20 dla GD)	• 3 komory do pomiaru tempa respiracji i 1 komora kontrolna, 4 temperatury: 4°C, 10°C, 17°C, 24°C (± 0.2°C) • pomiar za pomocą elektrod (YSI 5331 oxygen probe)

2.3 Wyniki i dyskusja

2.3.1 Parametry morfometryczne i wskaźnik kondycji

Sercówki z GD były najmniejsze z badanych populacji. Zjawisko to zwane pauperyzacją jest typowe dla zbiorników o niskim zasoleniu, jakim jest Morze Bałtyckie (Tabela 6.1) (IMGW 2009). Niskie zasolenie powoduje stres osmotyczny i zwiększony wydatek energetyczny na osmoregulację, co skutkuje m. in. spowolnieniem wzrostu rozmiarów ciała (Schmidt-Nielsen 1990) (Rys. 6.1). Różnice długości muszli i suchej masy ciała pomiędzy populacjami były istotne statystycznie ($p < 0,001$), natomiast w obrębie analizowanych populacji w różnych sezonach, nie stwierdzono różnic statystycznie istotnych ($p > 0,01$) (Załącznik 1). Wartości wskaźnika kondycji różniły się istotnie zarówno między populacjami ($p < 0,001$), jak i w obrębie populacji w różnych sezonach ($p < 0,001$; w przypadku BL $p < 0,01$). Na kondycję organizmu ma wpływ zarówno tempo wzrostu i poziom metabolizmu, jak i stadium cyklu rozrodczego (Lucas i Beninger 1985; Smaal i Stralen 1990; Hummel i in. 2000b). W GD obserwowano spadek wartości wskaźnika kondycji (*CI*) latem związany z uwolnieniem gamet i rozrodem. Jesienią wartość wskaźnika kondycji była wyższa, co można wiązać z intensywnym odżywianiem małży podczas jesiennego zakwitu okrzemek w Zatoce Gdańskiej (Pliński 1995). W LV pomiędzy zimą a wiosną wartość *CI* wzrosła w wyniku intensywnego rozwoju gamet i przygotowania do rozrodu. Wiosennego wzrostu wartości wskaźnika kondycji nie zaobserwowano w GD. U wielu gatunków małży z północnej Europy notowano najwyższe wartości wskaźnika kondycji wiosną, przed rozrodem (*C. glaucum*, Wołowicz 1991; *M. balthica*, Hummel i in. 2000b; *Mytilus trossulus*, Wołowicz i in. 2006). W LV latem wartość *CI* była niewielka, choć przeprowadzone badania stadium rozwoju gonad dowiodły, iż większość osobników znajdowała się w stadium rozrodczym (IV). Jednak należy przypomnieć, że stadium rozwoju gonad i wskaźnik kondycji analizowane były na różnych osobnikach, które w przypadku pomiarów wskaźnika kondycji mogły być już po rozrodzie. Wysoka wartość wskaźnika kondycji sercówki w BL zimą, może mieć związek ze sprzyjającymi warunkami troficznymi (Gouze i in. 2005).

2.3.2 Elementy biologii rozrodu

Udział płci w badanych populacjach nie różnił się statystycznie od 1 (χ^2, $p > 0,05$). Również zaobserwowane niewielkie różnice sezonowe w proporcji płci nie były istotne

statystycznie (p > 0,05). U niektórych gatunków małży stwierdzono zwiększoną śmiertelność samic po rozrodzie, związaną prawdopodobnie z wysokim kosztem energetycznym dojrzewania gonad i uwalniania gamet (Brokordt i Guderley 2004; Wołowicz i in. 2006). W populacjach północnych (GD, LV) rozród następował tylko raz w roku, latem, co potwierdzają wcześniejsze wyniki (Rygg 1970; Wołowicz 1991), przy czym w LV następował on później niż w GD (Rys. 6.3). W populacji południowej (BL) trudno wyodrębnić okres rozrodu, ponieważ rozradzające się sercówki obserwowano przez cały rok. Potwierdzają to badania Ivella (1979), który stwierdził, że w lagunach Morza Śródziemnego rozród może następować nawet 3 razy w ciągu roku. Rozród sercówki jest ściśle związany z temperaturą wody i rozpoczyna się dopiero, gdy osiągnie ona 15-17°C, co w akwenach północnych (GD, LV) następuje późną wiosną (Tabela 6.1) (IMGW 2009; RIKZ i RIZA 2009). A zatem im bardziej na północ występuje populacja, tym później następuje rozród (Kingston 1974). Oprócz temperatury wody, istotny wpływ na okres rozrodu ma również dostępność pożywienia (Kingston 1974; Honkoop i Van der Meer 1998).

2.3.3 Skład biochemiczny

Zmiany w składzie biochemicznym tkanek miękkich małży są powiązane ze zmianami stadium rozwoju gonad (Sastry 1979; Pazikowska i Szaniawska 1988; Pazos i in. 1997; Bawazir 2000). Sercówka należy do małży "oportunistycznych" (Bayne 1976), które zimą przechodzą okres rozrodczego "spoczynku", a gametogeneza rozpoczyna się wiosną, gdy w środowisku jest wystarczająco dużo pożywienia. Skład biochemiczny tkanek związany jest nie tylko ze stanem fizjologicznym organizmu, ale zależy również od aktualnych warunków troficznych. Gdy są one niesprzyjające, dochodzi najpierw do katabolizmu cukrów, potem tłuszczów, a na koniec białek strukturalnych (Beninger i Lucas 1984).

Zawartości białek (37,5-77,4%) i tłuszczów (5,7-13,6%) w tkankach miękkich (Rys. 6.5a-b) były zbliżone do wartości uzyskanych przez Wołowicza (1991). Największe wartości w populacjach z północnej Europy odnotowano wiosną, natomiast w populacjach z południa Europy sezonowe zmiany były niewielkie. Zawartość białek i tłuszczów w GD była większa przed rozrodem, a po rozrodzie malała. Prawdopodobnie wynika to z tego, iż związki te stanowią główny składnik oocytów (Gabbott 1975). Białka są syntezowane w

trakcie gametogenezy, podczas gdy tłuszcze pochodzą w większości z tkanek somatycznych (Holland 1978). W LV odnotowano niewielkie sezonowe zmiany zawartości tłuszczów. Ich zawartość nie wzrastała wiosną, pomimo intensywnego rozwoju gamet. W BL, mimo braku wyraźnego okresu rozrodczego, zawartość białek i tłuszczów malała między wiosną a latem, co może być wynikiem niekorzystnych warunków troficznych.

Zawartość cukrów wynosiła 4,1-16,1%, a glikogenu 2,8-13,3% (Rys. 6.5c-d). W GD najniższą zawartość cukrów i glikogenu w tkankach odnotowano latem, ponieważ wykorzystywane są one w procesie gametogenezy (Gabbott 1975). Powodem wzrostu zawartości cukrów w populacjach z północnej Europy (GD, LV) między latem, a jesienią były lepsze warunki troficzne, a zwłaszcza jesienny zakwit fitoplanktonu (Pliński 1995; Wasmund i in. 1998). W BL odnotowano jedynie niewielkie różnice sezonowe zawartości cukrów, związane najprawdopodobniej z chwilową zmianą nasilenia rozrodu oraz warunków troficznych (Wołowicz 1991). Mały zakres sezonowych zmian zawartości tłuszczów i cukrów w LV mógł być spowodowany faktem, iż analizy składu biochemicznego były wykonywane bez oznaczania płci osobników oraz bez podziału na grupy wiekowe (Newell i Bayne 1980; Navarro i in. 1989; Wołowicz i in. 2006).

Suma zawartości białek, tłuszczów oraz cukrów w tkankach miękkich nie wynosiła 100%, ponieważ pozostałą część stanowił popiół, kwasy nukleinowe, wolne aminokwasy tworzące 7-12% suchej masy (Pieters i in. 1980) oraz woda stanowiąca 2-3% suchej masy (Beukema i De Bruin 1979).

2.3.4 Tempo respiracji

Tempo respiracji odzwierciedla aktywność metaboliczną organizmu i jest proporcjonalne do chwilowego zapotrzebowania na ATP (Bayne i Newell 1983; Clarke 1991). Wpływ na aktywność metaboliczną, ma między innymi temperatura otoczenia (Clarke 1991), a także rozmiar ciała (Dame 1996). U niektórych gatunków małży, poza metabolizmem tlenowym, w sytuacji stresu energia może być pozyskiwana na drodze metabolizmu beztlenowego (Liu i in. 1990; Oeschger 1990).

W analizowanych populacjach tempo respiracji było najmniejsze w temperaturze $4^{o}C$. W populacjach LV i BL było ono zbliżone do wartości notowanych wcześniej u *C. glaucum* (Wilson i Elkaim 1997) oraz u innych małży (McMahon i Wilson 1981; Wilson i

Davis 1985; Wilson 1990; Wilson i Elkaim 1991; Hummel i in. 2000b). Natomiast w GD tempo respiracji było wyjątkowo wysokie (Rys. 6.6), czego powodem mogło być zwiększone tempo metabolizmu wywołane stresem osmotycznym związanym z niskim zasoleniem (Engel i in. 1975; Newell 1979). W GD i LV wiosną tempo respiracji było duże i małże były wrażliwe na wzrost temperatury wody. Intensywna gametogeneza w tych populacjach mogła skutkować wzrostem tempa metabolizmu wiosną. U małży w trakcie rozrodu tempo oddychania może wzrosnąć o 30-80% (De Vooys 1976; Iglesias i Navarro 1991). Zarówno w GD, jak i w LV tempo respiracji malało latem, gdy u większości osobników rozród został zakończony. Wzrastało ono ponownie jesienią, co mogło być spowodowane intensywną filtracją związaną z zakwitem fitoplanktonu (Wołowicz 1991). Zimą tempo oddychania w tych populacjach było małe. Tempo respiracji u innego gatunku sercówki *Cerastoderma edule* (z Anglii) było najniższe zimą, w okresie grudzień-marzec (Newell i Bayne 1980). Tempo respiracji w populacji BL, która doświadcza wysokich temperatur wody latem, było większe zimą niż latem (z wyjątkiem pomiaru w temperaturze 24°C), co również wykazano u *C. glaucum* z południowo-zachodniego wybrzeża Francji (Arcachon) i zinterpretowano jako adaptację mającą na celu zmniejszenie wydatków energetycznych w okresach wysokich temperatur wody (Wilson i Elkaim 1997). Z drugiej strony, w BL w temperaturze 24°C, która jest zbliżona do temperatury wody o tej porze roku, tempo respiracji było największe latem, a zatem wyniki tych badań nie udowadniają tej adaptacji u *C. glaucum*.

<u>*2.4 Wnioski*</u>

Cerastoderma glaucum jest gatunkiem eurytopowym zaadaptowanym nie tylko do ekstremalnych warunków środowiska, jak niskie zasolenie w Morzu Bałtyckim czy bardzo wysokie temperatury wody lagun śródziemnomorskich, ale przede wszystkim warunków niestabilnych i szybko zmiennych. Takie zmienne warunki środowiska w Lagunie Berre odpowiedzialne są za nagłe zmiany dominujących gatunków makrobentosowych, często również za ich wymieranie. Sercówka wydaje się stanowić stabilny element zoobentosu tej laguny (Stora i in. 1995; Stora i in. 2004), co może być dowodem odporności tego gatunku na ekstremalne lub niestabilne warunki środowiskowe. Wykazano istotne różnice fizjologiczne między populacjami *C. glaucum* (Wołowicz 1987; Wołowicz 1991; Wilson i Elkaim 1997) będące efektem aklimatyzacji i prawdopodobnie również lokalnych adaptacji genetycznych (Nevo 1978; Brock i Wołowicz 1994).

3 Struktura genetyczna populacji *Cerastoderma glaucum*

3.1 Wstęp

U gatunków morskich na zróżnicowanie genetyczne oraz przepływ genów między populacjami wpływa wiele czynników, takich jak tolerancja na warunki środowiska, cykl życiowy oraz historia geologiczna i klimatyczna obszaru występowania gatunku. Zatem nawet u gatunków z planktonową fazą życia w okresie ontogenezy, struktura genetyczna jest rzadko homogeniczna (Gilg i Hilbish 2003; Veliz i in. 2006). U gatunków występujących w północnej Europie, na obecną strukturę genetyczną istotny wpływ miały plejstoceńskie zlodowacenia (Quesada i in. 1995; Pannacciulli i in. 1997; Luttikhuizen i in. 2003a) (Rys. 9.1).

3.2 Materiały i metody

Struktura genetyczna 20 populacji sercówki (*C. glaucum*) (Rys. 10.1, Tabela 10.1) była badana w oparciu o mitochondrialne DNA i 4 loci mikrosatelitarne (Tabela B).

Tabela B. Zestawienie przeprowadzonych analiz genetycznych sercówki.

	Typ markera	
	Mitochondrialne DNA (Rys. 9.3)	**Mikrosatelitarne DNA** (Rys. 9.4, 9.5)
Ekstrakcja DNA	QIAamp DNA Mini Kit (QIAGEN); 22 - 31 osobników z każdej populacji (z wyjątkiem BK- 6 osobników)	
Startery	F:CTAYCTAGCTTTTTGAGCGGG R:CACCWCCCCCAACTGGATCGA	*Cg4*- F:GTGTTGGACTCGCCATACC* R:GACACAAGTAAAAACAATGTCT *Cg7*- F:GATCCAGCCGTTCAAGTCC* R:CGAAATAATGCGCGATGC *Cg9*- F:CCATATTACCACTGCCCACAC* R:TGACCCCCTCCAGTGATTC *Cg11*- F:GGGGCGATTCTGGAGTAGTAG* R:GTCAAACCAGGCGCTAAGTC (Pearson 2003) *startery znakowane fluorescencyjnie
Amplifikowany fragment	Fragment oksydazy cytochromowej (COI) o długości 580 par zasad (analizowano fragment o długości 514 par zasad)	*Cg4*- $(GT)_n$ *Cg7*- $(GT)_n$ *Cg9*- $(GACA)_n$ *Cg11*- $(C)_n(CA)_n$
Program PCR	4’94°C, 35×(30’’94°C, 30’’65°C, 1’72°C)	*Cg4*- 2’94°C, 25×(30’’94°C, 30’’50°C, 1’72°C), 20’72°C; *Cg7*- 2’94°C, 25×(30’’94°C, 30’’55°C, 1’72°C), 20’72°C; *Cg9*- 2’94°C, 30×(30’’94°C, 30’’45°C, 1’72°C), 20’72°C; *Cg11*- 2’94°C, 25×(30’’94°C, 30’’50°C, 1’72°C), 20’72°C;
Skład mieszaniny reakcyjnej PCR	Objętość 25 µl: 1.5 µl roztworu ekstraktu DNA, 1 µM każdego ze starterów, 265 µM trifosforanów deoksyrybonukleozydów, bufor 1×PCR, 2.5 mM $MgCl_2$, 0.65 jednostek polimerazy DNA GoTaq Flexi	Objętość 10 µl: 0.7 µl roztworu ekstraktu DNA, 1 µM każdego ze starterów, 265 µM trifosforanów deoksyrybonukleozydów, bufor 1×PCR, 1.675 mM (dla *Cg4* i *Cg7*) lub 2 mM (dla *Cg9* i *Cg11*) $MgCl_2$, 0.25 jednostek polimerazy DNA GoTaq Flexi

Kontynuacja **Tabeli B**

	Typ markera	
	Mitochondrialne DNA	**Mikrosatelitarne DNA**
Analizy statystyczne struktury genetycznej między populacjami	• sieć haplotypów- metoda łączenia wektorów mediany, program NETWORK v.4.5 (Bandelt i in. 1999) • F_{ST} (Weir i Hill 2002) dla par populacji, program ARLEQUIN v.3.1 (Excoffier i in. 2005) • reprezentacja graficzna F_{ST} za pomocą MDS (skalowanie wielowymiarowe), program XLstat 7.5.2 • wybranie najbardziej prawdopodobnego modelu ewolucyjnego dla mitochondrialnego DNA (Posada i Crandall 1998) • drzewo filogenetyczne haplotypów- metoda największego prawdopodobieństwa (Guindon i Gascuel 2003), korzeń- sekwencja *C. edule* • procent zróżnicowania między dwoma głównymi grupami haplotypów, program MEGA 4 (Tamura i in. 2007) • oszacowanie czasu dywergencji w obrębie blisko spokrewnionych grup haplotypów z odległych geograficznie populacji (symulacja Monte Carlo w oparciu o łańcuch Markowa), program IM (Nielsen i Wakeley 2001)	• nierównowaga sprzężeń pomiędzy parami loci mikrosatelitów (Weir 1979), program GENETIX v.4.02. (Belkhir i in. 1998) • drzewo filogenetyczne- metoda najbliższego sąsiada (Saitou i Nei 1987), dystans na podstawie modelu Cavalli-Sforza i Edwards (1967), zestaw programów PHYLIP 3.6 (Felsenstein 2005) • F_{ST} (Weir i Cockerham 1984) dla par populacji dla pojedynczych i wszystkich loci, program GENETIX v.4.02, korekta poziomu istotności metodą Benjamini i Hochberg (1995) • reprezentacja graficzna F_{ST} za pomocą MDS (skalowanie wielowymiarowe), program XLstat 7.5.2 • wykrywanie obecności selekcji (metoda "F_{ST}-outlier"), program LOSITAN (Beaumont i in. 1996; Antao i in. 2008) • badanie struktury genetycznej przy użyciu metody bayesowskiej, w której osobniki nie są przydzielane do populacji *a priori*, program STRUCTURE (Pritchard i in. 2000; Falush i in. 2003; Evanno i in. 2005); uśrednienie wyników z wielu powtórzeń, program CLUMPP v.1.1.1 (Jakobsson i Rosenberg 2007); graficzne opracowanie wyników, program DISTRUCT v.1.1 (Rosenberg 2004)

Kontynuacja **Tabeli B**

	Typ markera	
	Mitochondrialne DNA	**Mikrosatelitarne DNA**
Analizy statystyczne struktury genetycznej wewnątrz populacji	• liczba miejsc segregujących (*S*), różnorodność haplotypowa (H_d) (Nei 1987), różnorodność nukleotydowa (Π) (Nei 1987; Nei i Miller 1990), "testy neutralności" *D* Tajima (1989) oraz *D** i *F** (Fu i Li 1993), program DNASP v.4.10.9 (Rozas i in. 2003)	• obserwowana (H_{obs}) i oczekiwana heterozygotyczność (H_{nb}, estymacja nieobciążona) (Nei 1978) i F_{IS} (Weir i Cockerham 1984), program GENETIX v.4.02 • liczba alleli obecnych tylko w jednej populacji • test na obecność inbredu, program RMES (David i in. 2007)
Inne analizy	tłumaczenie sekwencji nukleotydów na aminokwasy, program MEGA 4	
Porównanie wyników z 2 typów markerów	• test istotności korelacji F_{ST} dla par populacji dla obydwu typów markerów- test Mantela (Mantel 1967; Smouse i in. 1986), program ARLEQUIN v.3.1 • analiza grup w obrębie badanych populacji i ich zgodność między dwoma typami markerów- analiza wariancji molekularnej (AMOVA, Excoffier i in. 1992)	

3.3 Wyniki i dyskusja

Nie wykryto nierównowagi sprzężeń pomiędzy loci mikrosatelitarnymi. Liczba alleli mikrosatelitów wynosiła 42 dla *Cg4*, 25 dla *Cg7*, 63 dla *Cg9* oraz 24 dla *Cg11* (Załącznik 5 i 6). W większości populacji zaobserwowano deficyt heterozygot (istotne statystycznie F_{IS}). Przynajmniej w pięciu populacjach, trzech z Morza Śródziemnego (SE, SM, TU) i dwóch z Morza Czarnego (RO, UK), ten deficyt nie był spowodowany obecnością alleli zerowych, lecz miał przyczyny biologiczne, jak inbred, efekt Wahlunda (Castric i in. 2002) lub selekcja eliminująca heterozygoty (Tabela 11.7). W małych, izolowanych populacjach często dochodzi do inbredu (Frankham 1995; Madsen i in. 1996; Lacy 1997) i według Pearson (2003) to właśnie fragmentacja habitatu jest przyczyną inbredu u sercówki.

Po przetłumaczeniu sekwencji mtDNA na aminokwasy wykazano, że wszystkie mutacje za wyjątkiem jednej były synonimiczne. Zaobserwowano 90 haplotypów będących wynikiem 100 mutacji w 92 miejscach polimorficznych (Załącznik 2, 3 i 4). Tempo dywergencji u sercówki oszacowane na podstawie mtDNA wyniosło 5% na milion lat, co oznacza, że tempo substytucji wynosi 2.5% na milion lat (Nikula i Väinölä 2003).

Wykazano duże zróżnicowanie genetyczne pomiędzy populacjami *C. glaucum*, sugerujące ograniczony przepływ genów, który może wynikać z izolacji geograficznej populacji (Rys. 11.1-11.4, Tabela 11.1-11.2). Początki dywergencji między haplogrupami sercówki mogły mieć miejsce jeszcze przed okresem zlodowaceń, a różnice zostały pogłębione przez brak przepływu genów między refugiami lodowcowymi, co zaobserwowano u wielu morskich gatunków (np. Coyer i in. 2003; Luttikhuizen i in. 2003a; Papadopoulos i in. 2005; Provan i in. 2005; Pérez-Losada i in. 2007).

Analizy oparte na dwóch typach markerów wykazały różnice w strukturze genetycznej populacji sercówki (Rys. 11.4, Tabela 11.1-11.2). Na podstawie badań mitochondrialnego DNA populacje z Morza Egejskiego oraz z rejonu ponto-kaspijskiego tworzyły odrębną haplogrupę wykazującą 6,6% różnicy w sekwencji DNA w stosunku do populacji zamieszkujących akweny położone bardziej na zachód (Rys. 11.1-11.3). Przepływ genów między tą grupą, a pozostałymi populacjami wydaje się bardzo ograniczony i może nawet stanowić podstawę wyodrębnienia jej jako odrębny podgatunek. Sercówka mogła skolonizować Morze Czarne pod koniec pliocenu (około 1,8 miliona lat temu), kiedy zostało uformowane połączenie między Morzem Egejskim, a Morzem Czarnym (Bacescu 1985). Dywergencja między tymi dwoma haplogrupami miała miejsce około 1,3 miliona lat temu przy zakładanym tempie dywergencji 5% na milion lat. Nikula i Väinölä (2003) tłumaczyli aktualną izolację sercówki z Morza Egejskiego i z Morza Czarnego warunkami hydrologicznymi w rejonie Morza Jońskiego. Jednak, rozprzestrzenianie się sercówki za pomocą przenoszonych z prądami morskimi planktonowych stadiów larwalnych wydaje się mało prawdopodobne między izolowanymi lagunami, więc warunki hydrologiczne mogą mieć ograniczone znaczenie dla przepływu genów u tego gatunku.

W sieci haplotypów utworzonej metodą łączenia wektorów mediany, sekwencja *C. edule* łączyła się z grupą haplotypów *C. glaucum*, do której należały populacje z Morza Egejskiego, z Morza Czarnego oraz z Morza Kaspijskiego (Rys. 11.2). Nie potwierdza to pierwotnego charakteru haplotypów z tego rejonu, gdyż powiązania filogenetyczne pomiędzy gatunkami są trudne do odtworzenia na podstawie sekwencji mtDNA (Ballard i Whitlock 2004; Moritz i Cicero 2004; Monaghan i in. 2006).

W obrębie haplotypów "atlantycko-śródziemnomorskich" wydzielono cztery wyraźne grupy haplotypów oraz heterogeniczną grupę haplotypów z Morza Jońskiego (Rys. 11.1-11.3). Centralna pozycja haplotypów z Morza Jońskiego w sieci haplotypów

oraz ich duża różnorodność nukleotydowa (Π), a także brak śladów ekspansji demograficznej sugerują, iż sercówka może pochodzić z tego akwenu. Populacje z Tunezji również wykazały wysoką różnorodność nukleotydową (Π), choć nie zajmowały centralnej pozycji w sieci haplotypów. Sercówka prawdopodobnie przetrwała w tych dwóch rejonach plejstoceńskie zlodowacenia.

DNA mikrosatelitarne wykazało dużą dywergencję między populacjami z Morza Jońskiego, a pozostałymi populacjami (Rys. 11.4b). Struktura genetyczna różni się dla każdego z 4 loci mikrosatelitarnych. Na odrębny charakter populacji z Morza Jońskiego wskazuje głównie locus *Cg11* (Rys. 11.6). Test, który porównuje wartości współczynnika utrwalenia (F_{ST}) z oczekiwaną heterozygotycznością (H_e) wykazał pozytywną selekcję działającą na locus *Cg11* (Rys. 11.5). Analizy wykonane po wykluczeniu tego locus potwierdziły ponto-kaspijski charakter jądrowego DNA sercówki z Morza Jońskiego (Rys. 11.4c), który już wcześniej zasugerowano na podstawie badań allozymów (Nikula i Väinölä 2003). Różnice w pochodzeniu dwóch genomów sercówki z Morza Jońskiego tłumaczono dawną introgresją "atlantycko-śródziemnomorskiego" mitochondrialnego DNA do jądrowego genomu pochodzenia ponto-kaspijskiego (Nikula i Väinölä 2003).

Uzyskane wyniki dowodzą, że nie ma podstaw do sugerowanego wcześniej podziału wewnątrz "kompleksu *C. glaucum*" na dwa gatunki: "śródziemnomorski" *C. glaucum* oraz "atlantycko-bałtycki" *C. lamarcki* (Brock 1987; Brock i Christiansen 1989) lub wyodrębnienia podgatunku *C. glaucum glaucum* i *C. glaucum lamarcki* (Brock 1991; Hummel i in. 1994). Wyniki te, podobnie jak badania Mariani i in. (2002) oraz Nikula i Väinölä (2003), dowodzą, że Cieśnina Gibraltarska nie stanowi głównej przeszkody dla przepływu genów między populacjami sercówki.

Na strukturę genetyczną w północnej części obszaru występowania sercówki miały wpływ zlodowacenia plejstoceńskie (Rys. 9.1). Dopiero po ich ustąpieniu rejon ten został skolonizowany. Świadczy o tym mniejsza różnorodność haplotypowa i nukleotydowa wewnątrz populacji z rejonów północnych w stosunku do populacji z rejonów południowych (mtDNA) (Rys. 11.1, Rys. 11.9, Tabela 11.5), a także większe zróżnicowanie genetyczne między populacjami północnymi w stosunku do populacji południowych widoczne w mikrosatelitach (Rys. 11.4b-c). Ponadto testy neutralności mające na celu wykrycie selekcji lub niestabilnego efektywnego rozmiaru populacji w trzech populacjach z Oceanu Atlantyckiego (PT, AR, AN) oraz w jednej z Morza Północnego (LV) dały istotne statystycznie wyniki (Tabela 11.5).

Struktura genetyczna opisana za pomocą mitochondrialnego DNA wykazała pewne nieciągłości geograficzne. Haplotypy z Portugalii (PT) i z Morza Bałtyckiego (GD, FI) należały do jednej monofiletycznej grupy. Również dywergencja między haplotypami z Sardynii (SA) i z Laguny Berre (BL), z atlantyckiego wybrzeża Francji (AN, AR) oraz z Morza Północnego (LV, AL) była bardzo niewielka (Rys. 11.1-11.3). Symulacje za pomocą programu IM wykazały, iż dywergencja pomiędzy tymi odległymi rejonami w obrębie grup haplotypów miała miejsce około 55 tysięcy lat temu. Jak wykazały badania Audzijonytė i Väinölä 2006; Waters i in. 2007 czy Peterson i Masel 2009, szacunek ten może być nieprecyzyjny, a do dywergencji mogło dojść prawdopodobnie w okresie po ustąpieniu lodowców. Wydaje się, że hipotezę o późniejszym, antropogenicznym transporcie sercówki należy odrzucić. Stwierdzone nieciągłości w strukturze genetycznej populacji mogą sugerować obecność refugiów w rejonach Portugalii i Sardynii w okresie lodowcowym. Z tych refugiów *C. glaucum* mogła skolonizować północną część swojego zasięgu występowania. Z drugiej strony, możliwe jest również, że haplotypy zbliżone do obecnych w Morzu Bałtyckim i Morzu Północnym występowały w innych allopatrycznych refugiach, z których materiałem badawczym nie dysponowano (Jaramillo-Correa i in. 2004). Struktura genetyczna opisana na podstawie badań mikrosatelitów (cztery niezależne loci w stosunku do tylko jednego locus mitochondrialnego) była dużo bardziej homogeniczna w obrębie badanych regionów (Rys. 11.8). Z badań mikrosatelitów wynika, że populacje północne są bardzo zróżnicowane genetycznie oraz, że ślady kolonizacji polodowcowej zostały zatarte. Jeżeli zatem struktura genetyczna uzyskana za pomocą badań mitochondrialnego DNA odzwierciedla drogi kolonizacji nowych obszarów, możliwe jest, że ptaki migrujące przetransportowały sercówkę między tak odległymi rejonami (Sanchez i in. 2006; Sanchez i in. 2007). W literaturze istnieją dowody na ważną rolę ptaków migrujących w transporcie bezkręgowców wodnych (De Gelas i De Meester 2005; Figuerola i in. 2005). Kolonizacja nowych habitatów poprzez planktonowe larwy sercówki nie wydaje się prawdopodobna, ze względu na nieciągłość obszaru występowania tego gatunku

Różnica w strukurze genetycznej opisanej przez dwa typy markerów może być spowodowana różnymi właściwościami tych markerów (Féral 2002; Chan i Levin 2005; Chenuil 2006). Zaletą badań sekwencji DNA, jest fakt, iż są one mniej podatne na homoplazję, niż w przypadku badań różnic długości fragmentów mikrosatelitarnego DNA między allelami i osobnikami (Estoup i Angers 1998) (Rys. 9.3-9.5).

Efektywny rozmiar populacji genomu mitochondrialnego jest cztery razy mniejszy niż genomu jądrowego (Birky i in. 1989; Mills i Allendorf 1996). Zatem różnice pomiędzy genomami w zróżnicowaniu genetycznym pomiędzy populacjami mogły być spowodowane różnymi efektami dryfu genetycznego i migracji (Ballard i Whitlock 2004). Jednak w przypadku sercówki również powiązania między populacjami różniły się (np. w genomie mitochondrialnym najbardziej odrębną grupę stanowiły populacje z Morza Egejskiego i rejonu ponto-kaspijskiego, a w genomie jądrowym, te z Morza Jońskiego), czego dowodem są wyniki testu Mantela i analizy wariancji molekularnej (AMOVA) (Tabela 11.4).

Mitochondrialne DNA z powodu braku rekombinacji jest bardziej podatne na wpływ selekcji (Gillespie 2001; Ballard i Whitlock 2004; Bazin i in. 2006) i introgresję (Currat i in. 2008) niż DNA jądrowe. Duże zróżnicowanie między haplogrupami mitochondrialnego DNA mogło być też spowodowane przez polimorfizm ancestralny, a następnie segregację linii ewolucyjnych (przypadkowe znikanie linii ewolucyjnych) (Avise i in. 1984, 1987). Ze względu na nieciągły obszar występowania tego gatunku, nie wydaje się prawdopodobne, by kiedykolwiek w Europie występowała homogeniczna populacja sercówki.

Mitochondrialne DNA jest dziedziczone w linii matki (poza nielicznymi wyjątkami, np. Gyllensten i in. 1991; Zouros i in. 1992, 1994), więc zjawiskiem, które mogło spowodować powstanie różnych struktur genetycznych w obydwu genomach mogą być różnice w zdolności rozprzestrzeniania się obu płci (Avise i in. 1987) wynikające w przypadku sercówki, na przykład z różnic w tolerancji ekspozycji na działanie powietrza lub brak pokarmu, które towarzyszą pasywnemu transportowi (np. przez ptaki migrujące lub przez działalność antropogeniczną). U innych gatunków małży różnice w strukturze genetycznej pochodzącej z dwóch genomów tłumaczono nierównymi proporcjami płci w populacjach oraz różnicami w sukcesie reprodukcyjnym między płciami (Arnaud-Haond i in. 2003b; Diaz-Almela i in. 2004).

4 Wnioski i perspektywy dla dalszych badań

4.1 Strategia umożliwiająca sercówce efektywną kolonizację i przetrwanie w ekstremalnych habitatach

Istnieją dwie główne strategie pozwalające organizmom na efektywną kolonizację nowych habitatów (Parker i in. 2003): obecność genotypów generalistycznych

(niewyspecjalizowanych) i związana z tym duża plastyczność fenotypowa (np. Bamber i Henderson 1988; Pearson i in. 2002; Lasota 2009) oraz zdolność szybkiej adaptacji (genotyp wyspecjalizowany).

Jeśli w przypadku sercówki przeważają genotypy generalistyczne, wówczas fizjologiczne różnice między populacjami żyjącymi w różnych warunkach środowiska wynikają z aklimatyzacji. Jeśli natomiast gatunek ten posiada zdolność szybkiej adaptacji, wtedy różnice te związane są również z różnicami genetycznymi.

Ze względu na dużą zmienność (nie tylko sezonową) warunków środowiskowych w habitatach sercówki, plastyczność fenotypowa organizmów jest niezbędna do przetrwania (Bamber i Henderson 1988; Bamber i in. 1992). Na przykład w Lagunie Berre *C. glaucum*, mimo częstych zmian warunków środowiskowych stanowi stały element zoobentosu (Stora i in. 1995; Stora i in. 2004). Ponadto, różnorodność genetyczna w obrębie badanych neutralnych loci tej populacji była duża, a więc nie została ona zredukowana przez niekorzystne warunki środowiska. Może to sugerować, iż podobna sytuacja ma miejsce dla reszty genomu. A zatem kierunkowa selekcja nie zredukowała różnorodności genetycznej populacji tej laguny, co świadczy o obecności genotypów generalistycznych. Jeżeli taka redukcja związana z selekcją miałaby miejsce, to różnorodność musiałaby być odtworzona przez przepływ genów z innych populacji, co w związku z izolacją laguny nie wydaje się prawdopodobne.

Ponadto, mała różnorodność haplotypowa i nukleotydowa w obrębie populacji północnych wydaje się skutkiem kolonizacji polodowcowej, powodującej "efekt założyciela". Z drugiej strony, adaptacja do lokalnych warunków środowiska skutkująca spadkiem polimorfizmu genetycznego w obrębie populacji północnych (np. z Morza Bałtyckiego) jest również możliwa (Johannesson i André 2006).

Badania tempa respiracji *C. glaucum* oraz *C. edule* wykazały, że *C. glaucum* ma większą zdolność do regulacji swojego metabolizmu niż *C. edule* (Wilson i Elkaim 1997). Obszar występowania *C. edule* jest bardziej ciągły, niż w przypadku *C. glaucum*, a warunki środowiska w typowych habitatach bardziej stabilne, niż w habitatach *C. glaucum*. Również w obecnych badaniach udowodniono wyraźne różnice fizjologiczne między populacjami sercówki, jak również w obrębie populacji między sezonami.

Z drugiej strony, populacje sercówki były bardzo zróżnicowane genetycznie (w neutralnych loci), co świadczy o tym, że możliwe są też lokalne adaptacje, ponieważ

przepływ genów między populacjami jest ograniczony. Pomimo tych adaptacji, duża plastyczność fenotypowa związana z genotypem generalistycznym jest niezbędna w przypadku nagłych zmian warunków środowiskowych w obrębie habitatów.

4.2 Podsumowanie najważniejszych wyników i wnioski

- Zróżnicowanie genetyczne pomiędzy populacjami atlantyckimi i śródziemnomorskimi jest zbyt małe, aby wyróżnić dwa gatunki lub podgatunki w obrębie *C. glaucum*.
- Struktura genetyczna populacji sercówki uzyskana za pomocą badań mitochondrialnego DNA różniła się od struktury genetycznej uzyskanej w badaniach jądrowego DNA. Może to być spowodowane różnymi właściwościami tych markerów, lecz to nie wystarczy do wytłumaczenia wykrytych różnic.
- Populacje z Morza Jońskiego charakteryzowały się mitochondrialnym DNA pochodzenia atlantycko-śródziemnomorskiego, a jądrowym DNA pochodzenia ponto-kaspijskigo.
- Struktura genetyczna populacji z Morza Jońskiego i z Tunezji sugeruje, że mogły to być obszary pochodzenia *C. glaucum*, skąd gatunek ten skolonizował resztę rejonu występowania.
- Możliwa selekcja została wykryta w jednym z locus (*Cg11*) w populacji z Morza Jońskiego.
- W niektórych populacjach wykryto inbred, którego przyczyną może być fragmentacja habitatu sercówki.
- Wykryto wyraźne, zróżnicowane grupy haplotypów mitochondrialnego DNA, co sugeruje, iż dywergencja między haplogrupami miała miejsce przed okresem zlodowaceń.
- Wykazano duże nieciągłości geograficzne w obrębie grup haplotypów.
- Polodowcowa kolonizacja akwenów północnej Europy spowodowała niższe zróżnicowanie genetyczne wewnątrz populacji z tych akwenów w stosunku do populacji z południowej Europy.

- Obszar występowania sercówki jest nieciągły, co ogranicza możliwości wymiany osobników między populacjami przez planktonowe larwy.
- Ptaki migrujące mogą odgrywać istotną rolę w przepływie genów między populacjami sercówki.
- Populacje północne rozradzały się raz w roku, a południowa kilka razy w roku.
- Tempo respiracji u sercówek z Zatoki Gdańskiej (GD) było najwyższe pośród badanych populacji, co może mieć związek ze zwiększonym tempem metabolizmu spowodowanym stresem osmotycznym.
- W izolowanych populacjach sercówki występują adaptacje do lokalnych warunków środowiska. Niezbędna jest również plastyczność fenotypowa umożliwiająca przetrwanie zmiennych warunków środowiskowych.

4.3 Kierunki dalszych badań

Badania wykonane w ramach tej pracy powinny być kontynuowane i rozszerzone, co umożliwiłoby lepsze zrozumienie funkcjonowania *C. glaucum* w ekosystemie. Dalsze badania mogłyby dotyczyć następujących zagadnień:

- translokacja osobników z odległych populacji pozwalająca ocenić zdolności aklimatyzacji sercówki
- badania przy użyciu nowych markerów genetycznych, na przykład EPIC (Exon Primed Intron Crossing) pozwalające na porównanie sekwencji jądrowego DNA,
- wspólne badania biologów morza i ornitologów mające na celu zbadanie możliwości przenoszenia *C. glaucum* przez ptaki,
- badania porównawcze *C. glaucum* i blisko spokrewnionego gatunku *C. edule*, żyjącego w bardziej stabilnych warunkach środowiska, mające na celu lepsze zrozumienie wpływu ekstremalnych habitatów na fizjologię i strukturę genetyczną,
- szczegółowe badania różnic morfometrycznych, fizjologicznych oraz genetycznych między populacjami sercówki: comiesięczne badania fizjologii (zamiast sezonowych) z uwzględnieniem większej liczby populacji, badanie fizjologii populacji z Morza Jońskiego oraz genetycznie bliskich populacji żyjących w różnych warunkach środowiska (np. z Portugalii i Morza Bałtyckiego).

Summary in French / Résumé

Structure génétique et variation physiologique chez *Cerastoderma glaucum* (Bivalvia) vivant dans des conditions environnementales différentes

1 Introduction générale

1.1 Objectifs

Le développement rapide de méthodes moléculaires a apporté beaucoup de nouvelles données dans les études en biologie marine, qui donnent des informations permettant d'expliquer des processus écologiques.

Les objectifs de ce projet étaient:

- d'examiner la structure génétique de *Cerastoderma glaucum* à l'aide de l'ADN mitochondrial et des microsatellites dans son aire de distribution quasi entière,
- de former un scénario concernant l'origine, les modes de dispersion, les refuges glaciaires, la colonisation postglaciaire et le flux de gènes contemporain chez cette espèce,
- de comparer des structures génétiques entre des régions de l'Europe du nord, colonisées après les glaciations et des régions de l'Europe du sud, d'où cette espèce vient et où elle a une histoire plus longue et ininterrompue,
- d'étudier les effets de la fragmentation de l'habitat sur la structure génétique et de construire des hypothèses concernant les vecteurs et facteurs ayant une influence sur la dispersion,
- d'étudier les différences dans les paramètres morphométriques et physiologiques parmi les trois populations qui vivent dans des conditions environnementales différentes, souvent extrêmes,
- d'analyser la stratégie de cette espèce permettant la colonisation et la survie dans les conditions extrêmes des lagunes.

1.2 Lagunes comme milieu spécial

Les lagunes côtières sont des bassins peu profonds séparés partialement de la mer, créés naturellement ou par l'homme (Kjerfve 1994). A cause des fortes variations des paramètres environnementaux comme la température, la salinité, la quantité d'oxygène ou pH, ces habitats sont considérés extrêmes (Bamber *et al*. 1992; Downie 1996). La colonisation des lagunes peut être effectuée par exemple par des oiseaux migrateurs ou par des actions anthropogéniques (Barnes 1988). Les lagunes sont souvent comparées aux îles, parce que les populations sont isolées et souvent formées à partir d'un petit nombre d'individus. Le flux de gènes limité peut provoquer la sélection locale, le dérive génétique ou la consanguinité (Slatkin 1981; Frankham 1995).

Les lagunes sont des éléments très précieux de la zone côtière, parce qu'elles consistent en assemblages d'espèces absentes dans d'autres écosystèmes (Barnes 1980; Barnes 1991; Bamber *et al.* 1992). Ce sont des régions où les oiseaux se nourrissent (Razinkovas *et al.* 2008) et elles sont également importantes du point de vue socio-économique. L'homme souvent perturbe les écosystèmes lagunaires directement par la destruction de la faune et de la flore ou indirectement par exemple par le tourisme (Spellerberg 1996). De plus, la pollution et les changements climatiques ont une grande influence sur des lagunes (Viaroli *et al.* 1996).

*1.3 La biologie, l'écologie, des origines et des fossiles de la coque (*C. glaucum*)*

La coque glauque, *Cerastoderma glaucum* (Fig. 3.1), est une espèce ayant une grande aire de répartition. Cette espèce est présente dans la Mer Baltique, la Mer du Nord, le long de la côte Atlantique de l'Europe de l'ouest, dans la Méditerranée, la Mer Noire, la Mer Caspienne et la Mer d'Aral (Høpner Petersen et Russell 1973). Elle tolère des températures entre 0°C jusqu'à plus que 45°C (Zaouali 1974) et des salinités entre 5 PSU jusqu'à 84 PSU (Rygg 1970; Labourg et Lasserre 1980). Pourtant, son aire de distribution est fragmentée. Cette espèce habite dans les bassins isolés ou semi-isolés sans marée, comme les lagunes, les estuaires, les baies et les lacs (Kingston 1974). La coque n'habite jamais dans le sable bien trié, exposé aux courants et aux vagues (Brock 1979).

C. glaucum est une espèce gonochorique. Le cycle reproductif varie entre les régions parce que la température joue un rôle important (Rygg 1970). La coque possède une larve planctonique qui reste dans l'eau pendant une semaine (ou plus, selon l'endroit) avant sa

métamorphose. Ce temps est beaucoup plus court par rapport à l'autre coque *Cerastoderma edule* dont la larve reste dans l'eau jusqu'aux cinq semaines (Barnes 1980).

La coque est un filtreur. Selon Sarà (2007) dans les bassins de la Méditerranée elle se nourrit surtout du détritus de la plante à fleurs marine, *Cymodocea nodosa*, de la macroalgue, *Ulva lactuca* et de la microflore benthique. Les diatomées sont considérées comme une source importante de la nourriture de *C. glaucum* (Høpner Petersen 1958; Beukema et Cadée 1996).

Les premiers fossiles du genre *Cerastoderma* viennent de l'Oligocène (il y a 33.9-23.0 millions d'années) (Keen 1969). *C. glaucum* a évolué dans la Mer Méditerranée avec son proche relatif *C. edule* quand la Mer Méditerranée a été séparée de l'Atlantique il y a environ 4 millions d'années entre le Miocène et le Pliocène (Rygg 1970). Les fossiles de la Libye et la Tunisie suggèrent que des bivalves morphologiquement indistinguables de *C. glaucum* étaient présents dans des lagunes sur le terrain de la Méditerranée actuelle pendant le Pliocène (Gaillard et Testud 1980; Wołowicz 1991). A cause de la séparation de l'Atlantique, la Méditerranée s'est divisée en plusieurs lagunes avec des salinités différentes (la crise de salinité mesinienne) (CIESM 2008). *C. glaucum* a survécu à des conditions difficiles, puis, quand la connexion entre la Méditerranée et l'Atlantique a été rétablie au début du Pliocène (Ruggieri 1967) elle ne se croisait plus avec *C. edule* qui est restée absente de Méditerranée.

2 La morphométrie et la physiologie des populations de la coque

2.1 Introduction

Les bivalves filtreurs sont souvent des espèces clés dans les lagunes, les estuaires et les habitats côtiers (Gili et Coma 1998). Ils jouent un rôle important dans le monitoring écologique à cause de leur position trophique, leur grande aire de distribution et leur sensibilité (Goldberg *et al.* 1978). Des changements dans le taux de croissance, dans la taille de population et dans l'aire de distribution peuvent avoir un effet en cascade sur les écosystèmes benthiques et pélagiques (Norkko *et al.* 2001; Dahlhoff *et al.* 2002; Ellis *et al.* 2002; Newell 2004). Les bivalves réagissent aux changements de conditions environnementales, comme le réchauffement climatique (Hughes 2000), la pollution ou l'eutrophisation (Cloern 2001). Les études sur leur physiologie peuvent permettre de prévoir les effets de ces changements.

2.2 Matériels et méthodes

Des paramètres morphométriques et physiologiques différents (Tableau I) ont été étudiés sur 30 individus pas saison (à l'exception du taux de respiration) de trois populations européennes de la coque (Fig. 5.1-5.5):

-de la Baie de Gdańsk (la Mer Baltique, la Pologne) (GD), (novembre 2005, février, mai, juillet et octobre 2006),

-du Lac Veere (la Mer du Nord, le Pays Bas) (LV), (novembre 2006, février, mai, et août 2007),

-de l'Etang de Berre (la Mer Méditerranée, la France) (BL), (février, mai, août et novembre 2007).

Tableau I. Méthodes appliquées pour étudier les paramètres morphométriques et physiologiques de la coque.

Paramètres environnementaux	• la mesure de la température et de la salinité à chaque site, chaque saison
Paramètres morphométriques	• la mesure de la longueur de coquille, par un vernier précis à 0.01 mm • le poids sec de chair- pesé après la lyophilisation • le poids sec de coquille- pesé après avoir séché les coquilles à 55°C • le rapport entre le poids sec de coquille/de tissu et la longueur pour chaque population; régression puissance: $W = a \times L^b$, *W*- poids sec de coquille/de tissu (g), *L*- longueur de coquille (mm), *a*, *b*- paramètres de l'équation
L'indice de condition	• $CI = (M_c \times 1000)/L^3$, M_c- poids sec de chair (mg), *L*- longueur de coquille (mm) (Beukema et De Bruin 1977)
Le cycle de reproduction	• le stade de maturité des gonades- une échelle de 5 stades (Wołowicz 1979; Wołowicz 1987) • l'indice gonadique *GI*= [∑ (le nombre d'individus au chaque stade de maturité des gonades × la valeur numérique de ce stade)/le nombre total des individus dans l'échantillon] (Tableau 5.1) (Chipperfield 1953)
La composition biochimique	• l'homogénéisation de chair lyophilisée • la teneur en protéines (Lowry *et al.* 1951), en lipides (l'extraction- Bligh et Dyer 1959, la mesure- De Marsh et Weinstein 1966), en glucides et en glycogène (Dubois *et al.* 1956)
La respiration (sur 5-10 individus par chambre pour LV et BL et environ 20 individus pour GD)	• 3 chambres de respiration et 1 chambre de contrôle, 4 températures: 4°C, 10°C, 17°C, 24°C • la mesure à l'aide d'électrodes (YSI 5331 oxygen probe) et l'enregistrement de résultats à l'aide du logiciel spécialisé • la température constante (± 0.2°C) maintenue avec un thermostat
Analyses statistiques (logiciel : STATISTICA v. 8.)	• test χ^2- teste la signification statistique de différences dans le sex-ratio • test de la normalité de données (test de Shapiro-Wilk) • test de la signification statistique des différences entre les poids secs de chair, les longueurs de coquille et entre les indices de condition entre les populations et entre les saisons à l'intérieur des populations (test de Kruskal-Wallis)

2.3 Résultats et discussion

2.3.1 Paramètres morphométriques et l'indice de condition

Les coques de GD étaient beaucoup plus petites par rapport aux coques de deux autres populations, en raison du stress osmotique provoqué par la basse salinité (Schmidt-Nielsen 1990) (Fig. 6.1). La salinité et la température de l'eau influencent la taille, la forme de la coquille ainsi que sa composition (ratio calcite/ aragonite) (Kennedy *et al.* 2008). Les différences dans les poids secs de chair et les longueurs de coquille étaient significatives ($p < 0.001$) entre les populations et pas significatives ($p > 0.01$) entre les saisons à l'intérieur des populations (Annexe 1). La condition est influencée par la croissance de la coque, son métabolisme et l'étape du cycle reproductif (Lucas et Beninger 1985; Smaal et Stralen 1990; Hummel *et al.* 2000b). Les différences dans les indices de condition étaient significatives entre les populations ($p < 0.001$) et entre les saisons à l'intérieur des populations ($p < 0.001$; dans le cas de BL $p < 0.01$). Des changements saisonniers de l'indice de condition dans des populations du nord (GD, LV) n'étaient pas tout à fait d'accord avec le pattern documenté pour des autres bivalves de ces régions ayant un mode de reproduction monocyclique, chez lesquels l'indice de condition était haut au printemps, avant le relâchement des gamètes (Wołowicz 1991; Hummel *et al.* 2000b; Wołowicz *et al.* 2006) (Fig. 6.2). Dans la population GD l'indice de condition a diminué en été parce que les gamètes ont été relâchés et il a augmenté en automne à cause de conditions trophiques favorables liées au bloom de diatomées (Pliński 1995). Dans LV entre l'hiver et le printemps *CI* a augmenté, ce qui était lié au développement des gamètes, qui n'a pas eu lieu dans GD. En été dans LV cet indice était bas. Pourtant des études sur les stades de développement de gonades suggéraient que la majorité de la population était dans le stade IV (reproduction). Le stade de développement des gonades et des indices de conditions ont été étudiés sur des individus différents, qui dans le cas de l'indice de condition ont pu avoir déjà pondu. L'indice de condition haut en hiver dans BL a pu être lié avec des conditions trophiques favorables. La présence de parasites dans le tissu influence aussi la condition, parce que *C. glaucum* sert d'hôte à metacercaria du genre *Meiogymnophallus* (Digenea: Gymnophallidae) (Bowers *et al.* 1996).

2.3.2 Reproduction

Les proportions de femelles et de mâles dans les populations étaient de 1 : 0.74 pour GD, 1 : 1.22 pour LV et BL et elles n'étaient pas statistiquement différentes de 1 : 1 ($p > 0.05$). La majorité de différences entre les saisons dans les proportions des femelles aux males n'étaient statistiquement pas significatives ($p > 0.05$). Chez certains bivalves une grande mortalité des femelles après la ponte a été observée, ce qui est probablement provoqué par le coût énergétique de l'émission de gamètes (Brokordt et Guderley 2004; Wołowicz *et al.* 2006). Les populations du nord (GD, LV) se sont reproduites une fois pendant l'année, entre mai et juillet (Rygg 1970; Wołowicz 1991) et dans LV la reproduction s'est déroulée plus tard que dans GD (Fig. 6.3). Dans la population du sud (BL) il est très difficile de spécifier la période de l'émission de gamètes parce que des coques en train de se reproduire étaient présentes dans la population tout au long de l'année. Il a été prouvé que les coques de la Méditerranée peuvent se reproduire plusieurs fois pendant l'année (Ivell 1979). La reproduction de la coque est liée à la température de l'eau et elle commence quand elle atteint 15-17°C, donc plus la population habite au nord, plus tard elle se reproduit (Kingston 1974). A part la température de l'eau, la quantité de nourriture influence aussi la reproduction (Kingston 1974; Honkoop et Van der Meer 1998).

2.3.3 Composition biochimique

Des changements dans la composition biochimique des bivalves sont liés au cycle reproductif (Sastry 1979; Pazikowska et Szaniawska 1988; Pazos *et al.* 1997; Bawazir 2000). La coque est une espèce "opportuniste" (Bayne 1976), qui fait une "pause" reproductive en hiver. La gamétogenèse recommence au printemps, quand dans le milieu il y a assez de nourriture pour assurer la biosynthèse. Donc le développement de gamètes et l'accumulation de réserves énergétiques sont temporairement synchronisés. Les conditions trophiques déterminent les changements saisonniers de la composition biochimique. Les conditions trophiques défavorables conduisent au catabolisme des glucides, après c'est au tour des lipides et enfin des protéines structurales (Beninger et Lucas 1984).

Les teneurs en protéines (37.5-77.4%) et en lipides (5.7-13.6%) (Fig. 6.5a-b) étaient comparables aux études précédentes de Wołowicz (1991). Chez Wołowicz (1991) la teneur la plus élevée en ces deux composants a été trouvée au printemps dans les populations du

nord et, pour les populations du sud, il n'y avait pas beaucoup de différence entre les saisons. Les teneurs en protéines et en lipides chez les femelles augmentent pendant le développement de gamètes, et diminuent après leur émission, parce que les protéines et les lipides sont les éléments les plus importants des oocytes (Gabbott 1975). Cette tendance était présente dans la population GD. Les protéines sont synthétisées pendant la gamétogenèse et les lipides sont partiellement redistribuées des tissus somatiques vers les gonades (Holland 1978). Dans LV il n'y avait pas des grandes différences entre les saisons dans la teneur en lipides, qui n'augmentait pas au printemps malgré le développement de gamètes. Ceci peut être lié au fait que les analyses biochimiques sur *C. glaucum* ont été faites sans la distinction en deux sexes et chez les mâles on n'attend pas de changement de la teneur en protéines lié au cycle reproductif (Navarro *et al.* 1989). Dans BL, malgré le fait qu'il n'y avait pas de période d'émission de gamètes précise, les teneurs en protéines et en lipides ont diminué entre le printemps et l'été, ce qui pourrait être lié aux conditions trophiques défavorables.

La teneur en glucides était entre 4.1% et 16.1% et la teneur en glycogène était entre 2.8% et 13.3% (Fig. 6.5 cd). Dans GD la teneur la plus basse en glucides et en glycogène dans les tissus a été trouvée en été, parce que le glycogène est utilisé dans le développement de gamètes et chez les femelles il est transformé en lipides dans les oocytes (Gabbott 1975). L'augmentation de la teneur en glucides dans les populations du nord entre l'été et l'automne paraît corrélée à la floraison du phytoplancton en automne (Wasmund *et al.* 1998). Dans BL il y avait que des petites différences entre les saisons dans les teneurs en glucides qui peuvent être dues aux changements des conditions trophiques (Wołowicz 1991). Dans LV les différences saisonnières étaient petites, peut-être parce que la mesure a été faite sans la division en deux sexes et selon l'âge (Newell et Bayne 1980; Navarro *et al.* 1989; Wołowicz *et al.* 2006).

La somme de la teneur en protéines, en lipides et en glucides n'est pas égale à 100%, parce que le reste c'est la cendre, des acides aminés qui forment 2-12% du poids de chair sec (Pieters *et al.* 1980) et de l'eau qui forme 2-3% du poids de chair sec (Beukema et De Bruin 1979).

2.3.4 Respiration

La consommation d'oxygène correspond à l'activité métabolique de l'organisme et est proportionnelle à la demande instantanée de l'ATP (Bayne et Newell 1983; Clarke 1991). La température ambiante influence l'activité métabolique (Clarke 1991). La diminution de la consommation de l'oxygène peut être dûe au stress ou à la diminution du taux de filtration (Akberali et Trueman 1985; Riisgård *et al.* 2003). La consommation d'oxygène dépend également de la taille du corps (Dame 1996). Etant exposés au stress, certains mollusques peuvent adopter un métabolisme anaérobie (Liu *et al.* 1990; Oeschger 1990).

Dans toutes les populations le taux de respiration était le plus bas à la température de 4°C. Le taux de respiration était semblable à celui trouvé dans des études précédentes sur *C. glaucum* (Wilson et Elkaim 1997) et sur d'autres bivalves (McMahon et Wilson 1981; Wilson et Davis 1985; Wilson 1990; Wilson et Elkaim 1991; Hummel *et al.* 2000b), sauf GD, où le taux de respiration était très haut (Fig. 6.6). Le taux de respiration haut dans GD avait pu être provoqué par le métabolisme accéléré à cause du coût énergétique lié au stress osmotique dû à la basse salinité de l'eau (Engel *et al.* 1975; Newell 1979). Dans GD et LV au printemps le niveau de métabolisme et la consommation d'oxygène étaient hauts et les coques étaient sensible à l'augmentation de la température, parce qu'elles avaient besoin d'énergie supplémentaire pour le développement de gamètes.

Chez les bivalves pendant la reproduction le taux de respiration peut augmenter de 30 à 80% (De Vooys 1976; Iglesias et Navarro 1991). Dans GD et LV le niveau de métabolisme a diminué en été quand la majorité d'individus avait déjà émis des gamètes. Dans GD et LV le niveau de métabolisme était haut en automne, à cause du bloom de phytoplancton et de l'activité intense de filtration (Wołowicz 1991). En hiver le taux de respiration chez ces populations était bas. La consommation d'oxygène chez l'autre coque *Cerastoderma edule* (de l'Angleterre) était la plus basse en hiver (entre décembre et mars) (Newell et Bayne 1980). Le taux de respiration de la population BL qui est exposée aux hautes températures en été, était plus haut en hiver qu'en été (sauf 24°C), qui a été trouvé aussi chez *C. glaucum* de la côte sud-ouest de la France (Arcachon) et a été interprété comme l'adaptation permettant d'économiser de l'énergie pendant les périodes de températures élevées (l'acclimatization inverse) (Wilson et Elkaim 1997). Cependant, chez les coques de BL mises à 24°C (ce qui est proche à la température ambiante dans cette

lagune en été) le taux de respiration était le plus haut en été. Donc ces données ne prouvent pas l'acclimatisation inverse chez *C. glaucum*.

2.4 Conclusions

C. glaucum est une spécialiste lagunaire eurytopique adaptée aux conditions environnementales extrêmes, comme la basse salinité de la Mer Baltique et des très hautes températures dans des lagunes de la Méditerranée, comme l'Etang de Berre. Les conditions environnementales changeantes dans l'Etang de Berre provoquent des changements rapides des espèces macrobenthiques dominantes et souvent leur extinction. Pourtant, *C. glaucum* parait un élément stable des communautés benthiques de cette lagune (Stora *et al.* 1995; Stora *et al.* 2004), qui prouve sa résistance contre les conditions environnementales extrêmes ou peu stables. Il y a des différences physiologiques entre les populations *C. glaucum* (Wołowicz 1987; Wołowicz 1991; Wilson et Elkaim 1997) liées à l'acclimatisation et probablement aux adaptations génétiques (Nevo 1978; Brock et Wołowicz 1994).

3 La structure génétique de la coque (*Cerastoderma glaucum*)

3.1 Le flux de gènes dans le milieu marin et entre les lagunes

Les structures phylogéographiques chez les espèces marines sont très diversifiées car elles sont influencées par plusieurs facteurs, comme la tolérance vers les conditions environnementales, le cycle de vie ainsi que le passé géologique et climatique dans le région de distribution de l'espèce. Donc même chez les espèces avec une phase larvaire planctonique, la structure génétique des populations est rarement homogène (Gilg et Hilbish 2003; Veliz *et al.* 2006). Chez les espèces dont l'aire de distribution atteint le nord de l'Europe la structure génétique actuelle est influencée par les glaciations du Pléistocène (Quesada *et al.* 1995; Pannacciulli *et al.* 1997; Luttikhuizen *et al.* 2003a) (Fig. 9.1).

3.2 Matériels et méthodes

La structure génétique de 20 populations de la coque (*C. glaucum*) (Fig. 10.1, Tableau 10.1) a été étudiée à l'aide de l'ADN mitochondrial et 4 loci microsatellites (Tableau II).

Tableau II. Les méthodes appliquées pour étudier la structure génétique de la coque.

	Le type de marquer	
	L'ADN mitochondrial (Fig. 9.3)	**Microsatellites** (Fig. 9.4, 9.5)
L'extraction de l'ADN	QIAamp DNA Mini Kit (QIAGEN); 22-31 individus par population (sauf BK-6 individus)	
Les amorces	F:CTAYCTAGCTTTTTGAGCGGG R:CACCWCCCCCAACTGGATCGA	*Cg4*- F:GTGTTGGACTCGCCATACC* R:GACACAAGTAAAAACAATGTCT *Cg7*- F:GATCCAGCCGTTCAAGTCC* R:CGAAATAATGCGCGATGC *Cg9*- F:CCATATTACCACTGCCCACAC* R:TGACCCCCTCCAGTGATTC *Cg11*- F:GGGGCGATTCTGGAGTAGTAG* R:GTCAAACCAGGCGCTAAGTC (Pearson 2003) *amorces fluorescentes
Le fragment amplifié	Le fragment de 580 paires de base de cytochrome oxidase I (le fragment analysé- 514 paires de base)	*Cg4*- $(GT)_n$ *Cg7*- $(GT)_n$ *Cg9*- $(GACA)_n$ *Cg11*- $(C)_n(CA)_n$
Le programme PCR	4'94°C, 35×(30''94°C, 30''65°C, 1'72°C)	*Cg4*-2'94°C, 25×(30''94°C, 30''50°C, 1'72°C), 20'72°C; *Cg7*-2' 94°C, 25×(30''94°C, 30''55°C, 1'72°C), 20' 72°C; *Cg9*-2'94°C, 30×(30''94°C, 30''45°C, 1'72°C), 20'72°C; *Cg11*-2'94°C, 25×(30''94°C, 30''50°C, 1'72°C), 20'72°C;
Le mélange réactionnel de PCR	Volume 25 µl: 1.5 µl de solution de l'ADN , 1 µM de chaque amorce, 265 µM dNTP, tampon 1×PCR, 2.5 mM $MgCl_2$, 0.65 U de polymérase GoTaq Flexi DNA	Volume 10 µl: 0.7 µl de solution de l'ADN, 1 µM de chaque amorce, 265 µM dNTP, tampon 1×PCR, 1.675 mM (pour *Cg4* et *Cg7*) ou 2 mM (pour *Cg9* et *Cg11*) de $MgCl_2$, 0.25 U de polymérase GoTaq Flexi DNA

Tableau II continuation

	Le type de marquer	
	L'ADN mitochondrial	**Microsatellites**
Analyses statistiques de la structure génétique entre les populations	• réseau d'haplotypes- méthode de l'union des vecteurs de médiane, logiciel NETWORK v.4.5 (Bandelt *et al*. 1999) • F_{ST} (Weir et Hill 2002) entre paires de populations, logiciel ARLEQUIN v.3.1 (Excoffier *et al.* 2005) • représentation graphique de F_{ST} à l'aide du positionnement multidimensionnel (MDS), XLstat 7.5.2 • le choix du modèle évolutif de l'ADN mitochondrial le plus probable (Posada et Crandall 1998) • arbre phylogénétique des haplotypes- la méthode de maximum de vraisemblance (Guindon et Gascuel 2003), racine fournie par la séquence de *C. edule* • pourcentage de divergence entre deux groups d'haplotypes, logiciel MEGA 4 (Tamura *et al.* 2007) • estimation du temps de la divergence au sein de groupes d' haplotypes proches venant des populations éloignées (Monte Carlo méthode basée sur la chaîne de Markov), logiciel IM (Nielsen et Wakeley 2001)	• déséquilibre des liaisons entre les paires de loci de microsatellites (Weir 1979), logiciel GENETIX v.4.02. (Belkhir *et al.* 1998) • arbre phylogénétique- la méthode du plus proche voisin (Saitou et Nei 1987), distance selon le modèle Cavalli-Sforza et Edwards (1967), le paquet PHYLIP 3.6 (Felsenstein 2005) • F_{ST} (Weir et Cockerham 1984) entre paires de populations pour chaque locus séparément et multiloci, logiciel GENETIX v.4.02; corrections du niveau de la significativité avec la méthode de Benjamini et Hochberg (1995) • représentation graphique de F_{ST} à l'aide du positionnement multidimensionnel (MDS), XLstat 7.5.2 • détection de la sélection (la méthode de "F_{ST}-outlier"), logiciel LOSITAN (Beaumont *et al.* 1996; Antao *et al.* 2008) • détection de la structure génétique avec la méthode de Bayes où des individus ne sont pas attribués aux populations *a priori*, logiciel STRUCTURE (Pritchard *et al.* 2000; Falush *et al.* 2003; Evanno *et al.* 2005); permutation des résultats de plusieurs répétitions- logiciel CLUMPP v.1.1.1 (Jakobsson et Rosenberg 2007), représentation graphique de résultats - logiciel DISTRUCT v.1.1 (Rosenberg 2004)

Tableau II continuation

<table>
<tr><th></th><th colspan="2">Le type de marquer</th></tr>
<tr><th></th><th>L'ADN mitochondrial</th><th>Microsatellites</th></tr>
<tr><td>Analyses statistiques de la structure génétique au sein des populations</td><td>• le nombre des sites ségrégeants (variables) (S), diversité haplotypique (H_{d}) (Nei 1987), diversité nucléotidique (Π) (Nei 1987; Nei et Miller 1990), tests de neutralité D Tajima (1989) et D^* et F^* (Fu et Li 1993), logiciel DNASP v.4.10.9 (Rozas et al. 2003)</td><td>• hétérozygotie attendue et observée (H_{nb}, H_{obs}) (Nei 1978) et F_{IS} (Weir et Cockerham 1984), logiciel GENETIX v.4.02
• nombre d'allèles privés dans chaque population
• test pour détecter la consanguinité, logiciel RMES (David et al. 2007)</td></tr>
<tr><td>Autres analyses</td><td>• la traduction des séquences des nucléotides en acides amines, logiciel MEGA 4</td><td></td></tr>
<tr><td>Comparaison des résultats obtenus avec les 2 types de marqueurs</td><td colspan="2">• le test de la corrélation des matrices de F_{ST} entre paires de populations obtenues avec les 2 types de marqueurs- test de Mantel (Mantel 1967; Smouse et al. 1986), logiciel ARLEQUIN v.3.1
• l'analyse des regroupements dans les populations étudiées et leurs conformité entre les 2 types de marqueurs- analyse de la variance moléculaire (AMOVA, Excoffier et al. 1992)</td></tr>
</table>

3.3 Résultats et discussion

Aucun déséquilibre des liaisons n'a été détecté entre les loci microsatellites. Le nombre d'allèles microsatellites était de 42 pour *Cg4*, 25 pour *Cg7*, 63 pour *Cg9* et 24 pour *Cg11* (Annexe 5 et 6). Dans la majorité des populations un déficit en hétérozygotes (F_{IS} significatif) a été détecté. Au moins dans cinq populations, trois de la Méditerranée (SE, SM, TU) et deux de la Mer Noire (RO, UK) ce déficit n'était pas lié à la présence d'allèles nuls, mais à des raisons biologiques, comme la consanguinité, l'effet Wahlund (la présence des sous-populations génétiquement diversifiées au sein d'une population) (Castric *et al.* 2002) ou la sélection contre les hétérozygotes (Tableau 11.7). Dans les études précédentes la consanguinité a été trouvée et expliquée par la fragmentation d'habitat chez la coque (Pearson 2003). La consanguinité existe souvent dans les petites populations isolées (Frankham 1995; Madsen *et al.* 1996; Lacy 1997).

Toutes les mutations sauf une étaient synonymes après la traduction de l'ADN mitochondrial en acides aminés. 90 haplotypes ont été observés venant de 100 mutations en 92 sites polymorphes (Annexe 2, 3 et 4). Le taux de divergence au sein de *C. glaucum*

estimé à l'aide de l'ADN mitochondrial était de 5% de divergence par million d'années, ce qui peut être traduit en un taux de substitution de 2.5% par un million d'années (Nikula et Väinölä 2003).

Une grande divergence entre les populations de *C. glaucum* a été trouvé, malgré la présence d'une larve planctonique (Fig. 11.1-11.4, Tableau 11.1-11.2). La forte structure génétique était probablement provoquée par la fragmentation de l'habitat limitant le flux de gènes. Les structures génétiques bien marquées ont été également trouvées chez d'autres espèces marines et elles ont été expliquées par la divergence allopatrique dans les refuges glaciaires (e.g. Coyer *et al.* 2003; Luttikhuizen *et al.* 2003a; Papadopoulos *et al.* 2005; Provan *et al.* 2005; Pérez-Losada *et al.* 2007), ce qui a pu avoir lieu également chez *C. glaucum*.

Les structures révélées avec les 2 types de marqueurs étaient différentes (Fig. 11.1-11.4, Tableau 11.1-11.2). Selon les résultats de l'ADN mitochondrial les populations de la Mer Égée et de la région ponto-caspienne forment un groupe très divergent avec une différence de 6.6% dans la séquence par rapport aux populations habitant plus à l'est (Fig. 11.1-11.3). Ce groupe de populations a évolué en premier et maintenant il semble y avoir un flux de gènes très faible entre ce groupe et les autres populations. La coque a pu coloniser la Mer Noire à la fin du Pliocène (il y a environ 1.8 millions d'années), quand la connexion entre la Mer Égée et la Mer Noire a été formée (Bacescu 1985). La divergence entre ces deux groupes a eu lieu il y a environ 1.3 millions d'années, si le taux de divergence était de 5% par million d'années. Nikula et Väinölä (2003) ont pensé que l'isolement de la coque de la Mer Égée et de la Mer Noire pouvait être du aux conditions hydrologiques dans la Mer Ionienne. Pourtant, cette espèce habite souvent dans les lagunes isolées, donc les conditions hydrologiques dans la mer n'ont probablement pas beaucoup d'influence sur le flux de gènes chez cette coque.

Dans le réseau d'haplotypes, la séquence de *C. edule* se branche avec le groupe des haplotypes de *C. glaucum* auquel appartiennent les populations de la Mer Égée, de la Mer Noire et de la Mer Caspienne (Fig. 11.2). Cependant, cela ne prouve pas le caractère ancestral des haplotypes de cette région, parce que les relations phylogénétiques entre des espèces sont difficiles à tracer avec les séquences de l'ADN mitochondrial (Ballard et Whitlock 2004; Moritz et Cicero 2004; Monaghan *et al.* 2006). Cela suggère tout de même que ce groupe est frère de tous les autres haplotypes éventuellement une sous-espèce.

Parmi les haplotypes “atlanto-méditerranée” quatre groupes peuvent être définis, ainsi qu’un groupe d’haplotypes de la Mer Ionienne très divergent (Fig. 11.1-11.3). La grande diversité nucléotidique des haplotypes de la Mer Ionienne sans traces d’expansion démographique, ainsi que leur position centrale dans le réseau des haplotypes indique que cela pourrait être le centre d’origine de *C. glaucum*. Les haplotypes de Tunisie ont également une grande diversité nucléotidique, mais ils n’ont pas la position centrale dans le réseau. La coque a probablement survécu aux cycles glaciaires du Pléistocène dans ces deux régions.

Selon les microsatellites les populations de la Mer Ionienne étaient les plus divergentes des autres populations (Fig. 11.4b). Pourtant les structures trouvées avec 4 loci de microsatellites étaient différentes et le caractère très spécial de la Mer Ionienne était demontré surtout grâce au locus *Cg11* (Fig. 11.6). Le test qui compare les valeurs de F_{ST} avec l’hétérozygotie attendue a mis en évidence la sélection positive possible agissant sur *Cg11* (Fig. 11.5). Les tests faits après l’exclusion de ce locus confirment le caractère ponto-caspien de l’ADN nucléaire de la coque de la Mer Ionienne (Fig. 11.4c), ce qui a été suggéré selon les études basées sur des allozymes (Nikula et Väinölä 2003). Les origines différentes des génomes de la coque de la Mer Ionienne ont été expliquées par l’introgression ancienne de l’ADN mitochondrial “atlanto-méditerranée” dans le génome nucléaire ponto-caspien (Nikula et Väinölä 2003).

Les structures génétiques trouvées à l’aide de l’ADN mitochondrial et des microsatellites ont prouvé que la séparation au sein du “complexe *Cerastoderma glaucum*” en deux espèces, *C. glaucum* de la Méditerranée et *C. lamarcki* de la region atlanto-baltique (Brock 1987; Brock et Christiansen 1989), ou sous-espèces, *C. glaucum glaucum* et *C. glaucum lamarcki* (Brock 1991; Hummel *et al.* 1994), n’est pas justifiée. Ces résultats sont en accord avec les résultats de Mariani *et al.* (2002) et Nikula et Väinölä (2003) et démontrent que le Détroit de Gibraltar ne forme pas la plus grande barrière contre le flux de gènes entre les populations de la coque.

La structure génétique au nord de l’aire de distribution a été influencée par les glaciations du Pléistocène (Fig. 9.1). Cette région a été colonisée quand les glaciers ont fondu. Dans les structures génétiques des populations de ces régions il y a les traces des goulots d’étranglement, des dérives génétiques et des effets fondateurs. Ces phénomènes sont prouvés par la plus grande diversité haplotypique et nucléotidique à l’intérieur de populations du sud par rapport aux populations du nord (l’ADN mitochondrial) (Fig. 11.1,

Fig. 11.9, Table 11.5) et également par la plus grande différentiation génétique en microsatellites entres les populations du nord de l'aire de distribution par rapport aux populations du sud (Fig. 11.4b-c). De plus dans le trois populations de l'Océan Atlantique (PT, AR, AN) et une population de la Mer du Nord (LV) les tests de neutralité qui ont pour but la détection de la sélection ou l'instabilité de l'effectif efficace ont donné les résultats significatifs (Tableau 11.5).

La structure génétique trouvée à l'aide de l'ADN mitochondrial démontre certaines discontinuités géographiques: les haplotypes du Portugal (PT) sont très proches des haplotypes de la Mer Baltique (GD, FI) et les haplotypes de la Sardaigne (SA) et deux haplotypes de l'Etang de Berre (BL) sont très proches des haplotypes de la côte Atlantique de la France (AN, AR) et de la Mer du Nord (LV, AL) (Fig. 11.1-11.3). Selon les simulations faites avec le logiciel IM, la divergence entre les régions éloignées à l'intérieur de ces deux groupes d'haplotypes a eu lieu il y a environ 55 mille ans. Cette estimation peut être imprécise (par exemple Audzijonytė et Väinölä 2006; Waters *et al.* 2007; Peterson et Masel 2009) et la divergence a eu probablement lieu dans la période juste après la dernière glaciation. L'hypothèse alternative, qui peut être rejetée, était une divergence plus récente provoquée par le transport anthropogénique. Ces discontinuités dans la structure génétique de ces populations suggèrent la présence des refuges glaciaires dans les régions du Portugal et de la Sardaigne. A partir de ces refuges *C. glaucum* a pu coloniser la partie nord de son aire de répartition. D'un autre côté, il est possible que les haplotypes présents dans la Mer Baltique et dans la Mer du Nord soient également présents dans les refuges allopatriques, donc ces populations ne viennent pas des régions du Portugal et de la Sardaigne, mais des autres refuges qui n'ont pas été échantillonnés (Jaramillo-Correa *et al.* 2004). La structure génétique des populations étudiée à l'aide des microsatellites (quatre loci indépendants versus un seul locus mitochondrial) est beaucoup plus homogène à l'intérieure des régions (Fig. 11.8). Toutefois, selon les microsatellites il y a beaucoup de divergence entre les populations du nord et les traces de la colonisation postglaciaire semblent effacées (Fig. 11.4b-c). La colonisation des nouveaux habitats par les larves planctoniques de *C. glaucum* ne semble pas probable à cause de l'isolement et de la fragmentation des habitats et les discontinuités géographiques entre les haplotypes mitochondriaux semblables. Si la structure génétique de l'ADN mitochondrial reflète les voies de la colonisation chez la coque il est probable que des oiseaux migrateurs aient pu transporter les coques entre les régions tellement éloignées (Sanchez *et al.* 2006, Sanchez

et al. 2007). Il y beaucoup de preuves que les oiseaux migrateurs jouent un rôle important dans le transport des invertébrés aquatiques (De Gelas et De Meester 2005; Figuerola *et al.* 2005).

Des différences entre les structures génétiques révélées par les deux types des marqueurs peuvent être provoquées par les propriétés différentes de ces marqueurs (Féral 2002; Chan et Levin 2005; Chenuil 2006). L'avantage des études basées sur l'ADN mitochondrial est le fait, que les séquences sont moins influencées par l'homoplasie, que dans le cas des études de microsatellites (Estoup et Angers 1998) (Fig. 9.3-9.5). La taille efficace de la population est quatre fois plus petite pour le génome mitochondrial par rapport au génome nucléaire (Birky *et al.* 1989; Mills et Allendorf 1996). Donc la différente force de la structure a pu être provoquée par les effets différents de la dérive génétique et de la migration sur le deux génomes (Ballard et Whitlock 2004). Toutefois, dans le cas présent, ce ne sont pas seulement les intensités des structures observées, mais les relations mêmes entre les populations relevées par le deux génomes ont été différentes (par exemple dans le cas de l'ADN mitochondrial, le groupe le plus divergent était les populations de la région ponto-caspienne et de la Mer Egée et dans le cas de microsatellites c'était les populations de la Mer Ionienne), ce qui a été confirmé par le test de Mantel et l'analyse de la variance moléculaire (AMOVA) (Tableau 11.4). Donc une autre explication est nécessaire. L'ADN mitochondrial ne recombine pas et il est plus influencé par le balayage sélectif (Gillespie 2001; Ballard et Whitlock 2004; Bazin *et al*. 2006) et l'introgression (Currat *et al.* 2008) que l'ADN nucléaire. Par ailleurs la divergence profonde entre les groupes des haplotypes de l'ADN mitochondrial a pu être provoquée par le polymorphisme ancestral et le tri des lignées évolutives (la perte des lignées évolutives au hasard) (Avise *et al.* 1984, 1987). Pourtant à cause de la fragmentation de l'aire de distribution, il semble très improbable qu'il y ait eu une population homogène de la coque à travers toute l'Europe par le passé.

L'ADN mitochondrial est transmis dans la lignée de la mère, sauf quelques exceptions (par exemple Gyllensten *et al.* 1991; Zouros *et al.* 1992, 1994) donc le phénomène qui a pu créer ces structures génétiques est une possible différence entre les sexes dans les capacités de la dispersion (Avise *et al.* 1987). Celles-ci pourraient venir chez la coque par exemple des différences dans la survie de l'exposition aérienne ou le manque de nourriture, qui peuvent avoir lieu pendant le transport passif (par exemple par les oiseaux migrateurs ou par les activités anthropogéniques). Chez d'autres bivalves des

différences dans les structures révélées par les deux génomes ont été expliquées par les différences dans les proportions de deux sexes et les différences entre les deux sexes dans le succès reproductif (Arnaud-Haond *et al.* 2003b; Diaz-Almela *et al.* 2004).

4 Conclusions générales et perspectives

4.1 La stratégie permettant la colonisation efficace et la persistance les lagunes

Il y a deux stratégies permettant la colonisation efficace de nouveaux habitats (Parker *et al.* 2003): la présence d'haplotypes généralistes (la grande plasticité phénotypique) (e.g. Bamber et Henderson 1988; Pearson *et al.* 2002; Lasota 2009) ou une capacité d'adaptation rapide.

Dans la première hypothèse, supposant que les génotypes généralistes dominent chez *C. glaucum*, les différences physiologiques observées entres populations habitant dans des conditions environnementales différentes seraient dues à l'acclimatisation. Dans la deuxièmre hypothèse, où cette espèce possède une capacité d'adaptation rapide, ces différences correspondraient à des différences génétiques).

La possibilité de changements très rapides des conditions environnementales dans les lagunes suggère une grande plasticité phénotypique de la coque dans ces habitats (Bamber et Henderson 1988; Bamber *et al.* 1992). Notamment, *C. glaucum* forme un élément stable dans l'habitat changeant de l'Etang de Berre (Stora *et al.* 1995; Stora *et al.* 2004). De plus, la variabilité génétique étudiée sur les loci neutres dans cette population est grande. Si la situation est la même pour le reste du génome, cela indique que la sélection directionnelle n'a pas réduit la variabilité, ce qui suggère la présence d'haplotypes généralistes dans cette population. S'il y avait de la réduction de la variabilité liée à la sélection, la variabilité génétique observée devrait avoir été rétablie grâce au flux de gènes en provenance d'autres populations, ce qui est improbable entre les populations isolées des lagunes.

Par ailleurs, les faibles variabilités haplotypiques et nucléotidiques des populations du nord s'expliquent bien par la colonisation postglaciaire ayant provoqué des goulets d'étranglement et des effets fondateurs. Il n'est donc pas nécessaire d'invoquer l'adaptation. Cependant il est également possible que des adaptations aux conditions locales (par exemple dans la Mer Baltique) aient eu lieu et aient provoqué la réduction de la variabilité génétique observée (Johannesson et André 2006).

L'étude du taux de la respiration chez la coque lagunaire C. *glaucum* et chez l'espèce marine *C. edule* a démontré que *C. glaucum* possède une plus grande capacité de réguler son métabolisme que *C. edule* (Wilson et Elkaim 1997). Dans cette étude les différences physiologiques entres les populations de la coque et aussi entre les saisons à l'intérieur des populations ont été également trouvées.

D'autre part, la structure génétique entre les populations de coques était très marquée (aux loci neutres), donc les adaptations locales sont possibles, parce que le flux de gènes est limité. Malgré cette possibilité des adaptations locales, la grande plasticité phénotypique liée aux génotypes généralistes est indispensable face aux changements rapides des conditions environnementales dans les habitats.

Afin de créer des stratégies de protection des lagunes il faut bien étudier les espèces qui y habitent et il faut également faire le suivi des conditions environnementales, parce que les lagunes sont très différentes entre elles (Gates 2006). Leur protection des impacts anthropogéniques est également indispensable (Marin-Guirao *et al.* 2005).

4.2 Le résumé des résultats les plus importants et les conclusions

- La division en deux espèces ou sous-espèces, atlantique et méditerranée, devrait être abandonnée, parce que le détroit de Gibraltar ne forme pas la plus grande barrière contre le flux de gènes.
- Les structures génétiques qui viennent de deux types de marqueurs étaient différentes, ce qui peut être provoqué par les propriétés différentes de ces marqueurs, mais cela ne suffit pas comme l'explication.
- Les populations de la Mer Ionienne avaient l'ADN mitochondrial d'origine atlanto-méditerranée et l'ADN nucléaire d'origine ponto-caspienne.
- Les structures génétiques des populations de la Mer Ionienne et de la Tunisie suggèrent que dans ces localités les cycles climatiques ont provoqué beaucoup moins (voire aucune) diminution de la diversité génétique qu'ailleurs, et que peut-être cette espèce provient de ces régions mêmes.
- La sélection positive possible a été détectée sur l'un des loci (*Cg11*) dans la Mer Ionienne.

- Dans quelques unes des populations la consanguinité a été détectée, qui peut être due à la fragmentation de l'habitat.
- Il y avait des groupes d'haplotypes de l'ADN mitochondrial bien séparés, ce qui suggère, que la divergence a eu lieu avant les glaciations.
- Il y avait des discontinuités géographiques au sein des groupes des haplotypes.
- Les régions au nord de l'aire de distribution de la coque ont été colonisées quand les glaciers ont fondu. Dans les structures génétiques des populations de ces régions il y a les traces des goulots d'étranglement, des dérives génétiques et des effets fondateurs.
- A cause de l'isolement des habitats de la coque, la dispersion de cette espèce par des larves planctoniques semble peu probable.
- Les oiseaux migrateurs peuvent jouer un rôle important dans le flux de gènes chez la coque dans le passé et actuellement.
- La reproduction chez les populations du nord a lieu une fois par an et chez les populations du sud- plusieurs fois par an.
- Le taux de respiration chez les coques de la Baie de Gdańsk était le plus élevé parmi les populations étudiées, ce qui peut être lié au métabolisme élevé provoqué par le stress osmotique.
- Les adaptations locales sont possibles chez la coque, parce que le flux de gènes est limité. Malgré cette possibilité des adaptations locales, la grande plasticité phénotypique liée aux génotypes généralistes est indispensable face aux changements rapides des conditions environnementales dans les habitats.

4.3 Les perspectives de recherche

Les recherches qui ont été faites dans le cadre de ce projet devraient être continuées et développées, ce qui pourrait permettre de mieux comprendre le fonctionnement de *C. glaucum* dans l'écosystème. Les recherches futures pourraient être focalisées sur certains sujets:

- les expériences de translocation des populations distantes, ce qui pourrait permettre d'étudier la capacité d'acclimatisation chez cette espèce (Hummel *et al.* 2000a),

- l'application de nouveaux marqueurs génétiques sur *C. glaucum* afin d'explorer les différences dans la structure génétique entre le génome mitochondrial et nucléaire; l'analyse avec des marqueurs EPIC (Exon Primed Intron Crossing) (Chenuil *et al.* 2009) pourrait donner une information nucléaire précise (séquences) sans l'homoplasie inhérente aux données microsatellites,
- des études menées par les biologistes marins en coopération avec les ornithologues focalisées sur la dispersion possible de *C. glaucum* par les oiseaux,
- des études comparatives de *C. glaucum* et *C. edule* afin de mieux comprendre l'influence de l'habitat lagunaire sur *C. glaucum*,
- des études détaillées des différences morphométriques, physiologiques et genetiques entre les populations: des études tous les mois, pas dans la manière saisonnière; les études de la physiologie des populations de la Mer Ionienne et des populations génétiquement proches vivant dans les conditions environnementales différentes (par exemple du Portugal et de la Mer Baltique).

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Appendix 1. Morphometric data for physiological analysis.

Appendix 1a. Gulf of Gdańsk (GD), Poland, winter, February 2006.

Individual number	**Shell length (mm)**	**Dry tissue weight (g)**	**Dry shell weight (g)**	**Dry tissue weight content in the total dry weight (%)**	**Condition index, *CI***
1	16.45	0.0430	0.5912	6.7802	9.66
2	17.11	0.0265	0.7276	3.5141	5.29
3	16.39	0.0465	0.5403	7.9243	10.56
4	15.42	0.0347	0.4524	7.1238	9.46
5	15.8	0.0411	0.6095	6.3172	10.42
6	16.29	0.0447	0.6154	6.7717	10.34
7	17.43	0.0612	0.7307	7.7282	11.56
8	15.68	0.0524	0.5892	8.1671	13.59
9	17.70	0.0677	0.683	9.0182	12.21
10	18.45	0.0819	0.883	8.4879	13.04
11	16.32	0.0531	0.4747	10.0606	12.22
12	14.98	0.0416	0.5448	7.0941	12.38
13	14.04	0.0353	0.3725	8.6562	12.75
14	15.50	0.0390	0.5023	7.2049	10.47
15	15.52	0.0413	0.5910	6.5317	11.05
16	15.06	0.0299	0.5094	5.5442	8.75
17	16.65	0.0576	0.8042	6.6837	12.48
18	16.19	0.0543	0.6552	7.6533	12.80
19	14.74	0.033	0.5542	5.6199	10.30
20	17.08	0.0525	0.6449	7.5280	10.54
21	15.15	0.0375	0.4482	7.7208	10.78
22	13.41	0.0336	0.3272	9.3126	13.93
23	19.48	0.0908	0.9736	8.5306	12.28
24	16.13	0.0470	0.5181	8.3171	11.20
25	20.33	0.0935	1.2404	7.0095	11.13
26	13.22	0.0302	0.4413	6.4051	13.07
27	14.11	0.0398	0.4919	7.4854	14.17
28	13.69	0.0355	0.4381	7.4958	13.84
29	13.72	0.0333	0.5626	5.5882	12.89
30	14.63	0.0426	0.4793	8.1625	13.60
Average	**15.89**	**0.0474**	**0.5999**	**7.3479**	**11.56**
SD	**1.71**	**0.0171**	**0.1872**	**1.3061**	**1.88**

Appendix 1b. Gulf of Gdańsk (GD), Poland, spring, May 2006.

Individual number	Shell length (mm)	Dry tissue weight (g)	Dry shell weight (g)	Dry tissue weight content in the total dry weight (%)	Condition index, *CI*
1	15.31	0.0262	0.4629	5.3568	7.30
2	15.68	0.0318	0.5783	5.2123	8.25
3	16.91	0.0504	0.6446	7.2518	10.42
4	19.55	0.0552	1.2449	4.2458	7.39
5	16.80	0.0589	0.8276	6.6441	12.42
6	14.54	0.0238	0.3168	6.9877	7.74
7	16.89	0.0386	0.7043	5.1959	8.01
8	17.73	0.0386	0.9742	3.8112	6.93
9	15.59	0.0432	0.4640	8.5174	11.40
10	15.58	0.0395	0.6587	5.6574	10.44
11	16.72	0.0531	0.6305	7.7677	11.36
12	18.11	0.0447	0.8770	4.8497	7.53
13	16.77	0.0384	0.5993	6.0216	8.14
14	16.19	0.0466	0.6246	6.9428	10.98
15	16.29	0.0458	0.7056	6.0953	10.60
16	15.24	0.0327	0.4148	7.3073	9.24
17	15.4	0.0369	0.4371	7.7848	10.10
18	17.19	0.0497	0.5460	8.3431	9.78
19	15.48	0.0233	0.5225	4.2690	6.28
20	16.65	0.0348	0.6351	5.1948	7.54
21	14.35	0.0219	0.4010	5.1785	7.41
22	16.13	0.0417	0.5930	6.5700	9.94
23	17.36	0.0586	0.6870	7.8594	11.20
24	16.54	0.0296	0.4787	5.8233	6.54
25	16.51	0.0445	0.5643	7.3095	9.89
26	17.18	0.0351	0.5099	6.4404	6.92
27	18.1	0.0536	0.6088	8.0918	9.04
28	16.28	0.0354	0.5035	6.5689	8.20
29	16.85	0.0356	0.5234	6.3685	7.44
30	16.67	0.0536	0.6985	7.1267	11.57
Average	**16.49**	**0.0407**	**0.6146**	**6.3598**	**9.00**
SD	**1.09**	**0.0105**	**0.1850**	**1.2725**	**1.76**

Appendix 1c. Gulf of Gdańsk (GD), Poland, summer, July 2006.

Individual number	Shell length (mm)	Dry tissue weight (g)	Dry shell weight (g)	Dry tissue weight content in the total dry weight (%)	Condition index, *CI*
1	13.73	0.0215	0.5451	3.7946	8.31
2	17.45	0.0412	0.8439	4.6548	7.75
3	15.65	0.0280	0.4719	5.6011	7.30
4	15.68	0.0314	0.7524	4.0061	8.15
5	13.93	0.0174	0.3536	4.6900	6.44
6	15.64	0.0180	0.5493	3.1729	4.71
7	15.89	0.0344	0.5914	5.4970	8.57
8	15.81	0.0174	0.6234	2.7154	4.40
9	15.36	0.0346	0.5036	6.4288	9.55
10	16.89	0.0326	0.7291	4.2799	6.77
11	14.50	0.0135	0.6016	2.1948	4.43
12	15.51	0.0264	0.5579	4.5182	7.08
13	16.56	0.0279	0.5066	5.2198	6.14
14	16.08	0.0288	0.7199	3.8467	6.93
15	16.29	0.0511	0.7455	6.4148	11.82
16	14.46	0.0341	0.5740	5.6076	11.28
17	16.17	0.0334	0.6109	5.1839	7.90
18	15.89	0.0397	0.4992	7.3669	9.90
19	16.45	0.0386	0.7526	4.8787	8.67
20	17.61	0.0525	0.8116	6.0757	9.61
21	15.27	0.0258	0.5529	4.4583	7.25
22	16.21	0.0340	0.7908	4.1222	7.98
23	16.33	0.0365	0.6285	5.4887	8.38
24	16.18	0.0364	0.7769	4.4756	8.59
25	15.50	0.0317	0.5593	5.3638	8.51
26	15.33	0.0177	0.6466	2.6645	4.91
27	17.41	0.0447	0.9633	4.4345	8.47
28	15.11	0.0159	0.5160	2.9893	4.61
29	13.16	0.0162	0.3673	4.2243	7.11
30	16.48	0.0428	0.9000	4.5397	9.56
Average	**15.75**	**0.0308**	**0.6348**	**4.6303**	**7.70**
SD	**1.05**	**0.0105**	**0.1484**	**1.1931**	**1.90**

Appendix 1d. Gulf of Gdańsk (GD), Poland, autumn, September 2005.

Individual number	**Shell length (mm)**	**Dry tissue weight (g)**	**Dry shell weight (g)**	**Dry tissue weight content in the total dry weight (%)**	**Condition index, *CI***
1	15.30	0.0435	0.3381	11.3994	12.15
2	13.66	0.0285	0.2615	9.8276	11.18
3	17.34	0.0530	0.5659	8.5636	10.17
4	15.96	0.0464	0.6287	6.8731	11.41
5	14.99	0.0494	0.3895	11.2554	14.67
6	15.82	0.0447	0.4149	9.7258	11.29
7	15.27	0.0402	0.4992	7.4527	11.29
8	12.90	0.0221	0.3074	6.7071	10.29
9	14.03	0.0332	0.3492	8.6820	12.02
10	14.88	0.0264	0.3436	7.1351	8.01
11	16.78	0.0410	0.5033	7.5326	8.68
12	17.69	0.0527	0.7391	6.6557	9.52
13	17.17	0.0525	0.6148	7.8675	10.37
14	13.49	0.0210	0.3392	5.8301	8.55
15	14.75	0.0422	0.3793	10.0119	13.15
16	14.02	0.0284	0.2960	8.7546	10.31
17	17.96	0.0699	0.6266	10.0359	12.07
18	14.47	0.0335	0.3486	8.7673	11.06
19	15.68	0.0408	0.3588	10.2102	10.58
20	12.8	0.0345	0.3308	9.4443	16.45
21	13.51	0.0353	0.3346	9.5431	14.32
22	13.22	0.0166	0.3131	5.0349	7.18
23	13.75	0.0267	0.2543	9.5018	10.27
24	15.69	0.0363	0.3346	9.7870	9.40
25	19.71	0.0622	0.9777	5.9813	8.12
26	14.87	0.0356	0.3790	8.5866	10.83
27	14.84	0.0367	0.2763	11.7252	11.23
28	14.38	0.0296	0.4162	6.6397	9.95
29	14.82	0.0377	0.3947	8.7188	11.58
30	15.62	0.0436	0.4070	9.6760	11.44
Average	**15.18**	**0.0388**	**0.4241**	**8.5975**	**10.92**
SD	**1.63**	**0.0120**	**0.1602**	**1.7191**	**1.99**

Appendix 1e. Gulf of Gdańsk (GD), Poland, autumn, October 2006.

Individual number	**Shell length (mm)**	**Dry tissue weight (g)**	**Dry shell weight (g)**	**Dry tissue weight content in the total dry weight (%)**	**Condition index, *CI***
1	17.25	0.0465	0.6489	6.6868	9.06
2	17.10	0.0487	0.7344	6.2189	9.74
3	16.54	0.0456	0.6394	6.6569	10.08
4	17.38	0.0620	0.8003	7.1901	11.81
5	19.96	0.0469	0.6006	7.2432	5.90
6	16.86	0.0513	0.6473	7.3433	10.70
7	16.54	0.0593	0.6173	8.7644	13.11
8	18.00	0.0503	0.688	6.8129	8.62
9	16.30	0.0426	0.4564	8.5371	9.84
10	15.52	0.0382	0.5453	6.5467	10.22
11	16.98	0.0482	0.7509	6.0318	9.85
12	16.00	0.0371	0.5908	5.9086	9.06
13	20.46	0.0823	1.1399	6.7338	9.61
14	16.42	0.0413	0.5434	7.0635	9.33
15	16.55	0.0468	0.5423	7.9443	10.32
16	16.93	0.0356	0.4401	7.4837	7.34
17	17.54	0.0607	0.7722	7.2878	11.25
18	17.44	0.0496	0.7643	6.0941	9.35
19	17.41	0.0533	0.7344	6.7665	10.10
20	17.64	0.0192	0.3663	4.9805	3.50
21	17.16	0.0570	0.7433	7.1223	11.28
22	19.81	0.0695	1.0823	6.0340	8.94
23	17.08	0.0511	0.4892	9.4577	10.26
24	16.41	0.0484	0.4902	8.9863	10.95
25	18.17	0.0606	0.678	8.2047	10.10
26	16.89	0.0478	0.4988	8.7450	9.92
27	17.02	0.0542	0.5823	8.5153	10.99
28	16.42	0.0479	0.6674	6.6965	10.82
29	15.87	0.0408	0.4193	8.8676	10.21
30	16.19	0.044	0.5671	7.2001	10.37
Average	**17.19**	**0.0496**	**0.6413**	**7.2708**	**9.75**
SD	**1.15**	**0.0114**	**0.1715**	**1.0870**	**1.76**

Appendix 1f. Lake Veere (LV), the Netherlands, winter, February 2006.

Individual number	**Shell length (mm)**	**Dry tissue weight (g)**	**Dry shell weight (g)**	**Dry tissue weight content in the total dry weight (%)**	**Condition index, *CI***
1	29.00	0.2212	3.0702	6.7205	9.07
2	29.00	0.1926	3.1813	5.7085	7.90
3	29.20	0.2997	3.8061	7.2994	12.04
4	29.80	0.2682	3.3535	7.4054	10.13
5	29.00	0.2357	3.9666	5.6088	9.66
6	28.10	0.1946	3.3714	5.4571	8.77
7	26.00	0.1711	2.3750	6.7201	9.73
8	28.50	0.2743	3.4669	7.3319	11.85
9	30.50	0.2554	3.8458	6.2274	9.00
10	26.00	0.1774	2.8757	5.8105	10.09
11	26.00	0.1842	4.3786	4.0370	10.48
12	31.50	0.2709	3.1274	7.9716	8.67
13	28.80	0.2512	3.2065	7.2649	10.52
14	27.00	0.1668	2.6634	5.8936	8.47
15	28.00	0.2083	3.4641	5.6720	9.49
16	27.00	0.1808	3.2864	5.2146	9.19
17	30.00	0.2636	4.2705	5.8137	9.76
18	31.50	0.3325	4.6243	6.7080	10.64
19	28.00	0.2642	3.8670	6.3952	12.04
20	32.20	0.3146	4.6775	6.3020	9.42
21	29.00	0.2713	3.4536	7.2834	11.12
22	31.00	0.3547	4.3305	7.5706	11.91
23	31.50	0.2525	3.6121	6.5337	8.08
24	31.50	0.3476	4.3315	7.4288	11.12
25	32.00	0.3601	5.8239	5.8231	10.99
26	27.00	0.2003	3.1869	5.9134	10.18
27	28.00	0.1750	3.0681	5.3961	7.97
28	26.00	0.2181	3.3932	6.0394	12.41
29	29.50	0.2844	4.5527	5.8796	11.08
30	32.00	0.2124	2.8892	6.8481	6.48
Average	**29.09**	**0.2468**	**3.6507**	**6.3426**	**9.94**
SD	**1.98**	**0.0576**	**0.7294**	**0.8712**	**1.44**

Appendix 1g. Lake Veere (LV), the Netherlands, spring, May 2006.

Individual number	Shell length (mm)	Dry tissue weight (g)	Dry shell weight (g)	Dry tissue weight content in the total dry weight (%)	Condition index, *CI*
1	26.00	0.2708	4.5575	5.6086	15.41
2	25.50	0.3077	4.2115	6.8087	18.56
3	29.00	0.3415	5.5500	5.7965	14.00
4	29.00	0.3461	4.9901	6.4859	14.19
5	25.00	0.2835	3.1582	8.2372	18.14
6	23.00	0.1613	3.7879	4.0844	13.26
7	24.00	0.1991	2.3805	7.7183	14.40
8	24.00	0.2646	3.6258	6.8014	19.14
9	26.00	0.3820	4.1896	8.3559	21.73
10	22.00	0.1770	2.1884	7.4829	16.62
11	27.00	0.3767	4.9915	7.0172	19.14
12	25.00	0.2732	4.3511	5.9079	17.48
13	22.50	0.2262	2.8447	7.3659	19.86
14	22.00	0.1827	2.7889	6.1482	17.16
15	21.00	0.1349	2.8238	4.5594	14.57
16	18.00	0.1687	2.1762	7.1943	28.93
17	21.00	0.1824	3.4408	5.0342	19.70
18	24.00	0.2327	3.9831	5.5197	16.83
19	25.50	0.2645	4.9589	5.0638	15.95
20	24.00	0.2920	3.8613	7.0306	21.12
21	22.00	0.1589	3.4788	4.3681	14.92
22	22.50	0.1874	2.8166	6.2383	16.45
23	25.00	0.4768	4.6111	9.3713	30.52
24	28.00	0.4416	5.4715	7.4682	20.12
25	26.00	0.4802	5.2511	8.3786	27.32
26	27.00	0.2287	3.2958	6.4889	11.62
27	29.00	0.3804	4.8104	7.3284	15.60
28	21.00	0.1648	2.9960	5.2139	17.80
29	24.50	0.2862	3.4885	7.5821	19.46
30	28.00	0.2715	3.7699	6.7180	12.37
Average	**24.55**	**0.2715**	**3.8283**	**6.5792**	**18.08**
SD	**2.73**	**0.0965**	**0.9792**	**1.2880**	**4.49**

Appendix 1h. Lake Veere (LV), the Netherlands, summer, July 2006.

Individual number	Shell length (mm)	Dry tissue weight (g)	Dry shell weight (g)	Dry tissue weight content in the total dry weight (%)	Condition index, *CI*
1	30.50	0.4570	5.5571	7.5988	16.11
2	27.50	0.2388	3.0537	7.2528	11.48
3	27.00	0.1580	3.2443	4.6439	8.03
4	30.50	0.2998	4.6503	6.0564	10.57
5	30.00	0.2840	4.3795	6.0898	10.52
6	27.80	0.2468	4.2600	5.4762	11.49
7	28.20	0.3046	4.0875	6.9352	13.58
8	27.20	0.2316	2.8946	7.4084	11.51
9	30.00	0.2159	4.4600	4.6173	8.00
10	28.00	0.2218	3.6644	5.7074	10.10
11	25.50	0.1555	3.2322	4.5901	9.38
12	27.20	0.1627	3.9642	3.9424	8.09
13	25.00	0.1496	2.3270	6.0405	9.57
14	30.00	0.1925	5.0561	3.6676	7.13
15	31.50	0.4725	5.9217	7.3895	15.12
16	31.00	0.6217	6.3469	8.9214	20.87
17	32.00	0.2873	4.4627	6.0484	8.77
18	32.00	0.5185	5.9067	8.0698	15.82
19	29.80	0.1950	3.5311	5.2334	7.37
20	33.00	0.3147	5.9472	5.0256	8.76
21	32.30	0.4612	6.8569	6.3022	13.69
22	31.00	0.2334	4.7484	4.6851	7.83
23	38.00	0.7059	9.525	6.8997	12.86
24	33.20	0.4210	5.9144	6.6452	11.50
25	34.00	0.4362	5.5151	7.3295	11.10
26	32.00	0.4520	4.8835	8.4716	13.79
27	33.80	0.4059	5.2767	7.1429	10.51
28	30.80	0.4639	5.0765	8.3730	15.88
29	31.00	0.2730	5.1425	5.0411	9.16
30	28.50	0.3052	2.0368	13.0316	13.18
Average	**30.28**	**0.3295**	**4.7308**	**6.4879**	**11.39**
SD	**2.79**	**0.1438**	**1.5036**	**1.8582**	**3.20**

Appendix 1i. Lake Veere (LV), the Netherlands, autumn, November 2006.

Individual number	**Shell length (mm)**	**Dry tissue weight (g)**	**Dry shell weight (g)**	**Dry tissue weight content in the total dry weight (%)**	**Condition index, *CI***
1	25.50	0.2787	2.6018	9.6754	16.81
2	28.00	0.2725	2.8626	8.6919	12.41
3	26.00	0.3081	2.7652	10.0251	17.53
4	26.00	0.2666	2.7871	8.7304	15.17
5	29.00	0.3502	2.8231	11.0358	14.36
6	21.50	0.2453	2.6092	8.5934	24.68
7	25.80	0.2494	2.7691	8.2624	14.52
8	29.00	0.3656	3.2852	10.0142	14.99
9	23.00	0.2350	2.2509	9.4533	19.31
10	25.00	0.2468	2.3077	9.6614	15.80
11	26.00	0.1943	2.0889	8.5100	11.05
12	29.50	0.3405	3.6708	8.4885	13.26
13	28.00	0.2716	2.9921	8.3218	12.37
14	26.50	0.1795	2.5505	6.5751	9.65
15	23.50	0.1821	2.4496	6.9195	14.03
16	29.50	0.362	3.3648	9.7134	14.10
17	27.00	0.2252	2.8881	7.2335	11.44
18	26.00	0.2588	2.5482	9.2198	14.72
19	25.50	0.1907	2.7179	6.5564	11.50
20	30.50	0.2381	3.0416	7.2598	8.39
21	29.20	0.2979	3.6614	7.5241	11.97
22	30.50	0.2853	3.4319	7.6751	10.06
23	28.50	0.3287	3.8444	7.8766	14.20
24	29.40	0.2789	3.676	7.0520	10.98
25	29.80	0.2868	3.8965	6.8558	10.84
26	28.50	0.2704	3.3799	7.4076	11.68
27	29.00	0.3333	3.5045	8.6847	13.67
28	27.00	0.2343	2.9331	7.3972	11.90
29	29.00	0.3104	3.6346	7.8682	12.73
30	27.50	0.2784	3.4901	7.3876	13.39
Average	**27.31**	**0.2722**	**3.0276**	**8.2890**	**13.58**
SD	**2.26**	**0.0512**	**0.5077**	**1.1679**	**3.18**

Appendix 1j. Berre Lagoon (BL), France, winter, February 2006.

Individual number	Shell length (mm)	Dry tissue weight (g)	Dry shell weight (g)	Dry tissue weight content in the total dry weight (%)	Condition index, *CI*
1	24.00	0.2467	3.2979	6.9599	17.85
2	24.00	0.2083	2.2363	8.5208	15.07
3	16.00	0.0918	0.7435	10.9901	22.41
4	19.00	0.1344	1.4682	8.3864	19.59
5	18.50	0.1283	1.1348	10.1575	20.26
6	17.00	0.0773	0.8135	8.6776	15.73
7	20.00	0.1352	1.3397	9.1667	16.90
8	19.50	0.2810	3.0472	8.4430	37.90
9	24.50	0.1472	2.8560	4.9014	10.01
10	18.00	0.1282	1.3075	8.9294	21.98
11	22.00	0.1482	1.7680	7.7341	13.92
12	20.50	0.1455	1.5917	8.3755	16.89
13	19.00	0.1136	1.1628	8.9000	16.56
14	21.00	0.1490	1.1456	11.5093	16.09
15	24.00	0.2310	2.2942	9.1478	16.71
16	17.20	0.0712	0.6628	9.7003	13.99
17	24.50	0.2211	1.9298	10.2794	15.03
18	18.00	0.0882	0.9796	8.2600	15.12
19	25.00	0.2211	2.2167	9.0697	14.15
20	23.00	0.2059	2.1175	8.8620	16.92
21	22.50	0.1820	2.0319	8.2208	15.98
22	19.80	0.1160	1.1506	9.1584	14.94
23	18.00	0.1068	1.1047	8.8155	18.31
24	17.00	0.0789	0.7087	10.0178	16.06
25	17.00	0.1064	1.1107	8.7421	21.66
26	17.50	0.0847	1.6093	5.0000	15.80
27	22.50	0.1798	2.2908	7.2776	15.78
28	17.50	0.1084	1.0761	9.1515	20.23
29	17.00	0.1110	0.734	13.1361	22.59
30	18.00	0.0910	1.1333	7.4328	15.60
Average	**20.05**	**0.1446**	**1.5688**	**8.7975**	**17.67**
SD	**2.83**	**0.0567**	**0.7188**	**1.6340**	**4.77**

Appendix 1k. Berre Lagoon (BL), France, spring, May 2006.

Individual number	**Shell length (mm)**	**Dry tissue weight (g)**	**Dry shell weight (g)**	**Dry tissue weight content in the total dry weight (%)**	**Condition index, *CI***
1	19.39	0.1278	1.7439	6.8280	17.53
2	20.20	0.1489	1.9998	6.9298	18.07
3	21.66	0.1157	1.7999	6.0399	11.39
4	22.29	0.1974	3.0426	6.0926	17.82
5	21.89	0.1588	1.9825	7.4161	15.14
6	18.63	0.1065	1.4106	7.0200	16.47
7	20.94	0.1246	1.6516	7.0150	13.57
8	22.68	0.1342	2.9181	4.3967	11.50
9	20.20	0.1453	2.3651	5.7879	17.63
10	22.67	0.1364	2.4065	5.3640	11.71
11	21.46	0.1601	1.9896	7.4476	16.20
12	21.10	0.1499	1.9370	7.1829	15.96
13	21.21	0.1556	1.6385	8.6729	16.31
14	21.86	0.1198	1.8658	6.0334	11.47
15	24.09	0.1558	2.0116	7.1883	11.14
16	22.71	0.1671	2.0670	7.4795	14.27
17	20.72	0.1988	2.6946	6.8708	22.35
18	20.94	0.1298	1.8493	6.5585	14.14
19	21.00	0.0877	1.2619	6.4982	9.47
20	18.59	0.1153	1.4342	7.4411	17.95
21	19.72	0.1034	1.2121	7.8601	13.48
22	16.79	0.1479	2.2161	6.2563	31.25
23	21.52	0.1390	2.0837	6.2537	13.95
24	21.87	0.1189	1.7539	6.3488	11.37
25	17.32	0.0908	1.0203	8.1721	17.48
26	22.91	0.2111	3.0665	6.4407	17.56
27	18.25	0.1008	1.2813	7.2932	16.58
28	20.36	0.1232	1.6964	6.7707	14.60
29	15.26	0.0498	0.6906	6.7261	14.01
30	23.03	0.1999	2.9158	6.4159	16.37
Average	**20.71**	**0.1373**	**1.9336**	**6.7600**	**15.56**
SD	**2.01**	**0.0360**	**0.5928**	**0.8419**	**4.10**

Appendix 1l. Berre Lagoon (BL), France, summer, July 2006.

Individual number	Shell length (mm)	Dry tissue weight (g)	Dry shell weight (g)	Dry tissue weight content in the total dry weight (%)	Condition index, *CI*
1	18.43	0.1019	1.3172	7.1806	16.28
2	22.50	0.1504	1.7666	7.8456	13.20
3	28.13	0.2840	3.2437	8.0506	12.76
4	20.17	0.1079	1.3260	7.5249	13.15
5	27.27	0.2576	3.4281	6.9892	12.70
6	23.07	0.1983	2.5390	7.2444	16.15
7	15.49	0.0608	0.6488	8.5682	16.36
8	21.33	0.1430	1.5718	8.3392	14.74
9	20.28	0.1189	1.2272	8.8329	14.26
10	23.13	0.1624	2.7368	5.6015	13.12
11	21.63	0.1622	2.0866	7.2127	16.03
12	20.28	0.1122	1.4166	7.3391	13.45
13	16.18	0.0944	0.9951	8.6645	22.29
14	24.55	0.1934	2.7973	6.4667	13.07
15	23.53	0.1932	2.4053	7.4351	14.83
16	28.51	0.2423	3.6800	6.1775	10.46
17	23.02	0.1944	2.2662	7.9005	15.94
18	22.95	0.1722	1.9691	8.0418	14.25
19	21.38	0.1424	1.4840	8.7555	14.57
20	23.07	0.1434	2.3693	5.7070	11.68
21	24.54	0.1923	2.5039	7.1323	13.01
22	19.93	0.1643	1.7170	8.7333	20.75
23	21.12	0.1343	1.6448	7.5488	14.26
24	21.72	0.1342	1.8267	6.8438	13.10
25	21.72	0.1324	1.8043	6.8383	12.92
26	20.94	0.1522	2.0885	6.7925	16.58
27	21.94	0.1534	2.0429	6.9845	14.52
28	24.83	0.1922	2.4854	7.1781	12.56
29	20.42	0.1323	1.2886	9.3110	15.54
30	21.70	0.1223	1.6688	6.8282	11.97
Average	**22.13**	**0.1582**	**2.0115**	**7.4689**	**14.48**
SD	**2.90**	**0.0485**	**0.7154**	**0.9230**	**2.46**

Appendix 1m. Berre Lagoon (BL), France, autumn, November 2006.

Individual number	**Shell length (mm)**	**Dry tissue weight (g)**	**Dry shell weight (g)**	**Dry tissue weight content in the total dry weight (%)**	**Condition index, *CI***
1	19.20	0.1151	1.7501	6.1709	16.26
2	21.00	0.1347	1.7828	7.0248	14.54
3	23.80	0.1643	2.6689	5.7991	12.19
4	23.20	0.155	2.3600	6.1630	12.41
5	21.00	0.1032	2.1717	4.5365	11.14
6	24.00	0.1642	2.2902	6.6900	11.88
7	21.00	0.0898	1.3288	6.3302	9.70
8	21.00	0.1346	2.1058	6.0079	14.53
9	20.00	0.1444	1.8300	7.3136	18.05
10	20.50	0.1075	2.1922	4.6745	12.48
11	22.00	0.1490	2.0287	6.8421	13.99
12	20.00	0.1119	1.5561	6.7086	13.99
13	21.50	0.1798	1.7179	9.4746	18.09
14	20.00	0.1234	1.8974	6.1065	15.43
15	24.00	0.1842	2.6377	6.5275	13.32
16	20.00	0.1358	1.9390	6.5452	16.98
17	20.20	0.1089	1.9913	5.1852	13.21
18	20.10	0.1208	1.8081	6.2626	14.88
19	19.00	0.1279	1.8797	6.3708	18.65
20	20.00	0.1143	1.3044	8.0567	14.29
21	19.70	0.1265	1.8909	6.2704	16.55
22	23.00	0.2047	3.4112	5.6611	16.82
23	22.30	0.1668	2.3959	6.5088	15.04
24	20.00	0.1449	1.8369	7.3115	18.11
25	19.50	0.1211	1.5572	7.2156	16.33
26	19.50	0.1173	1.7269	6.3605	15.82
27	21.20	0.1543	2.2230	6.4906	16.19
28	20.90	0.0714	2.6513	2.6224	7.82
29	21.00	0.1332	1.9295	6.4576	14.38
30	19.00	0.1114	1.4730	7.0311	16.24
Average	**20.92**	**0.1340**	**2.0112**	**6.3573**	**14.64**
SD	**1.47**	**0.0291**	**0.4461**	**1.1625**	**2.56**

Appendix 2. The sequence of the haplotype H1 and its translation into amino acids.

Reading frame for the translation into amino acids starts at the second position of the DNA sequence.

```
1     TAATCATGGG   CTATACAATC   TTATTATTAC   AAGGCACGCT   TTGATCATAA
51    TTTTCTTTAT   GGTGATGCCA   GTGATAATGG   GTGGTTTTGG   TAATTGGTTA
101   GTTCCGTTAA   TATTGATGGT   ACCTGATATG   CATTTCCCCC   GGTTGAACAA
151   TATAAGATTT   TGGTTTGTTC   CAAATGCTTT   AATCCTGTTA   GCATTTTCTG
201   GATTCGTAGA   AGGTGGTGTT   GGGGCTGGTT   GAACTATTTA   TCCCCCTTTA
251   ACTTCGATTG   AATTCTTGGG   GGACCCTTCA   ATAGACTTGG   CTATTTTTGC
301   GCTACACCTA   GGGGGAATCT   CTTCTATTGC   AGCAAGTCTG   AATTTCTGTT
351    CGACTGCTAT  TAATATGCGA   CAGAGACAAC   GATGGGTGCA   CAAAATTCCA
401   ATATTACCAA   TTTCTCTAGC   GATTACAGCT   CTTCTATTGA   TTATTGCTAT
451   GCCGGTTCTG   GCTGGAGCTC   TAACTATACT   TCTTTTAGAT   CGGAATTTTG
501   CTACAAGATT   TTTC
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1     NHGLYNLIIT   SHALIMIFFM   VMPVMMGGFG   NWLVPLMLMV   PDMHFPRLNN
51    MSFWFVPNAL   ILLAFSGFVE   GGVGAGWTIY   PPLTSIEFLG   DPSMDLAIFA
101   LHLGGISSIA   ASLNFCSTAI   NMRQSQRWVH   KIPMLPISLA   ITALLLIIAM
151   PVLAGALTML   LLDRNFATSF   F
```

Appendix 3. Observed mtDNA COI haplotype frequencies

Haplotypes were grouped according to haplotype network (Fig. 11.2). See Table 10.1 for sites abbreviations.

Haplogroup	Haplotype number	FI	GD	AL	LV	AR	AN	PT	BL	SA	TU	SE	SM	SS	GI	ET	PA	GR	RO	UK	BK	TOTAL
I	1	18	15		1																	34
	2	3																				3
	3	2																				2
	4		2																			2
	5	1																				1
	6		1																			1
	7							17														17
	8							1														1
	9							1														1
	10							1														1
	11							1														1
	12							1														1
III	13			6	17	10	12															45
	14			5																		5
	15			8																		8
	16				1																	1
	17									10												10
	18									1												1
	19									2												2
	20									1												1
	21									2												2
	22									1												1
	23									1												1
	24									1												1
	25									1												1
	26									1												1
	27								1													1
	28								1													1
	29						1															1
	30						1															1
	31						1															1
	32						1															1
	33					1																1
	34					1																1
	35					1																1
	36					1																1
	37					1																1
	38					1																1
III	39								9			1	2	3								15
	40												1	5								6
	41													1								1
	42													1								1
	43								1													1
	44												1	1								2
	45								1													1
	46								1													1
	47								3													3
	48								1													1
	49								1			1										2
	50								1													1
	51								1													1
IV	52										8											8
	53										4											4
	54										4											4
	55										2											2
	56										2											2
	57										1											1
	58										1											1
	59										1											1
	60										1											1
	61										1											1
V	62														7							7
	63														1							1
	64														1	1	1					3
	65																1					1
	66															1						1
	67																1					1
	68														2	3	1					6

	69															1						1
	70															1						1
	71															2						2
	72																6					6
	73															1						1
	74																1					1
VI	75																	8	6	12	3	29
	76																	1				1
	77																	1				1
	78																		2		1	3
	79																		2			2
	80																			1		1
	81																			1		1
	82																	1				1
	83																	2				2
	84																	1				1
	85																	1				1
	86																			1		1
	87																			2		2
	88																		9	3	1	13
	89																		1			1
	90																			1		1
	Sum	24	18	19	19	16	16	22	21	21	25	2	4	11	11	10	11	15	20	21	5	311

Appendix 4. Variable sites in the 90 haplotypes of *C. glaucum*

Dots correspond to the same base pair as in haplotype H1

```
     41111133455678999111111111111111111112222222222222222222333333333333333344444444444444444444
      0136947358132147002223333445677888890012234444556778889000111344567778900011112334667788999
                      361470369581089157802570921478065170378247039169540698746912561495360845036
  1  TGCACTGCGCTGGTTTGTGATTGTCCGCTTTTACGTAACTTTATCTTAGCGTAATTCACAGCACTTTAAAGTTAATTTCGCGTTACATTTGT
  2  .....................................................................G......................
  3  ..........................................................................................T.
  4  ....................................................................G.......................
  5  .....................................G......................................................
  6  .............................................................................C..............
  7  ......A..............................G......................................................
  8  ......A............................C.G...A..................................................
  9  ......A...........................C..G......................................................
 10  ..T...A..............................G.....C................................................
 11  ......A.A............................G......................................................
 12  ...G..A..............................G......................................................
 13  .....CA...............A..T.....C.............................................C..T......C....
 14  ...G.CA...............A..T.....C.............................................C..T......C....
 15  .....CA...............A..T.....C.............................................CT.T......C....
 16  .....CA...............A..T....CC.............................................C..T......C....
 17  .....CA...........T...A..T.....C.............................................C..T......C....
 18  .....CA...........T...A..T.....C.........C...................................C..T......C....
 19  .....CA...........T...A..T.....C.............................................C..T...G..C....
 20  .....CA...........T...A..T.....C...........................................C.C..T......C....
 21  .....CA...........T...A..T.....C...............................T.............C..T......C....
 22  .....CA...........T...A..T.....C.............................................C..T.C....C....
 23  ..T..CA...............A..T.....C.............................................C..T......C....
 24  ..T..CA...............A........C.............................................C..T......C....
 25  ..T..CA...............A........C......T......................................C..T......C....
 26  ..T..CAT..............A........C.............................................C..T......C....
 27  .....CA...............A..T.....C............................................CC..T......C....
 28  .....CA...............A..T.....C.............C..............................CC..T......C....
 29  .....C................A..T.....C.............................................C..T......C....
 30  .....CA.............C.A..T.....C.............................................C..T......C....
 31  .....CA...............A..T.....C.....................G.......................C..T......C....
 32  .....CA...............A..T.....C.....G.......................................C..T......C....
 33  .....CA...............A..T.....C...........................G.................C..T......C....
 34  .....CA...............A..T.....C.............................................C..T...........
 35  .....CA...............A..T.....C.............................................C..T......A....
 36  .....CA...............A..T.....C..........................................T..C..T......C....
 37  .....CA...............A..T...C.C.............................................C..T......C.C..
 38  .....CA........C......A..T.....C.............................................C..T......C....
 39  ......A...............A.T..................C..............T.T................C.........C...C
 40  ......A...............A.T..................CT.............T.T................C.........C...C
 41  C.....A...............A.T..................CT.............T.T................C.........C...C
 42  ......A.........A.....A.T..................C..............T.T................C.........C...C
 43  ......A...............A.T.................................T.T................C.........C...C
 44  ......A...............A.T..................C..............T.T................C...A.....C...C
 45  ......A...............A.T..................C............T.T.T................C.........C...C
 46  ......A...............A.T..................C..............T.T...........C....C.........C...C
 47  ......A...............A.T.................GC..............T.T................C.........C...C
 48  ......A...............A.T...............C..C..............T.T......T.........C.........C...C
 49  ......A........C......A.T..................C..............T.T................C.........C...C
 50  ......A...............A.T..................C..C...........T.T................C.........C...C
 51  .T....A...............A.T..................C..............T.T................C.........C...C
 52  .....CA......C.C..A...A.T......C..A........C.............................G...C..T..C...C....
 53  .....CA......C.C......A.T......C..A........C.............................G...C..T..C...C....
 54  .....CA......C.C......A........C..A........C.............................G...C..T..C...C....
 55  ....TCA......C.....G..A........C..A........C........G....................G...C..T..C...C....
 56  ..T.TCA......C........A..T.....C..A......................................G...C..T..C...C....
 57  ....TCA.....AC...G.G..A........C..A........CT............................G...C..T......C....
 58  ....TCA.....AC........A.T......C..A........CT............................G...C..T......C....
 59  ....TCA.....AC........A........C..A........CT............................G......T......C....
 60  .....CA......C.C...G..A..T.....C..A....C...C.............................G...C..T..C...C....
 61  .....CA......C.C...G..A.T......C..A........C.............................G...C..T..C...C....
 62  ......A.....A...AC....A.T....................................................C.........C....
 63  ......A..T..A...AC....A.T....................................................C.........C....
 64  ......A.........AC....A..T...................................................C.........C....
 65  ......A..........C....A..T......G............................................C.........C....
 66  ......A.....A...AC.G..A....................C.................................C.........C....
 67  ......A.....A...AC.G..A....................C.....................C...........C.........C....
 68 ......A..........C....A.T......C.................T....................A......C.........C....
 69  ......A..........C....A.T......C.................T....C...............A......C.........C....
 70  ......A..........C....A........C.................T.C.........................C............T.
 71  ......A..........C....A........C...........C.....T..............C............C.........C..T.
 72  ......A........C.C....A........C.................T..............C............C.........C..T.
 73  ......A..T.......C....A........C.................T..............C............C.........C..T.
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74   ......A..T.....C.C....A........C.................T..............C............C.........C..T.
75   ..........CA.........CAC..ATCC.C..A.........CG.........G...G...TG.....G..CC.G..CTAT....TG.C.A.
76   ..........CA.........CAC..ATCC.C..A.........CG.........G...G...TG.....G..CC.G..CTAT....TG.C...
77   ..........CA.........CAC..ATCC.C..A.........CG.........G...G...TG.....G..CC.G..CTAT....TG.....
78   ..........CA.........CAC..ATCC.C..A.G.......CG.........G...G...TG.....G..CC.G..CTAT....TG.C...
79   ..........CA.........CAC..ATCC.C..A.........CG.....A..G...G...TG.....G..CC.G..CTAT....TG.C.A.
80   ..........CA.........CAC..ATCC.C..A.........CG.........G...G...TG...C.G..CC.G..CTAT....TG.C.A.
81   ..........CA..A......CAC..ATCC.C..A.........CG.........G...G...TG.....G..CC.G..CTAT....TG.C.A.
82   ..........CA.........CAC..ATCC.C..A....C...CG.........G.C.G...TG.....G..CC.G..CTAT....TG.C.A.
83   ..........CA.........CAC..AT.C.C..A.........CG.........G...G...TG.....G..CC.G..CTAT....TG.C.A.
84   ..........CA.........CAC..ATCC.C..A.........CG....T...G...G...TG.....G..CC.G..CTAT....TG.C.A.
85   ..........CA.........CAC..ATCC.C..A.........CG..G.....G...G...TG.....G..CC.G..CTAT....TG.C.A.
86   ..........CA.........CAC..ATCC.C..A..........G.........G...G...TG.....G..CC.G..CTAT....TG.C.A.
87   ..........CA.........CAC..ATCC.C.TA..........G.........G...G...TG.....G..CC.G..CTAT....TG.C.A.
88   ..........CA.........CAC..ATCC.C..A.........CG...T....G...G...TG.....G..CC.G..CTAT....TG.C.A.
89   ..........CA.........CA...ATCC.C..A.........CG...T....G...G...TG.....G..CC.G..CTAT....TG.C.A.
90   .........TCA.........CAC..ATCC.C..A.........CG...T....G...G...TG.....G..CC.G..CTAT....TG.C.A.
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Appendix 5. Microsatellite genotypes found for each individual and each locus.

Two alleles for each locus are marked in the same column (without space) with three digits. "0" means that there was no amplification at the given locus. Pop- population, Ind- individual. See Table 10.1 for sites abbreviations.

Pop	**Ind**	*Cg4*	*Cg7*	*Cg9*	*Cg11*	**Pop**	**Ind**	*Cg4*	*Cg7*	*Cg9*	*Cg11*
FI	1	162166	117119	225225	096099	**GD**	1	162195	113125	229229	082082
	2	166166	119121	225225	095098		2	195228	113119	225225	082096
	3	162166	119123	229229	095096		3	166205	113119	215229	095095
	4	166166	117117	0	082095		4	162166	119119	284284	096097
	5	166166	113119	225225	083096		5	162199	113119	225225	097097
	6	162166	117119	225225	083097		6	162166	119119	225225	082098
	7	162166	119121	225225	083096		7	225235	119119	0	0
	8	166166	119119	215225	083094		8	225235	119119	225225	083099
	9	162166	119125	225225	083096		9	166210	113119	225225	083098
	10	162166	119119	225225	083098		10	166208	113125	217225	097097
	11	166166	119199	225225	096098		11	162228	119119	217223	095095
	12	166166	115121	225225	095097		12	166210	119121	223223	097097
	13	166166	119119	229229	0		13	162208	119123	225225	096097
	14	166166	113117	225229	095098		14	162166	119119	0	082094
	15	166166	119119	225225	095098		15	162162	113119	225225	082082
	16	162166	119121	225225	082098		16	162222	117123	225225	094098
	17	166166	119121	225225	083099		17	162166	113123	217217	083083
	18	166166	117123	225225	096096		18	176176	119119	225225	082100
	19	162166	117119	284284	096097		19	162201	119119	217217	097099
	20	166166	119119	225225	082083		20	162162	113123	225225	082098
	21	166166	117119	225225	096097		21	193210	119119	225225	083098
	22	166166	117121	0	094094		22	162222	113121	225225	097097
	23	162166	119125	225225	083094		23	184197	119123	217217	094097
	24	166166	119119	225225	083095		24	162218	119119	225225	097097
	25	162166	117119	225229	083097		25	195237	113121	225225	097097
	26	162166	119119	225225	094094		26	166197	117119	225225	094097
	27	154166	117119	225225	083099		27	162166	113119	202217	082096
	28	162166	123123	225225	103111		28	162166	119119	225225	082094
	29	162166	117121	225225	082097		29	162164	113119	225225	097098
							30	162164	119119	225225	082097

Appendix 5. continuation.

Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*	Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*
AL	1	162166	113119	194217	098098	**LV**	1	162166	113119	229229	082083
	2	166166	119119	217229	082098		2	162162	113113	225229	083094
	3	162166	119119	217217	096097		3	162162	113113	229229	082094
	4	166166	113119	194217	095099		4	162166	113121	217229	082096
	5	162174	119119	194194	096099		5	162162	113113	229229	095097
	6	172174	119119	217217	083094		6	162166	119119	217229	083099
	7	162162	113119	211217	096098		7	166166	117121	217229	082082
	8	162174	119119	217217	098098		8	162166	121121	229229	082097
	9	162162	119119	217217	083096		9	162166	119125	225229	082097
	10	162174	119119	217217	083098		10	162166	119121	225229	083094
	11	162162	119119	217217	083098		11	162166	119121	229229	083094
	12	162162	113119	217217	096098		12	162166	119121	217229	097100
	13	162166	119119	194217	098100		13	166166	119119	0	083083
	14	166166	119119	194217	083098		14	162162	113117	225229	082098
	15	166166	113119	217217	096098		15	162166	119119	0	0
	16	162166	113119	198217	096098		16	162166	113121	229229	082098
	17	166166	0	217217	094098		17	162162	113123	0	082097
	18	162162	113119	211211	096096		18	162166	119119	0	076082
	19	166174	119119	194217	094098		19	162166	119121	217229	082099
	20	162162	113119	194225	083098		20	166166	119121	217229	082097
	21	162166	113119	217217	083096		21	162166	119121	225229	082082
	22	166172	119119	198217	095098		22	162166	113113	225229	083096
	23	166166	119119	198217	094097		23	162162	117119	217225	083098
	24	162166	119119	194217	096098		24	162166	113117	229229	082094
	25	166166	119119	194217	094098		25	162166	119119	217229	096099
	26	166172	119119	194225	096096		26	162166	113113	229229	082095
	27	162172	119119	217217	098099		27	162166	113121	225229	082094
	28	162166	119119	217229	083099		28	162162	113113	229229	082098
							29	162166	115119	0	0
							30	162166	113119	225229	097098

Appendix 5. continuation.

Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*	Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*
AR	1	162162	112113	225229	094094	**AN**	1	0	113121	225229	094097
	2	162162	113117	227229	094094		2	0	112113	229229	094099
	3	162164	112119	229229	094102		3	162172	113121	229229	093100
	4	162172	112120	225225	095095		4	162172	112114	225233	097097
	5	162172	113119	229229	094096		5	162162	113119	229229	095096
	6	162172	113120	229229	083094		6	172174	113119	229229	095102
	7	162172	113115	0	083094		7	162172	112120	229229	083095
	8	162172	112113	225229	095100		8	162162	112113	229229	097097
	9	162162	118119	229229	083098		9	162172	112113	225225	095095
	10	162162	112118	225225	082095		10	162162	114118	229229	083095
	11	162162	113113	229229	095095		11	162164	112113	229229	097098
	12	162162	112113	229229	083096		12	168172	113113	229229	095097
	13	162172	112113	229229	095095		13	162172	112113	229229	093095
	14	162174	112113	225229	093094		14	162162	121121	229229	097097
	15	162162	114118	225229	095099		15	172174	112113	187229	095099
	16	162172	113115	229233	097097		16	162162	113113	229229	095099
	17	155162	112113	229229	096103		17	162162	112113	225229	098098
	18	162162	113113	225225	096097		18	172176	112113	0	095096
	19	162172	112112	225225	095097		19	162162	112116	229229	095095
	20	162174	112113	229229	094098		20	162172	113121	229229	095097
	21	162172	112113	225227	083095		21	162162	113119	225229	095095
	22	162162	112118	229233	096098		22	162162	113113	229229	095101
	23	162162	112118	225229	095095		23	162164	114118	227229	094096
	24	162162	112116	229229	083099		24	162174	112118	225229	094094

Appendix 5. continuation.

Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*	Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*
PT	1	162162	119119	227227	0	**BL**	1	150162	111119	217225	099101
	2	162178	119123	227227	097097		2	162162	111113	229272	083095
	3	162162	121123	227227	083097		3	162162	111121	214214	097100
	4	172180	119121	229229	0		4	162162	113121	217229	082096
	5	162162	117121	225229	0		5	160162	109109	214225	082097
	6	162162	128130	227227	097099		6	162162	111121	225233	083098
	7	162162	119119	187229	099099		7	162162	113115	217229	083083
	8	162162	123123	187225	097097		8	160162	111123	214214	099099
	9	172172	119119	225225	096096		9	162184	111119	214229	081095
	10	162178	119119	225227	0		10	162174	111113	217217	083097
	11	162162	117123	0	0		11	162162	111121	217217	087095
	12	172172	121123	225225	100101		12	162162	111111	214229	082097
	13	162176	123128	225229	096100		13	162172	111117	229229	083094
	14	162172	123123	187225	098101		14	162174	111111	214217	098099
	15	162162	119121	187225	096097		15	162193	113121	194198	083100
	16	162162	119121	187225	0		16	162162	113113	217221	083083
	17	162176	123123	187187	099099		17	162172	121123	0	082096
	18	162162	119119	187187	0		18	162162	113121	217225	094094
	19	172174	123123	225227	095097		19	162172	111121	217229	083083
	20	162172	117119	187229	090097		20	162162	121125	198214	082100
	21	162176	119121	187229	083098		21	160162	111128	214229	095098
	22	172176	119119	225229	097100		22	162162	115119	215229	082095
	23	162162	119119	0	097097		23	162182	111119	229229	101101
	24	0	0	0	097097		24	162162	111117	221229	083097
	25	162172	121125	225225	0		25	162178	117125	217219	099099
	26	164174	113119	225225	0		26	162164	111119	198214	081095
	27	162162	125125	187229	0		27	162172	111113	214217	083096
	28	162174	119123	210210	0		28	162178	111119	198214	083083
	29	162162	119121	227229	097097		29	162162	119121	225225	083096

Appendix 5. continuation.

Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*
SA	1	162170	111111	229229	097098
	2	162162	111128	198229	095097
	3	162170	111130	198217	094098
	4	170174	111115	198217	094094
	5	162172	111111	203217	098098
	6	164168	113119	229229	094097
	7	170176	113125	191229	083083
	8	162172	115121	229229	098098
	9	164170	111123	198280	092094
	10	162162	111121	217217	095095
	11	162162	113119	229276	089101
	12	162162	111128	217229	094096
	13	164170	111125	292292	097098
	14	158164	117130	203229	095095
	15	162170	115121	229229	097101
	16	162230	115117	225225	083099
	17	162162	111115	205276	095101
	18	170170	111113	225229	095097
	19	162162	113115	217229	087096
	20	162170	117128	217217	083083
	21	170170	111111	198225	097098
	22	162162	111111	229264	083098
	23	162162	113128	217225	0

Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*
TU	1	0	123128	217229	083097
	2	162162	111113	225225	083096
	3	162170	111113	198229	083100
	4	0	0	229229	083100
	5	162162	111113	229229	083095
	6	162162	113113	225225	083101
	7	162170	111113	202202	098100
	8	162162	121125	229229	083103
	9	162170	111138	198217	0
	10	162174	121125	198198	082095
	11	170170	111119	198227	098098
	12	162162	104125	191229	083097
	13	0	111111	198229	0
	14	162162	113121	225229	083089
	15	162162	111121	198229	096099
	16	162162	111121	229229	097097
	17	162172	113119	217229	093098
	18	162162	111121	225229	095097
	19	162162	113113	213213	099099
	20	162174	123125	198225	098098
	21	162174	113115	225225	095095
	22	162162	111111	198198	098098
	23	174176	111111	229229	095095
	24	0	121121	225225	095095
	25	162176	111113	229229	100101
	26	162180	111113	198209	083097
	27	162186	115115	198226	0

Appendix 5. continuation.

Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*
SE	1	150162	111121	0	089096
	2	162170	111121	0	085089
	3	162162	111123	187229	0
	4	0	119128	0	0
	5	0	121123	0	0
	6	150162	123128	0	082101
	7	162164	111123	217221	095097
	8	150162	111119	0	094097
	9	162162	123128	0	097098
	10	0	121123	0	082082
	11	0	0	0	095097
	12	162162	0	0	075082
	13	166170	117125	202202	082085
	14	0	121128	213229	075082
	15	150162	115123	202217	0
	16	162170	113119	0	0
	17	0	113121	0	0
	18	162162	121121	0	075082
	19	0	119119	0	084099
	20	0	111121	0	075082
	21	162166	113123	0	0
	22	0	0	202217	0
	23	150162	128128	0	098098
	24	170176	111119	209225	075082
	25	162170	117125	225225	0
	26	162166	117119	229229	082082
SM	1	150162	111123	0	082097
	2	162162	119123	0	082098
	3	170216	119123	217217	086086
	4	162162	119121	0	082082
	5	166166	111121	0	082098
	6	162162	119123	0	098098
	7	0	111111	198198	082082
	8	160170	111121	0	0
	9	162162	119121	0	097097
	10	166170	0	0	097097
	11	0	115119	0	097097
	12	162162	115119	0	095096
	13	162166	119121	0	0
	14	0	113119	0	082098
	15	0	119121	0	097097
	16	162172	113119	198229	082082
	17	166172	113119	229229	095095
	18	160170	121121	0	082082
	19	162176	119121	221229	082082
	20	0	0	187187	082082
	21	162170	121121	221221	095096
	22	162162	117117	217217	082082
	23	150172	115125	0	0
	24	150162	0	0	076082
	25	0	123123	0	082096
	26	162162	119119	0	082082
	27	162164	119119	0	096097

Appendix 5. continuation.

Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*
SS	1	150162	111123	225225	082097
	2	150162	0	0	082082
	3	170216	119123	217217	0
	4	162162	119121	217217	103103
	5	166199	111121	0	082098
	6	162162	119123	0	098098
	7	162162	119119	225225	095095
	8	162205	111113	0	095095
	9	162166	0	187225	098099
	10	162170	119125	225225	0
	11	168170	119121	187217	082082
	12	162170	125128	198198	082082
	13	0	119121	225229	082082
	14	162162	123128	225229	095098
	15	150170	111125	191191	087087
	16	162170	123130	191225	094095
	17	162170	119121	198221	095095
	18	162199	128128	217229	099099
	19	162170	111111	191217	085097
	20	162162	119119	202202	095095
	21	170178	123125	217225	082082
	22	162170	125125	229229	082099
	23	0	115119	183198	098098
	24	150162	111128	199237	082082
	25	0	121121	217248	094094
	26	150162	123123	225225	082082
	27	0	117119	217225	097098

Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*
GI	1	205214	111125	201203	082082
	2	188214	109111	200200	082082
	3	212214	111111	194201	082082
	4	182189	109125	201265	082082
	5	182182	111111	203265	082082
	6	182212	113125	201219	082082
	7	216216	109125	219248	082092
	8	191216	111113	203219	082082
	9	189193	111111	230248	082082
	10	214216	111134	248268	082092
	11	182184	111111	203248	082082
	12	189216	111125	194203	082096
	13	189208	111113	207265	082096
	14	201205	125132	203219	082092
	15	184189	111115	203246	082082
	16	0	109111	194201	082082
	17	189193	125134	201219	082082
	18	189193	113125	201203	082082
	19	189212	111111	207248	082082
	20	182189	0	194201	082082
	21	184205	109109	194268	082082
	22	0	109125	219265	082082
	23	214216	0	230248	075082
	24	189205	111132	219248	082092

Appendix 5. continuation.

Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*	Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*
ET	1	172186	111130	242265	082082	**PA**	1	191210	111115	225229	082082
	2	193193	125136	226226	082083		2	189191	111113	203229	082082
	3	193210	111125	211211	082082		3	180195	111111	199207	082082
	4	203210	119125	194261	082082		4	164187	111128	203285	082082
	5	180180	111128	219260	082082		5	189189	115128	198217	082082
	6	0	121125	199265	082082		6	162189	111123	227229	082092
	7	191205	111117	217269	082082		7	0	111111	207213	082082
	8	201203	111111	207273	082082		8	187199	125128	234253	082082
	9	0	0	215258	082082		9	182205	113115	199288	082089
	10	184184	119125	198233	082083		10	162220	105113	238238	082082
	11	0	111125	207221	082092		11	162182	125125	191191	082082
	12	201220	111111	207207	082094		12	162162	111123	225229	082082
	13	191193	111119	187238	082083		13	162182	113125	210225	082089
	14	0	125125	246246	082082		14	184193	113125	246246	082095
	15	172216	123123	219229	082082		15	203210	111125	199216	082092
	16	174197	128128	240246	082083		16	191191	113123	219228	082082
	17	180197	106128	250289	082082		17	195203	111125	246246	082092
	18	184208	109125	194277	082082		18	182193	123128	219252	082082
	19	0	125125	0	082082		19	180222	111125	208208	082082
	20	172216	111113	211211	082082		20	189220	123125	199203	082082
	21	176203	125128	221234	082082		21	189189	0	225229	075082
	22	168222	111125	168225	082082		22	189191	109113	225291	082082

Appendix 5. continuation.

Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*	Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*
GR	1	172174	111111	210210	082082	**RO**	1	170176	113117	217229	082095
	2	170172	111117	191225	093097		2	168178	113123	217229	094101
	3	162166	125125	217229	085085		3	170172	111111	213229	082085
	4	170176	113123	183198	094097		4	166170	119119	229229	082097
	5	168172	115115	205225	085100		5	168174	0	202202	0
	6	170170	111115	229244	096096		6	168168	106106	217229	082093
	7	168168	0	0	0		7	164164	109121	217229	093096
	8	162174	109109	218218	085085		8	168170	111115	202284	082098
	9	168168	111111	205225	085085		9	174176	113121	217229	085099
	10	170178	123123	210225	093096		10	168168	113115	0	082098
	11	174174	111111	0	086100		11	168170	113115	217229	082097
	12	164174	115115	225225	082094		12	162162	111111	0	098098
	13	164174	109111	210229	085086		13	174180	111125	0	098101
	14	170172	113115	214214	085097		14	174178	109119	225225	095097
	15	168174	115115	217217	093095		15	0	111111	0	0
	16	0	111123	0	082086		16	168172	113119	225268	0
	17	168172	111117	191226	082082		17	164170	106111	202284	097098
	18	170172	111128	205225	086086		18	0	0	213217	099099
	19	168174	119130	213213	086086		19	170174	113121	213217	082085
	20	168168	106123	210225	083096		20	168174	115119	202217	082098
	21	174180	0	221260	082093		21	168168	113113	218229	100100
	22	170170	106106	187217	084085		22	168172	115115	272272	082082
	23	164170	106117	210210	082085		23	164170	111111	205210	085099
	24	174184	113113	225225	082086						
	25	166170	111123	205225	0						
	26	172174	111119	253253	097103						
	27	168176	111130	198217	085099						
	28	162168	115115	225268	093096						
	29	168168	111113	210225	094094						
	30	0	111113	205225	082096						

Appendix 5. continuation.

Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*
UK	1	168172	113113	225225	082098
	2	168172	0	225225	0
	3	162170	0	0	082099
	4	0	0	0	096099
	5	0	0	229229	0
	6	164170	113115	221225	096098
	7	0	117119	0	0
	8	170178	117119	205233	096100
	9	0	119119	0	0
	10	0	119119	205264	0
	11	0	0	210229	0
	12	168168	119123	217225	082098
	13	162164	119119	227227	082085
	14	174178	113113	205233	098099
	15	168170	113113	210241	085098
	16	0	111111	198198	0
	17	172178	105140	205217	082098
	18	168174	111111	229229	082097
	19	164178	113121	205272	097098
	20	164168	121121	205205	082095

Pop	Ind	*Cg4*	*Cg7*	*Cg9*	*Cg11*
BK	1	170172	105105	205217	097101
	2	168172	105111	221288	097099
	3	0	113119	237244	0
	4	168174	111111	226276	082095
	5	168174	111119	217217	095099
	6	170174	111113	217240	082098

Appendix 6. Microsatellite allele frequencies.

Appendix 6a. Microsatellite allele frequencies in locus *Cg4*

Population	FI	GD	AL	LV	AR	AN	PT	BL	SA	TU	SE	SM	SS	GI	ET	PA	GR	RO	UK	BK
N / Allele	29	30	27	30	24	24	28	29	23	26	23	24	25	22	21	21	28	21	15	6
150	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.15	0.07	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00
154	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
155	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
158	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
160	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
162	0.22	0.33	0.41	0.57	0.73	0.61	0.63	0.72	0.50	0.70	0.56	0.50	0.50	0.00	0.00	0.14	0.05	0.05	0.08	0.00
164	0.00	0.03	0.00	0.00	0.02	0.05	0.02	0.02	0.09	0.00	0.03	0.02	0.00	0.00	0.00	0.02	0.05	0.10	0.15	0.00
166	0.76	0.18	0.43	0.43	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.12	0.04	0.00	0.00	0.00	0.04	0.02	0.00	0.00
168	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.02	0.00	0.00	0.00	0.02	0.00	0.03	0.00	0.25	0.31	0.27	0.30
170	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.11	0.15	0.12	0.22	0.00	0.00	0.00	0.20	0.19	0.15	0.20
172	0.00	0.00	0.07	0.00	0.19	0.23	0.18	0.07	0.04	0.02	0.00	0.07	0.00	0.00	0.09	0.00	0.13	0.07	0.12	0.20
174	0.00	0.00	0.09	0.00	0.04	0.07	0.05	0.03	0.02	0.09	0.00	0.00	0.00	0.00	0.03	0.00	0.20	0.14	0.08	0.30
176	0.00	0.03	0.00	0.00	0.00	0.02	0.07	0.00	0.02	0.04	0.03	0.02	0.00	0.00	0.03	0.00	0.04	0.05	0.00	0.00
178	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.03	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.02	0.05	0.15	0.00
180	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.09	0.05	0.02	0.02	0.00	0.00
182	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.10	0.00	0.00	0.00	0.00
184	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.07	0.09	0.02	0.02	0.00	0.00	0.00
186	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
187	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
188	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00
189	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.00	0.19	0.00	0.00	0.00	0.00
191	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.06	0.12	0.00	0.00	0.00	0.00
193	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.07	0.12	0.05	0.00	0.00	0.00	0.00
195	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
197	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00
199	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.02	0.00	0.00	0.00	0.00
201	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.06	0.00	0.00	0.00	0.00	0.00
203	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.05	0.00	0.00	0.00	0.00
205	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.09	0.03	0.02	0.00	0.00	0.00	0.00
208	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.03	0.00	0.00	0.00	0.00	0.00

210	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.05	0.00	0.00	0.00	0.00
212	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00
214	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00
216	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.14	0.06	0.00	0.00	0.00	0.00	0.00
218	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
220	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.05	0.00	0.00	0.00	0.00
222	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.02	0.00	0.00	0.00	0.00
225	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
228	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
230	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
235	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
237	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H_e	0.37	0.84	0.63	0.49	0.43	0.56	0.57	0.46	0.67	0.49	0.63	0.71	0.69	0.87	0.93	0.90	0.84	0.83	0.83	0.74
H_{nb}	0.38	0.85	0.65	0.50	0.44	0.58	0.58	0.47	0.69	0.50	0.65	0.72	0.70	0.89	0.96	0.92	0.85	0.85	0.86	0.82
H_{obs}	0.48	0.90	0.54	0.67	0.54	0.59	0.46	0.55	0.57	0.48	0.76	0.62	0.78	0.91	0.82	0.81	0.75	0.76	0.92	1.00

Appendix 6b. Microsatellite allele frequencies in locus *Cg7*

Population	FI	GD	AL	LV	AR	AN	PT	BL	SA	TU	SE	SM	SS	GI	ET	PA	GR	RO	UK	BK
N / Allele	29	30	27	30	24	24	28	29	23	26	23	24	25	22	21	21	28	21	15	6
104	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
105	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.03	0.25
106	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.07	0.07	0.00	0.00
109	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.16	0.02	0.02	0.05	0.05	0.00	0.00
111	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.34	0.37	0.33	0.15	0.10	0.14	0.43	0.29	0.29	0.30	0.26	0.13	0.42
112	0.00	0.00	0.00	0.00	0.33	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
113	0.03	0.22	0.17	0.33	0.35	0.42	0.02	0.16	0.13	0.25	0.07	0.06	0.02	0.09	0.02	0.17	0.11	0.21	0.27	0.17
114	0.00	0.00	0.00	0.00	0.02	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
115	0.02	0.00	0.00	0.02	0.04	0.00	0.00	0.03	0.13	0.06	0.02	0.06	0.02	0.02	0.00	0.07	0.18	0.14	0.03	0.00
116	0.00	0.00	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
117	0.21	0.03	0.00	0.07	0.02	0.00	0.05	0.05	0.07	0.00	0.07	0.04	0.02	0.00	0.02	0.00	0.05	0.02	0.07	0.00
118	0.00	0.00	0.00	0.00	0.10	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
119	0.50	0.58	0.83	0.35	0.06	0.06	0.41	0.12	0.04	0.04	0.15	0.35	0.26	0.00	0.07	0.00	0.04	0.12	0.30	0.17
120	0.00	0.00	0.00	0.00	0.04	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
121	0.12	0.05	0.00	0.20	0.00	0.10	0.16	0.17	0.07	0.15	0.20	0.23	0.14	0.00	0.02	0.00	0.00	0.07	0.10	0.00
123	0.07	0.08	0.00	0.02	0.00	0.00	0.25	0.03	0.02	0.04	0.17	0.13	0.16	0.00	0.05	0.12	0.11	0.02	0.03	0.00
125	0.03	0.03	0.00	0.02	0.00	0.00	0.05	0.03	0.04	0.08	0.04	0.02	0.12	0.20	0.31	0.21	0.04	0.02	0.00	0.00
128	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.02	0.09	0.02	0.13	0.00	0.10	0.00	0.12	0.10	0.02	0.00	0.00	0.00
130	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.04	0.00	0.00	0.00	0.02	0.00	0.02	0.00	0.04	0.00	0.00	0.00
132	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00
134	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00
136	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
138	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
140	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00
199	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H_e	0.68	0.60	0.28	0.72	0.74	0.74	0.74	0.81	0.81	0.79	0.86	0.79	0.84	0.73	0.80	0.82	0.84	0.84	0.80	0.71
H_{nb}	0.70	0.61	0.28	0.73	0.76	0.76	0.75	0.82	0.83	0.81	0.88	0.80	0.86	0.75	0.82	0.84	0.85	0.86	0.83	0.77
H_{obs}	0.69	0.60	0.33	0.60	0.88	0.83	0.57	0.86	0.83	0.73	0.87	0.71	0.72	0.73	0.71	0.86	0.57	0.62	0.40	0.67

Appendix 6c. Microsatellite allele frequencies in locus *Cg9*

Population	FI	G D	AL	LV	AR	AN	PT	BL	SA	TU	SE	SM	SS	GI	ET	PA	GR	RO	UK	BK
N / Allele	27	28	28	25	23	23	26	28	23	27	9	8	23	24	21	22	27	19	16	6
168	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
183	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.02	0.00	0.00	0.00
187	0.00	0.00	0.00	0.00	0.00	0.02	0.23	0.00	0.00	0.00	0.06	0.13	0.04	0.00	0.02	0.00	0.02	0.00	0.00	0.00
191	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.00	0.00	0.09	0.00	0.00	0.05	0.04	0.00	0.00	0.00
194	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.10	0.05	0.00	0.00	0.00	0.00	0.00
198	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.07	0.11	0.22	0.00	0.19	0.09	0.00	0.02	0.02	0.04	0.00	0.06	0.00
199	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.02	0.09	0.00	0.00	0.00	0.00
200	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00
201	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00
202	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.22	0.00	0.04	0.00	0.00	0.00	0.00	0.13	0.00	0.00
203	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.17	0.00	0.07	0.00	0.00	0.00	0.00
205	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.03	0.22	0.08
207	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.10	0.05	0.00	0.00	0.00	0.00
208	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
209	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
210	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.15	0.03	0.06	0.00
211	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00
213	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.06	0.00	0.00	0.00	0.00	0.02	0.04	0.08	0.00	0.00
214	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00
215	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
216	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
217	0.00	0.16	0.63	0.16	0.00	0.00	0.00	0.23	0.22	0.06	0.17	0.25	0.22	0.00	0.02	0.02	0.09	0.24	0.06	0.33
218	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.03	0.00	0.00
219	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.15	0.05	0.05	0.00	0.00	0.00	0.00
221	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.06	0.19	0.02	0.00	0.05	0.00	0.02	0.00	0.03	0.08
223	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
225	0.83	0.66	0.04	0.18	0.30	0.15	0.33	0.11	0.11	0.20	0.17	0.00	0.30	0.00	0.02	0.11	0.26	0.08	0.19	0.00
226	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.05	0.00	0.02	0.00	0.00	0.08
227	0.00	0.00	0.00	0.00	0.04	0.02	0.21	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.06	0.00
228	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
229	0.11	0.05	0.04	0.66	0.61	0.78	0.19	0.23	0.35	0.37	0.22	0.25	0.11	0.00	0.02	0.11	0.06	0.26	0.16	0.00
230	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00

233	0.00	0.00	0.00	0.00	0.04	0.02	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.06	0.00
234	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.00
237	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.08
238	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.05	0.00	0.00	0.00	0.00
240	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.08
241	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00
242	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
244	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.08
246	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.07	0.09	0.00	0.00	0.00	0.00
248	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.15	0.00	0.00	0.00	0.00	0.00	0.00
250	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
252	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
253	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.04	0.00	0.00	0.00
258	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
260	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.02	0.00	0.00	0.00
261	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
264	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00
265	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.05	0.00	0.00	0.00	0.00	0.00
268	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.02	0.03	0.00	0.00
269	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
272	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.03	0.00
273	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
276	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08
277	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
280	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
284	0.04	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00
285	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
288	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.08
289	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
291	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
292	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H_e	0.29	0.53	0.56	0.51	0.53	0.36	0.76	0.82	0.80	0.76	0.83	0.79	0.83	0.88	0.95	0.94	0.88	0.84	0.87	0.83
H_{nb}	0.30	0.54	0.57	0.52	0.54	0.37	0.77	0.83	0.82	0.78	0.88	0.84	0.85	0.90	0.98	0.96	0.90	0.86	0.90	0.91
H_{obs}	0.11	0.14	0.54	0.64	0.39	0.30	0.54	0.75	0.65	0.48	0.67	0.25	0.57	0.96	0.76	0.77	0.67	0.79	0.56	0.83

Appendix 6d. Microsatellite allele frequencies in locus *Cg11*

Population	FI	GD	AL	LV	AR	AN	PT	BL	SA	TU	SE	SM	SS	GI	ET	PA	GR	RO	UK	BK
N / Allele	28	29	28	28	24	24	18	29	22	24	17	24	25	24	22	22	28	20	13	5
75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.02	0.00	0.02	0.00	0.00	0.00	0.00
76	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
81	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
82	0.07	0.21	0.02	0.34	0.02	0.00	0.00	0.10	0.00	0.02	0.32	0.46	0.34	0.85	0.86	0.84	0.18	0.28	0.27	0.20
83	0.21	0.09	0.14	0.16	0.13	0.04	0.06	0.28	0.14	0.21	0.00	0.00	0.00	0.00	0.09	0.00	0.02	0.00	0.00	0.00
84	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
85	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.02	0.00	0.00	0.00	0.21	0.10	0.08	0.00
86	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.00
87	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00
89	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.06	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
90	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
92	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.08	0.02	0.07	0.00	0.00	0.00	0.00
93	0.00	0.00	0.00	0.00	0.02	0.04	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.05	0.00	0.00
94	0.11	0.09	0.09	0.11	0.21	0.10	0.00	0.05	0.14	0.00	0.03	0.00	0.06	0.00	0.02	0.00	0.07	0.03	0.00	0.00
95	0.13	0.07	0.04	0.04	0.27	0.35	0.03	0.10	0.16	0.19	0.06	0.08	0.20	0.00	0.00	0.02	0.02	0.05	0.04	0.20
96	0.18	0.07	0.23	0.05	0.10	0.06	0.11	0.07	0.05	0.04	0.03	0.08	0.00	0.04	0.00	0.00	0.11	0.03	0.12	0.00
97	0.11	0.33	0.04	0.13	0.08	0.21	0.44	0.09	0.16	0.13	0.12	0.21	0.06	0.00	0.00	0.00	0.07	0.10	0.08	0.20
98	0.11	0.10	0.36	0.09	0.06	0.06	0.06	0.05	0.20	0.17	0.09	0.10	0.16	0.00	0.00	0.00	0.00	0.18	0.27	0.10
99	0.05	0.03	0.07	0.05	0.04	0.06	0.14	0.10	0.02	0.06	0.03	0.00	0.08	0.00	0.00	0.00	0.02	0.10	0.12	0.20
100	0.00	0.02	0.02	0.02	0.02	0.02	0.08	0.05	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.05	0.04	0.00
101	0.00	0.00	0.00	0.00	0.00	0.02	0.06	0.05	0.07	0.04	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.10
102	0.00	0.00	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
103	0.02	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.04	0.00	0.00	0.00	0.02	0.00	0.00	0.00
111	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H_e	0.86	0.81	0.78	0.82	0.84	0.80	0.75	0.87	0.86	0.86	0.84	0.72	0.80	0.26	0.24	0.29	0.87	0.85	0.81	0.82
H_{nb}	0.88	0.83	0.80	0.83	0.86	0.82	0.77	0.88	0.88	0.88	0.86	0.73	0.82	0.27	0.25	0.29	0.89	0.87	0.85	0.91
H_{obs}	0.89	0.62	0.86	0.89	0.71	0.67	0.56	0.72	0.68	0.67	0.82	0.38	0.32	0.29	0.27	0.32	0.68	0.80	1.00	1.00