Genetička i morfološka varijabilnost plemenite periske (Pinna nobilis Linnaeus, 1758) u Parku prirode Telašćica i Nacionalnom parku Mljet

Ankon, Pavel

University of Zagreb

Faculty of Science

Division of Biology

Pavel Ankon

Genetic and morphological variability of the noble pen shell (*Pinna nobilis* Linnaeus, 1758) in Nature Park Telašćica and National Park Mljet

Graduation thesis

Zagreb, 2017.

This thesis had been conducted at the University of Sassari, Department of Sciences for Nature and Environmental Resources, in Sassari (Italy), in collaboration with the University of Zagreb, Faculty of Science, Division of Biology, under the guidance of Dr. Marco Casu, Assoc. Prof. and Dr. Petar Kružić, Assoc. Prof. The thesis was submitted for evaluation to the Division of Biology, Faculty of Science, University of Zagreb to acquire the academic title of Master of ecology and nature preservation.

Acknowledgement

First, I would like to thank Assoc. Prof. Marco Casu who gave me this remarkable opportunity to work as a part of his amazing research group. I am very grateful for his advice and guidance in completing this task, as well as hosting me on the University of Sassari. Many thanks to my cosupervisor Assoc. Prof. Petar Kružić for his care, support and guidance throughout my research. His enthusiasm cheered me on up through all the endless challenges we came across. I could have not asked for better mentors; as they have given me the rare opportunity to pursue my own research interests.

I would like to express my special thanks of gratitude to Daria Sanna, my undercover supervisor. She was there for me on every step of this thesis project. Her assistance in experimental work, data analysis, statistical analyses was irreplaceable. I cannot thank her enough for all her help. She was endlessly patient with me as she introduced me to genetics and she taught me one important thing: “Sempre i pensieri positivi”. Grazie Daria!

Special appreciation to Hrvoje Čižmek for his unlimited help (especially with maps), advice, valuable comments and suggestions. Thank you for your support as well as for the laughs and the good times.

I am really thankful to Marine Explorers Society 20000 League for organizing logistics for sampling in Nature Park Telašćica and National Park Mljet. Specially thank to Andrea, Tinče and Ines for help in sample collection and funny memories on Krilavi.

I would also like to thank everybody in the group of prof. Marco for their great company, advice and support during my stay in Sassari. I really enjoyed working with you.

I want to thank doc. Sunčica Bosak for her help with statistical analyses. Thanks to Stjepan, Petra, Sonja, Soža and Boris for helping me in collecting morphology data on Mljet and nice memories from fieldwork. A big thanks to my Štetočina, who helped me a lot with English spell check and was the first (and probably the last) person in family who read this thesis completely.

Thanks to all my friends and family who have never given up in giving their support to me in all aspects of life. Especially Marko, Una, Vanja, Mirna, Nevena, Borna, Lada, Grga for all the nice times and great support in tough ones. Also to all members of BIUS, they made my study years much more interesting, funnier and unforgettable.

Last but not the least, I would like to thank my parents, Igor and Sanja for having them by my side. Their encouragement when the times got rough is inexpressible. They are indorsing me in following my dreams and supporting me with all means possible. Thank you for everything.

BASIC DOCUMENTATION CARD

University of Zagreb

Faculty of Science

Division of Biology

Graduation Thesis

GENETIC AND MORPHOLOGICAL VARIABILITY OF THE NOBLE PEN SHELL (*Pinna nobilis* Linnaeus, 1758) IN NATURE PARK TELAŠĆICA AND NATIONAL PARK MLJET

Pavel Ankon

Rooselveltov trg 6, 10000 Zagreb, Croatia

Pinna nobilis is one of the most imposing member of Mediterranean bivalve mollusks, reaching a size up to 120 cm and with reported maximum age of 27 years. It is endangered and protected species under both local and EU laws with recent demographic expansions. The aim of this study was to provide a comprehensive information on conservation status of noble pen shell on Croatian coast: morphology of species with focus on the population density, structure, spatial distribution and size, together with appraisal of the genetic structure and population connectivity with its spatial genetic variation in the Adriatic Sea. Two populations from Nature Park Telašćica and National Park Mljet were surveyed using COI and 16S mtDNA markers. Population density of both MPA areas were higher than average found in Mediterranean with population structure mainly consists of adults and old individuals, possible from 8 to 15 years old. Genetic analysis revealed genetic structuring between Venetian and Croatian populations within Adriatic Sea, as well as between Aegean population and populations from Adriatic Sea. High levels of haplotype sharing between Sardinia and Croatia populations suggests the phenomena of a constant genetic flow between western Mediterranean and southern Adriatic Sea that has never been described before.

(55 pages, 19 figures, 7 tables, 94 references, original in: English)

Thesis deposited in the Central biological library

Key words: *Pinna nobilis*, population density, population structure, genetic structure, 16S, COI, Adriatic Sea

Supervisor: Dr. Marco Casu, Assoc. Prof.

Cosupervisor: Dr. Petar Kružić, Assoc. Prof.

Reviewers: Dr. Petar Kružić, Assoc. Prof., Dr. Zlatko Liber, Prof., Dr. Zrinka Ljubešić, Assoc. Prof.

Thesis accepted: 21.6.2017

TEMELJNA DOKUMENTACIJSKA KARTICA

Sveučilište u Zagrebu

Prirodoslovno – matematički fakultet

Biološki odsjek Diplomski rad

GENETIČKA I MORFOLOŠKA VARIJABILNOST PLEMENITE PERISKE (*Pinna nobilis* Linnaeus, 1758) U PARKU PRIRODE TELAŠĆICA I NACIONALNOM PARKU MLJET

Pavel Ankon

Rooselveltov trg 6, 10000 Zagreb, Croatia

Pinna nobilis jedan je od najimpozantnijih članova Sredozemnih školjkaša, može doseći veličinu od 120 cm i starost od 27 godina. Ugrožena je i zaštićena vrsta, ne samo zakonom nego i propisima na razini EU, s nedavnim demografskim oporavkom. Cilj ovog istraživanja je predočiti sveobuhvatnu informaciju o konzervacijskom statusu plemenite periske uzduž hrvatske obale: morfologiju vrste s naglaskom na gustoću populacije, njezinu strukturu, prostornu rasprostranjenost i veličinu te procjenu genetičke strukture i povezanosti s njezinom prostornom genetskom varijacijom u Jadranskom moru. Dvije populacije, po jedna iz Parka prirode Telašćica i Nacionalnog parka Mljet su obrađene korištenjem COI i 16S mtDNA markera. Gustoća populacije za oba MPA područja je bila gušća nego prosječna gustoća navedena za Mediteran sa populacijskom strukturom koja se sastoji većinom od odraslih i starijih jedinki, vjerojatne starosti između 8 - 15 godina. Genetske analize otkrile su postojanje genetičke strukturiranosti između venecijanskih i hrvatskih populacija unutar Jadranskog mora, kao i između egejske populacije i Jadranskog mora. Visoka razina dijeljenja haplotipova između sardinijskih i hrvatskih populacija navodi na fenomen o konstantnom protoku gena između zapadnog Mediterana i južnog Jadrana koji do sada nije bio otkriven.

(55 stranica, 19 slika, 7 tablica, 94 literaturnih navoda, jezik izvornika: engleski)

Rad je pohranjen u Središnjoj biološkoj knjižnici

Ključne riječi: *Pinna nobilis*, gustoća populacije, populacijska struktura, genetička struktura 16S, COI, Jadransko more

Voditelj: dr. sc. Marco Casu, izv. prof.

Suvoditelj: dr. sc. Petar Kružić, izv. prof.

Ocjenitelji: dr. sc. Petar Kružić, izv. prof., dr. sc. Zlatko Liber, red. prof., dr. sc. Zrinka Ljubešić, izv. prof.

Rad prihvaćen: 21.6.2017.

Table of contents

1. Introduction

The noble pen shell, *Pinna nobilis* Linnaeus, 1758 (Mollusca: Bivalvia: Pinnidae) has been used by mankind for hundreds of years. Indeed, since the time of Egyptians and Romans, it has been exploited as a valued resource for high-value fabric from its byssus threads, the so-called "sea silk". Furthermore, the pen shell has been commonly use in traditional cooking in some Mediterranean regions (Greenwald, 1996; Katsanevakis *et al.*, 2011). Problems affecting this species are caused by habitat degradation, illegal trawling, coastal construction, boat anchoring, illegal extraction and pollution (Basso *et al.*, 2015). These anthropogenic and environmental threats have contributed to accelerating the decline of populations of this species in the Mediterranean. This decline has in turn led the pen shell to be listed as an endangered and protected species in the ANNEX IV of European Council Directive 92/43/EEC (Habitat Directive) in 1992 and in the ANNEX II of Barcelona Convention in 1995. The species is also under protection by local laws in all European Union Mediterranean countries.

1.1 Biology and ecology

Pinna nobilis is one of the most imposing member of Mediterranean bivalve mollusks. Reaching a size up to 120 cm (Zavodnik, Hrs-Brenko and Legac, 1991; Richardson *et al.*, 1999; García-March *et al.*, 2007), it is considered one of the biggest living clams. *Pinna nobilis* is a long-lived species, with suggested maximum age of 20 years (Butler, Vicente and de Gaulejac, 1993), although a maximum reported age of 27 years was found (Galinou-Mitsoudi, Vlahavas and Papoutsi, 2006).

The genus *Pinna*, along with genera *Atrina* and *Streptopinna* (widespread in tropical west America), are a part of family Pinnidae, superfamily Pinnoidea, subclass Pteriomorphia, under the class Bivalvia, phylum Mollusca. As all pinnids, it is roughly triangular with a tapered anterior end. The triangular shape and their anysomiarian condition (the reduction of the anterior adductor muscle with respect to the posterior adductor muscle) are consequences of the adaptation to tethering to the substrate by byssus threads (Basso *et al.*, 2015) (Figure 1). Their enormous size is due to the great posterior extensions of mantle and shell, probably because of the semi-infaunal habitat in soft substrates (Yonge, 1953). The exterior colour can range from gold to redder, which is not noticeable due to the fact it is strongly overgrown by epibionts (Zavodnik, 1967).

Czihag and Dierl (1961) reported two different forms of *P. nobilis* shell, "crassa" and "papyracea", based on the first description of Linnaeus 1758. The most significant difference between the two forms is the ventral curvature, but it is difficult to distinguish the differences in colour, distal peeling or outer edge (García-March, 2005). Thereafter, García-March and Márquez-Aliaga (2006) noted that the morphologic types of the pen mussel are more diverse and they distinguished three

main morphologic types, i.e. “straight and wide”, “straight and narrow”, and “combed”. Even though there is a clear morphometric distinction between shell forms in *P. nobilis* (Rabaoui, Tlig-Zouari, *et al.*, 2011), study using the mtDNA COI sequences did not reveal any genetic differentiation between individuals (Rabaoui, Mejri, *et al.*, 2011).

The shell is composed of two main layers; the outer is formed by a microstructure of large calcite prisms present in the whole shell, while the inner layer is made up of aragonitic nacre present only in the anterior part of the shell. The ligament of *P. nobilis* is sub marginal elongated, duplivincular, opistodethic overlain dorsally by the calcitic prismatic outer shell layer, except where has been removed by erosion. Instead of having just one lamellar and fibrous couplet, as for the superfamily Pinnacea, this species has as many as four. (García-March and Vicente, 2006). Also, ligament is atypically hard and mineralized, and its function is holding the valves together. Gaping is produced by flexion of the posterior part of the shell, while the ligament remains immobile (Habdija *et al.*, 2011). The mantle is not fused to the shell, and this confers it an enormous capacity of retraction (García-March and Vicente, 2006). The byssus system, a complex structure specialized for strong fixation to the substrate, is found in *P. nobilis*. Byssus threads exit through a narrow hole in the antero-ventral part of the valves. These keratin filaments, that the animal secretes by its byssus gland, can have more than 20,000 filaments attached to the substrate (García-March and Vicente, 2006). About 25 cm length, they are not only glued to tiny particles, roots and rhizomes of the seagrass *Posidonia oceanica* (Linnaeus), Delile with the adhesion plates, but are also tied between them and with the roots and debris of the sediment (García-March and Vicente, 2006). Cerruti (1938) reports that entire byssus system needs up to 6 months for its regeneration, although Mihailinović (1955) suggested for Croatian waters *P. nobilis* needed 4–5 months to regenerate the dissected byssus. A detailed description of noble pen shell anatomy can be found in Czihag and Dierl (1961).

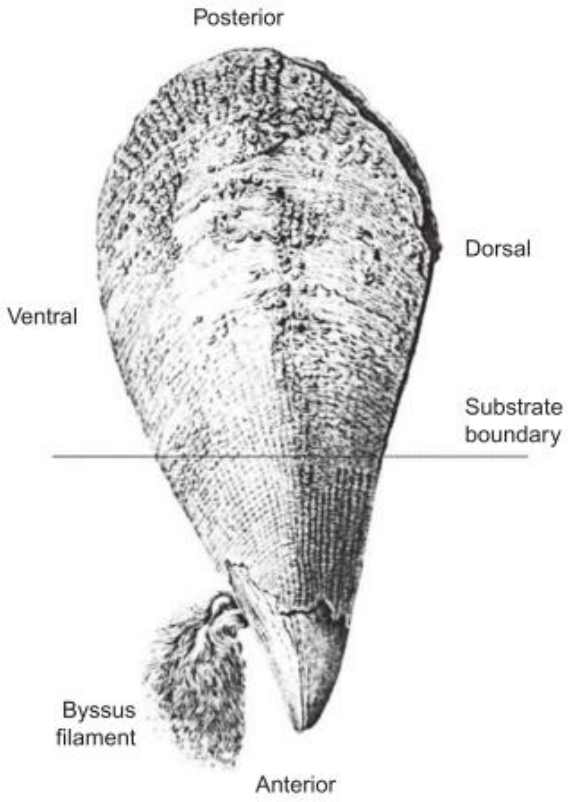


Figure 1: General drawing of an adult individual noble pen shell, Pinna nobilis, with the tapered anterior third of the shell buried in the sediment and attached to the substratum by byssus threads. Redrawn and modified from Czihag and Dierl (1961).

Sexual maturity is reached by the age 1-2 years (Butler, Vicente and de Gaulejac, 1993; De Gaulejac, Henry and Vicente, 1995). Spawning of the noble pen shell occurs during late summer to early autumn (De Gaulejac, 1993; Richardson *et al.*, 1999) and its veliger stage larvae drift in the water column 5 to 10 days (De Gaulejac and Vicente, 1990; Butler, Vicente and de Gaulejac, 1993) before differentiation into juvenile pinnids which settle in the sediment (Rabaoui, Mejri, *et al.*, 2011). At settlement the post-larva is a little over 1 mm in length (Butler, Vicente and de Gaulejac, 1993). At this stage, these animals are vulnerable to a range risks; including those of being ingested by deposit feeders such as holothurians, of being eaten by predators such as naticid gastropods, octopods, asteroids and fish, and of being unable to feed effectively because of movement of the sediment or because the supply of suspended food is too dilute (Butler, Vicente and de Gaulejac, 1993). The combination of the sessile adult lifestyle with the low dispersal potential for this species leads to the prediction of marked and discernible population structure (Rabaoui, Mejri, *et al.*, 2011)

The ecological role of *P. nobilis* is of importance as it filters substantial amounts of detritus and retains a high percentage of its organic matter, contributing to water clarity. It also provides a hard type of substrate in soft-bottom areas, thus increasing the variety of environments and providing a

surface that can be colonized by other (floral and faunal) benthic species (Basso *et al.*, 2015). In the mantle chamber of *Pinna nobilis*, near the gills, live several species of commensal shrimps of the genera *Pontonia (P. pinnophylax*) and crabs of the genera *Pinnotheres* (*P. pinnotheres* and *P. pisum*) (García-March and Vicente, 2006). *P. pinnophylax* is usually found in pairs, male and female (Richardson *et al.*, 1997).

Pinna nobilis inhabits the Mediterranean Sea, where it is endemic, since the end of the Miocene (MacDonald and Barrett, 2008). It is found primarily in coastal regions at depths ranging from 0.5 - 60m (Butler, Vicente and de Gaulejac, 1993; Templado *et al.*, 2004). The species is commonly found living in soft-sediment areas (Katsanevakis, 2005) in meadows of seagrass *P. oceanica* (Vicente, 1990; Ramos, 1998) and *Cymodocea nodosa* (Ucria) Ascherson, 1870 (Zavodnik, 1967; Zavodnik, Hrs-Brenko and Legac, 1991; Richardson *et al.*, 1999; Siletic and Peharda, 2003), where it lives partially buried in the sand upright with the anterior portion of its shell (Zavodnik, Hrs-Brenko and Legac, 1991; Richardson *et al.*, 1999; Templado *et al.*, 2004). Nevertheless, it can also be found in maërl beds, among boulders, and half buried in muddy substrates (Vicente, 1990; García-March, 2003). Seagrass meadows are unique, productive, and highly diverse ecosystems, which provide habitat and food for organisms (Beer, 2001). In particular, their leaves hide noble pen shell juveniles from *Octopus vulgaris* (Mollusca: Cephalopoda), its main predators (Fiorito and Gherardi, 1999; García-March *et al.*, 2007). Predation pressure on *P. nobilis* may have increased through a cascade of food web impacts, where the dusky grouper, *Epinephelus marginatus* (Teleostea: Serranidae), the main predator of octopus in the Mediterranean has declined greatly due to commercial and recreational overfishing (Basso *et al.*, 2015). Posidonia meadows have experienced widespread degradation across the Mediterranean (Marba, Díaz-Almela and Duarte, 2014). Loss of its primary habitat, may possibly be the largest driver, followed by damage from anchors, and food web alterations leading to increased predatory pressure along with, in the past, fishing pressure (Basso *et al.*, 2015).

1.2 Population dynamics

Pinna nobilis occurs over a range of depths and substrates, but has only been studied intensively in a few zones within the Mediterranean Basin (Basso *et al.*, 2015). According to the literature, the density of *P. nobilis* ranges between 0.001 (Centoducati *et al.*, 2007) and 600 ind 100 m^{-2} (De Gaulejac and Vicente, 1990; Catsiki and Katsilieri, 1992), but typical values are quite low (García-March *et al.*, 2007), and 1 ind 100 m^{-2} is the most widespread value found in the Mediterranean populations (Siletic and Peharda, 2003).

Among Mediterranean ecoregions, the Aegean Sea had the highest mean density of 14.30 $\pm$ 9.14 ind 100 m^{-2} (n = 16; $\pm$ SE), followed by the Adriatic Sea with 11.30 $\pm$ 2.17 ind 100 m^{-2} (n = 10; $\pm$ SE), the Tunisian plateau with 10.09 $\pm$ 5.10 ind 100 m^2 (n = 11), Algero Provencal Basin with 7.90

$\pm$ 2.16 ind 100 m^{-2} (n = 23; $\pm$ SE), Thyrrhenian sea 6.25 $\pm$ 2.52 ind 100 m^{-2} (n = 8) and last the Ionian Sea with 0.004 $\pm$ 0.004 ind 100 m^{-2} (n = 2) (Basso *et al.*, 2015).

Studies on population density and the conservation status of this endangered species should be evaluated in different habitats, in different regions of the Mediterranean Basin and at different time scales to identify probable, common or peculiar, sources of mortality within the Mediterranean Basin (Basso *et al.*, 2015).

1.3 Genetic structure

Molecular approaches can be particularly useful for determining the influence of ecological parameters on gene flow and spatial genetic structure (Knutsen *et al.*, 2003). The spatial genetic structure of a species may reflect not only its current dispersal capability, but also its phylogeographic history. The knowledge on the level of genetic variability and distribution in space and time is crucial for a correct diagnosis of the endangerment and viability of populations. (Escudero, Iriondo and Torres, 2003; Frankham, 2007).

Mitochondrial DNA (mtDNA) has been considered as a potential genetic marker in phylogeny, population and evolutionary studies for a wide variety of animal taxa (Saccone *et al.*, 1999). The characterization of mitochondrial genes has contributed to the identification of informative sequences that have improved our understanding of organism evolution and the diversity of the mitochondrial genome of *P. nobilis* (Katsares *et al.*, 2008).

Cytochrome oxidase subunit I (COI), a gene of the mitochondrial DNA (mtDNA), shows varying degree of conservation throughout its sequence, and a range of nucleotide substitution rates that can be used for different evolutionary analyses. The COI gene, an essential gene coding for a protein used in cellular respiration, is well conserved within species but is variable enough between species that closely related species could be distinguished. It has been extensively used for evolutionary studies in bivalves (Kojima *et al.*, 1995, 1997; Matsumoto, 2003; Cho *et al.*, 2007).

16S rDNA mtDNA gene is generally successful as an interspecific taxonomic marker and was used to resolve taxonomic problems in the family Mytilidae (Rawson and Hilbish, 1995), Veneridae (Canapa *et al.*, 1996) and Pectinidae (Canapa *et al.*, 2000; Saavedra and Peña, 2004) (Katsares *et al.*, 2008).

Microsatellite markers (simple sequence repeats, SSR) are one of the most valued genetic markers because of their high variability, codominance and repeatability. Using a tract of repetitive DNA in which certain DNA motifs (ranging in length from 2–5 base pairs) are repeated,

(typically 5–50 times), they are used in population genetics to measure levels of relatedness between subspecies, groups and individuals (Reid, 1998).

While morphological studies on *P. nobilis* concerning biology, population structure, mortality, growth have been conducted in Adriatic Sea (Mihailinović, 1955; Zavodnik, 1967; Zavodnik, Hrs-Brenko and Legac, 1991; Siletic and Peharda, 2003; Richardson *et al.*, 2004), the use of molecular tools has only become widely available and affordable in recent years. Although few genetic analyses were performed in Mediterranean on *P. nobilis* (Katsares *et al.*, 2008; Rabaoui, Mejri, *et al.*, 2011; Sanna *et al.*, 2013, 2014; González-Wangüemert *et al.*, 2015), so far only one of them has included a one of the populations in the Adriatic Sea (Sanna *et al.*, 2013).

1.4 Aim of work

The aim of this study is to investigate a *P. nobilis* populations in Nature Park Telašćica (middle-east Adriatic, Croatia) and National Park Mljet (south-east Adriatic, Croatia), confirming if there are connections within populations. The preliminary goal is to analyse the morphology of species with focus on the population density and structure, spatial distribution and size distribution. Furthermore, genetical aims are appraisal of the genetic structure and variation in Croatian populations of *P. nobilis* from the Adriatic Sea with potential hints on the relationship with other Mediterranean populations. We surveyed populations from Telašćica and Mljet and compared these populations with the available data from the Aegean Sea (Greece), Sardinian region and Venice (Italy).

2 Study area

2.1 Nature Park Telašćica

Nature Park Telašćica is bay located in the central part of the eastern coast of the Adriatic Sea, in the south-eastern part of the island of Dugi Otok, surrounded by 13 islands and islets, together with 6 islets inside the bay itself (Figure 2). It covers an area of 25.95 km^2 of the land (Dugi Otok and belonging islets), and 44.55 km^2 of the sea (PP Telašćica). Telašćica was proclaimed a nature park in 1988 (Official Gazette 14/88) after being separated from National Park Kornati (Magaš, 1998). The main natural phenomena that have been protected in the Park are: imposing maritime cliffs rising up to a height of 161 m above the sea level and reaching down to a depth of 90 m below the sea level, the Telašćica Bay with its autochthonous vegetation and unique salt lake Mir formed in karstic depression with its curative characteristics (Magaš, 1998). In the Telašćica bay and on the open-sea side of the Park there are 19 registered biocoenoses. The most wide-spread biocoenoses are: biocoenosis of supralittoral rocks, biocoenosis of supralittoral sands, biocoenosis of littoral upper and lower rocks, biocoenosis of littoral sands, biocoenosis of fine surface-sands, biocoenosis of fine homogenous sands, biocoenosis of infralittoral pebbles, biocoenosis of photophyllic algae, biocoenosis of *Cymodocea* meadows, biocoenosis of *Posidonia* meadows, biocoenosis of silted sands of the protected coasts, biocoenosis of silted detritus, precoralligenous aspect of the coralligenous biocoenosis, coralligenous biocoenosis, biocoenosis of semi-dark caves and biocoenosis of coastal detrital bottoms and biocoenosis of caves and passages in the complete darkness (Kružić, 2007). There is no evidence of recorded detailed survey of *Pinna nobilis* in literature for this area.

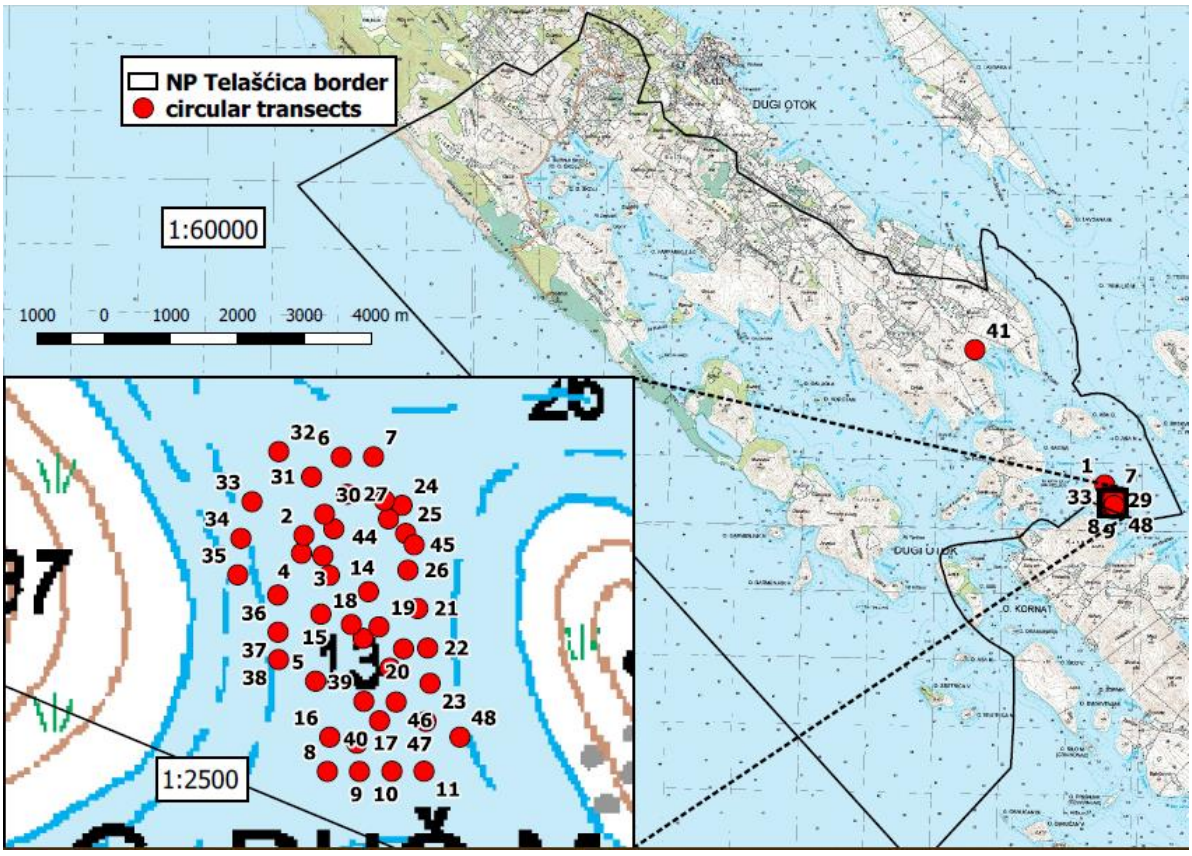


Figure 2: A map of Nature Park Telašćica marked with black line and GPS positions of transects marked with red circles and numbers (1-48)

2.2 National Park Mljet

National park Mljet is situated on northwest part of island Mljet, in southeast Adriatic, stretched over 53.75 km^2 including a marine area of 500 meters from the coast, islands and cliffs, and therefore spans over almost a third of the island (NP Mljet). Two deep bays filled with seawater in postglacial period (Juračić *et al.*, 1995), known as Malo Jezero and Veliko Jezero, are the most famous locations of this area and an important geological and oceanographical phenomenon (Figure 3) (NP Mljet). These semi-enclosed, relatively deep depressions are connected with the open sea by the narrow, shallow Soline Channel (Wunsam, Schmidt and Müller, 1999). Being connected with the sea, they contain marine water and, therefore, are not true lakes. However, due to their maximum depth (46 and 29 m respectively) they can hardly be termed lagoons, which are usually defined as shallow semi-enclosed water bodies (Phelger F.B., 1969) (Wunsam, Schmidt and Müller, 1999). Wunsam, Schmidt and Müller (1999) propose that such isolated marine water bodies should be called *marine lakes*. Both lakes are characterized by the input of runoff from the surrounding terrestrial area and by restricted communication with the open sea (Benović *et al.*, 2000). Detailed descriptions of the environmental conditions in these two inlets are given in Benović *et al.* (2000). The presence of *P. nobilis* in Mljet was recorded for the very first time by Draganović (1980) in Veliko jezero only. Almost

20 years after, Orepić *et al.*, (1997) and Žerlić (1999) conducted a new survey and found especially dense population in Malo jezero. Further, high *P. nobilis* density and spatial distribution have been noted in Malo jezero by Peharda (2000), Peharda *et al.* (2002), Siletic and Peharda (2003) and Richardson *et al.* (2004).

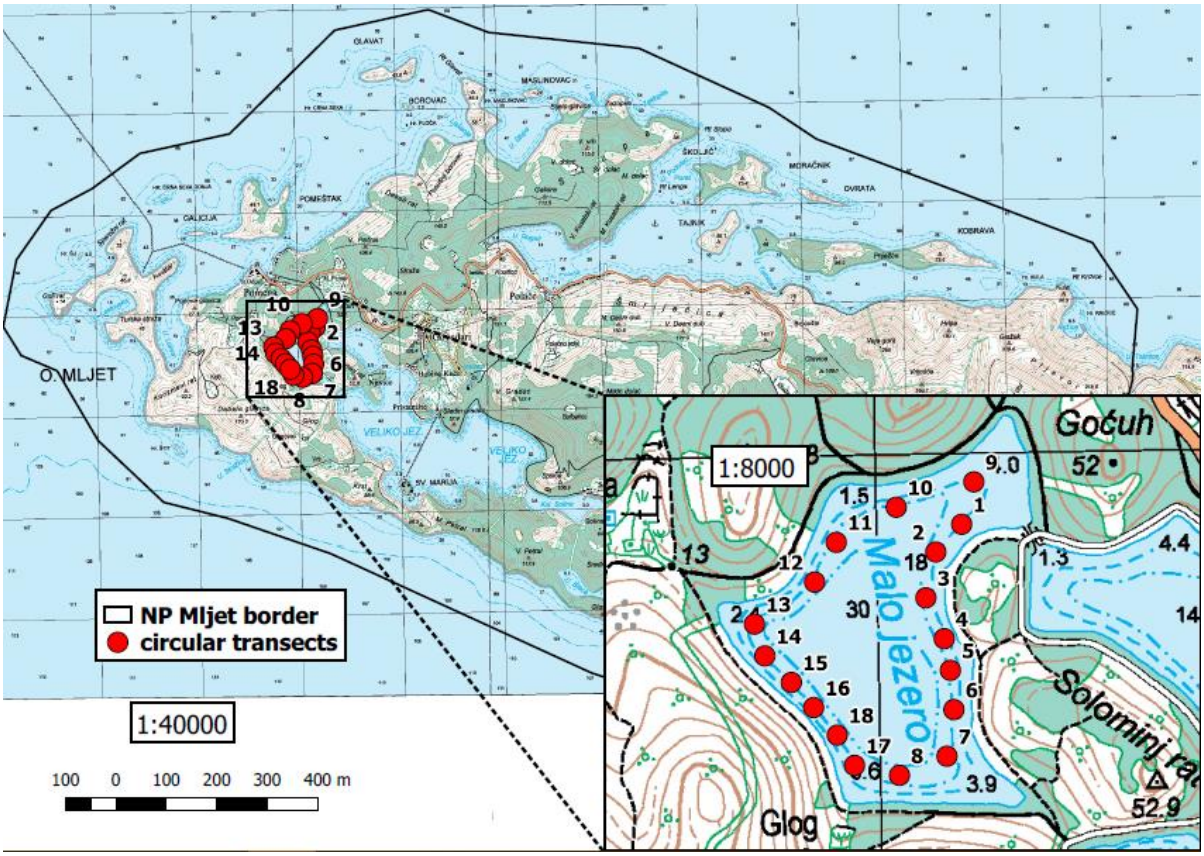


Figure 3: A map of National Park Mljet marked with black line and GPS positions of transects marked with red circles and numbers (1-18)

3 Materials and methods

3.1 Ethic statement

Since *P. nobilis* is strictly protected species (the Annex IV of EEC, 1992) on European level, as well as listed in Ordinance on strictly protected species in Croatia (Official Gazette 144/13, 73/16), no field studies involving impacting manipulation, dislocation or removal of *Pinna nobilis* individuals were performed. For both locations, Nature Park Telašćica and National Park Mljet, necessary permit was obtained for the sampling activities by the Ministry of Environment and Energy (KLASA: UP/I-612-07/16-48/103, URBROJ: 517-07-1-1-1-16-4).

3.2 Collection methods

Noble pen shells were sampled from two Marine Protected Areas (MPA) in the Adriatic Sea: Nature Park Telašćica (TEL) (Figure 2) and National park Mljet (MLJT) (Figure 3). In Telašćica, sampling for genetic analysis was performed in December 2015, while sampling for morphological analysis was performed in June 2016. In Mljet, sampling for morphological analysis was performed in October 2013 while data collection for genetic analysis was performed in August 2016. The samples were collected at depth range from 3 to 20 meters, in the seagrass meadow area of *Posidonia oceanica* and *Cymodocea nodosa*, using SCUBA.

For morphological analysis, survey has been carried applying circular sampling techniques (García-March and Vicente, 2006) within a radius of 10 meters. Diving buoy was used to mark the centre of the sampling circle on surface with GPS floating in the waterproof box to record position of each circle. Straight anchor line of diving buoy was outreaching the bottom where stainless rod is driven in seabed (ca 20 cm) to represent the centre of sampling circle on the seabed. To delimit the circle, a nylon line (diving reel) 10 meters long was used, attached to the rod. The starting point of the exploration was marked with a small diving buoy floating over the *Posidonia oceanica* meadow. The line was marked with water-resistant paint marker every meter, and divided into 5-m segments, with water-resistant numbered signs (Katsanevakis, 2007a). Each segment was surveyed by one SCUBA diver. The data were sampled making concentric rings while holding the line to mark the radius as straight as possible (Figure 4).

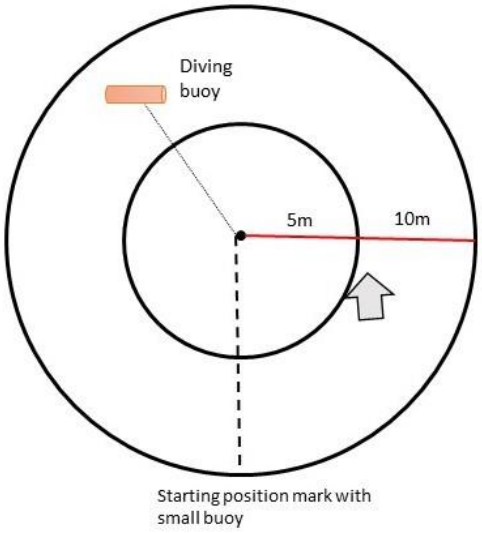
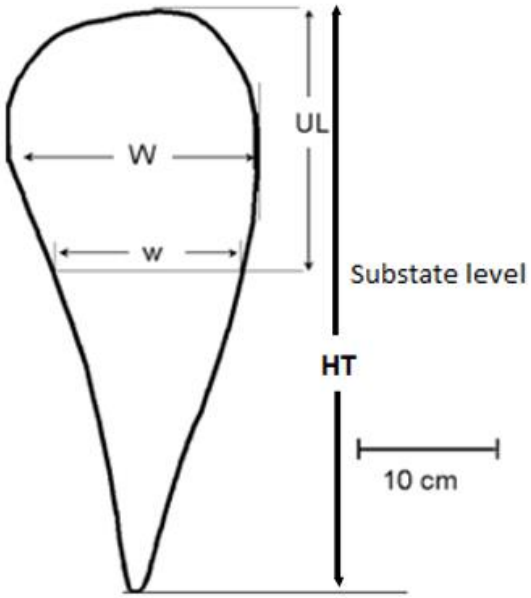


Figure 4: Schematic representation of a circle of 10 m radius with two rings, red line is representing diving reel, arrow mark direction of sampling

For each detected individual, all data were recorded in situ, written on diving slate with pencil, to avoid unnecessary removal and disturbance. Tree calliper and self-retracting tape measure were used. For each individual were recorded: corresponding circle in which was found, biometry data and substrate type according to visual assessment. Studies on *Pinna nobilis* biometry require three measurements of the unburied part of the valve: (1) the maximum dorso-ventral height or maximum width (W), (2) the minimum dorso-ventral height or minimum width (w), and (3) the unburied antero-posterior length or maximum unburied length (UL) (García-March, García-Carrascosa and Pena, 2002). Maximum width is measured at the point of maximum dorso ventral length of the shell (García-March and Vicente, 2006). Minimum width is measure at the interface of the shell with the sediment (García-March and Vicente, 2006). Unburied length is measured from the line of the w considered, to the most posterior extreme of the shell (García-March and Vicente, 2006). Biometry of *P. nobilis* is shown in Figure 5.

Figure 5. Biometry of Pinna nobilis. W: maximum width; w: minimum width; UL: unburied length; HT: Total shell height. Modified from (García-March, García-Carrascosa and Pena, 2002)



Sampling for genetic analysis was performed using developed non-lethal sampling method by Sanna *et al.*, which does not cause significant damages to the shell and soft tissues of *P. nobilis*. The valves of a given individual were held open with a wooden stick (diameter = 0.5 cm), put in proximity (4 – 5 cm) of the hinge ligament, and around 50 mg sample of mantle tissue was excised using pincers. The stick was then removed, and the tissue sample stored in a 5 mL tube. On surface, the tissue sample was then preserved in 5 ml tube with 96 % ethanol and transferred to the laboratory in a refrigerated box. This method ensures the survival of the sampled individuals (Sanna *et al.*, 2013).

In Nature Park Telašćica 14 individuals were sampled for genetic analysis and 451 individuals for morphological analysis, while in National Park Mljet 35 individuals were sampled for genetic analysis and 453 individuals for morphological analysis.

3.3 Morphological analysis

The population density was calculated for each transect by dividing the sum of all recorded individuals in transect by its area. The area of all circular transects used in density calculations were $10^2 \pi = 314.16$ m^2. The number of dead shells was recorded and ration between dead and live shell is given.

Total shell height (HT) (Figure 5) used in all analyses was calculated by the following equation: HT = (1.79 w + 0.5 ± 0.2) + UL given in García-March and Ferrer Ferrer (1995). Although this formula

was originally used for the calculation of *P. nobilis* total shell height from a different area, it was considered suitable for this study since it contains two shell size parameters which, when used together, eliminate potential problems with total shell height calculation that might occur because of differences in shell morphology or burial depth (Siletic and Peharda, 2003).

There are three more equations found in literature for total shell height (HT) calculation, and there is no data recorded in literature for their mutual comparison. Data comparison among populations were done confronting results of HT for each individual calculated with all four literature available equations. Except equation given in García-March and Ferrer Ferrer (1995), which contains maximum unburied length (UL) and minimum width (w), the equation listed in de Gaulejac and Vicente (1990), García-March and Vicente (2006) and Tempesta, Del Piero and Ciriaco (2013) which contains maximum width (W), were used.

Firstly, data distribution was tested using Shapiro – Wilk test. Since the given p-value (W = 0.098374; p = 0,00000) was less than 0.05, the null hypothesis (the population is normally distributed) was rejected and there is evidence that the data tested non follow normal distribution. Since the morphometric data of noble pen shells were not normally distributed, non - parametric Mann-Whitney U and Kruskal-Wallis tests were used for between - transects and between - populations comparison of population density and total shell. All analyses were performed in Statistica 13.1 software.

3.4 Genetic analysis

DNA was isolated using a Macherey-Nagel NucleoSpin® Tissue kit and the concentration were measured as well as purity ratio using a NanoDrop 8000 spectrophotometer. Mitochondrial regions were amplified using specific primers. The cytochrome C oxidase subunit I (COI) was amplified using the following primers: (L: 5'-GGTTGAACTATHTATCCNCC-3' and H: 5'-GAAATCATYCCAAAAGC-3') designed by Sanna *et al.* (2013) because those provided by Katsares *et al.* (2008) for Aegean populations did not yield satisfactory results on our samples, a possible consequence of high variability occurring in the annealing region that led to mispriming in PCR. As well, mitochondrial region of 16S ribosomal RNA (16S) was amplified using following primers: (L: 5'-TGCTCAATGCCCAAGGGGTAAAT-3' and H: 5'-AACTCAGATCACGTAGGG-3') designed by Sanna *et al.* (2013) because those provided by Katsares *et al.* (2008) for Aegean populations did not yield satisfactory results. A 6 µL of PCR products were electrophorized and visualized under UV on 2 % agarose/ 1× SBA gel stained with ethidium bromide (10 mg mL^{-1}) at 4 V/cm for 20 min (Sanna *et al.*, 2013). The PCR products were purified using ExoSAP-IT (USB Corporation) and sequenced using an external sequencing core service (Macrogen Europe). Each region was sequenced in both forward and reverse direction with the same primers used for PCR. Dual peaks of similar height, interpreted as evidence of mitochondrial pseudogenes in the nucleus (Numts) (Bensasson *et al.*, 2001) or heteroplasmy (see Passamonti (2007) for details), were not observed in the

electropherograms of mitochondrial markers. In addition, no evidence of non-specific DNA bands was detected on the agarose gel, thus excluding the possibility of multiple nuclear mitochondrial DNA-like sequences (Bensasson *et al.*, 2001). The detailed methods for molecular analysis are described below.

3.4.1 DNA Extraction

Upon removal from fridge, a small sample of mantle tissue (about 30 mg) was cut into small pieces using a sterile scissors and scalpel and placed in an eppendorf tube. After mechanical homogenization, DNA extraction was carried out by means of the Macherey-Nagel NucleoSpin® Tissue kit according to the protocol provided by the supplier and below reported. 180 µL of Buffer T1 and 25 µL of Proteinase K solution were added, and each sample was vortexted for 10 seconds. The samples were incubated in a thermostated bath at 56 ˚C overnight.

After incubation, samples were first vortexed, then 200 µL of Buffer B3 were added to each test tube and all test tubes were vortexted again vigorously. Following step was incubation of all test tubes at 72 ˚C for 10 minutes. After incubation, samples were vortexed again briefly. 210 µL of ethanol (96 %) were added to each test tube and the samples were immediately vortexed. Each sample was applied to the one NucleoSpin® Tissue Column into a collection tube and centrifuged for 1 minute at 12000 rpm. Flow-through was thrown away, leaving behind the DNA. With the DNA bound to the silica membrane, two wash phases were used to remove residue of lysed cells from the spin columns. In the first wash step, 500 µL of Buffer BW were added to each spin column and the samples were centrifuged at 12000 rpm for 1 minute; the flow-through was discarded. In the second wash step, 600 µL of Buffer B5 were added to each spin column and the samples were centrifuged for 1 minute at 12000 rpm; the flow-through was discarded. All samples were centrifuged for 1 minute at 12000 rpm to remove residual ethanol.

The spin columns with DNA were transferred to clean Eppendorf tubes and 100 µL of prewarmed (70 ˚C) elution buffer (Buffer BE) were added to the filter. The samples were incubated at room temperature for 1 minute, during which the elution buffer detaches the DNA from silica membrane. By centrifuging the samples at 12000 rpm for 1 minute, DNA and elution buffer were collected into the Eppendorf tube. The elution step was repeated twice for each specimen sample, and the DNA samples were tested for concentrations and purity by spectrophotometric device. All DNA samples were kept at 4 ˚C until PCR.

3.4.2 Spectrophotometric analysis

A Nanodrop spectrophotometer 8000 was used to measure the total of genomic DNA isolated from the samples (ng μL^{-1}) as well as the purity of the samples. The measurements should not be treated as exact since only 1 µL of sample was used for the reading, notwithstanding, this method is a

useful tool as a quick test of success of extracted DNA isolation. The DNA concentrations, as well as purity ratio (A260 and A260/280) of samples are listed in Table 1.

Table 1. DNA concentrations, letter P in the code of the sample indicates that this specimen was used as test sample during the standardization of the PCR procedure.

Date	Population	Sample	Nuclear acid	Concentration [ng μl^{-1}]	Factor	A260 (10 mm)	A260/A280
5.12.2016	Telašćica	TEL1	dsDNA	58.3	50	1.167	2.07
5.12.2016	Telašćica	TEL2	dsDNA	70.6	50	1.413	2.11
5.12.2016	Telašćica	TEL3	dsDNA	88.3	50	1,766	2.09
5.12.2016	Telašćica	TEL4	dsDNA	60.8	50	1.216	2.09
5.12.2016	Telašćica	TEL5	dsDNA	95.7	50	1.915	2.09
5.12.2016	Telašćica	TEL6	dsDNA	66.7	50	1.334	2.03
5.12.2016	Telašćica	TEL7	dsDNA	35.8	50	0.716	1.96
5.12.2016	Telašćica	TEL8	dsDNA	38.1	50	0.762	2.03
5.12.2016	Telašćica	TEL9	dsDNA	89.5	50	1.790	2.15
5.12.2016	Telašćica	TEL10	dsDNA	135.4	50	2.709	2.09
5.12.2016	Telašćica	TEL11	dsDNA	42.7	50	0.853	2.03
5.12.2016	Telašćica	TEL12	dsDNA	81.9	50	1.638	2.08
5.12.2016	Telašćica	TEL13	dsDNA	132.3	50	2.645	2.13
5.12.2016	Telašćica	TEL14	dsDNA	25.5	50	0.510	1.99
5.12.2016	Telašćica	PTEL1	dsDNA	14.7	50	0.294	2.13
5.12.2016	Telašćica	PTEL2	dsDNA	20.1	50	0.402	2.10
5.12.2016	Telašćica	PTEL3	dsDNA	39.8	50	0.796	2.13
5.12.2016	Telašćica	PTEL4	dsDNA	46.2	50	0.924	2.15
5.12.2016	Mljet	PMLJ3	dsDNA	42.7	50	0.854	2.05
5.12.2016	Mljet	PMLJ8	dsDNA	49.4	50	0.989	1.96
5.12.2016	Mljet	MLJ1	dsDNA	35.0	50	0.700	1.97
5.12.2016	Mljet	MLJ2	dsDNA	56.1	50	1.123	1.93
5.12.2016	Mljet	MLJ3	dsDNA	63.2	50	1.264	2.04
5.12.2016	Mljet	MLJ4	dsDNA	42.0	50	0.839	2.09
5.12.2016	Mljet	MLJ5	dsDNA	54.5	50	1.090	1.90
5.12.2016	Mljet	MLJ6	dsDNA	33.9	50	0.677	1.96
5.12.2016	Mljet	MLJ7	dsDNA	45.1	50	0.900	1.98
5.12.2016	Mljet	MLJ8	dsDNA	45.9	50	0.918	2.02
5.12.2016	Mljet	MLJ9	dsDNA	47.5	50	0.949	1.98
5.12.2016	Mljet	MLJ10	dsDNA	43.0	50	0.861	1.98
5.12.2016	Mljet	MLJ11	dsDNA	34.1	50	0.683	1.99
5.12.2016	Mljet	MLJ12	dsDNA	47.8	50	0.957	2.08
5.12.2016	Mljet	MLJ13	dsDNA	99.4	50	1.987	2.02
5.12.2016	Mljet	MLJ14	dsDNA	38.8	50	0.776	1.96
5.12.2016	Mljet	MLJ15	dsDNA	100.4	50	2.008	2.18
5.12.2016	Mljet	MLJ16	dsDNA	70.3	50	1.406	2.10

5.12.2016	Mljet	MLJ17	dsDNA	40.0	50	0.799	2.05
5.12.2016	Mljet	MLJ18	dsDNA	27.2	50	0.544	2.03
5.12.2016	Mljet	MLJ19	dsDNA	33.6	50	0.673	1.94
5.12.2016	Mljet	MLJ20	dsDNA	34.3	50	0.686	1.99
5.12.2016	Mljet	MLJ21	dsDNA	21.3	50	0.426	1.98
5.12.2016	Mljet	MLJ22	dsDNA	20.4	50	0.409	2.02
5.12.2016	Mljet	MLJ23	dsDNA	23.0	50	0.460	1.92
5.12.2016	Mljet	MLJ24	dsDNA	37.9	50	0.759	2.04
5.12.2016	Mljet	MLJ25	dsDNA	52.1	50	1.042	2.06
5.12.2016	Mljet	MLJ26	dsDNA	75.7	50	1.515	2.13
5.12.2016	Mljet	MLJ27	dsDNA	47.3	50	0.946	2.00
5.12.2016	Mljet	MLJ28	dsDNA	42.8	50	0.857	2.04
5.12.2016	Mljet	MLJ29	dsDNA	26.3	50	0.526	2.02
5.12.2016	Mljet	MLJ30	dsDNA	37.6	50	0.752	2.03
5.12.2016	Mljet	MLJ31	dsDNA	54.5	50	1.089	2.04
5.12.2016	Mljet	MLJ32	dsDNA	38.9	50	0.779	2.07
5.12.2016	Mljet	MLJ33	dsDNA	29.1	50	0.581	2.04
5.12.2016	Mljet	MLJ34	dsDNA	35.1	50	0.702	1.92
5.12.2016	Mljet	MLJ35	dsDNA	34.3	50	0.686	1.98

3.4.3 Polymerase chain reaction and gel electrophoresis for COI and 16S

PCR reactions were carried out in 25 µL volumes with 20 µL of made master mix (including polymerase, buffer, $MgCl_2$, dNTPs, primers forward and reverse, BSA and water) and 5 µL of sample DNA (Table 2).

Table 2. PCR reaction components and volumes used for amplification of the COI and 16S gene in P. nobilis

Component	**Volume (µL)**
DNA	5
Buffer 10×	2.5
dNTPs 200mM	2
Forward primer 10µM	0.8
Reverse primer 10µM	0.8
$MgCl_2$ 50mM	1.5
Taq DNA Polymerase SIGMA - Aldrich 5U/µL	0.5
Nuclease-free water	9.4
BSA 5µg/µL	2.5
Total	25

PCR amplifications were performed in a Thermocycler (BioRad DNA Engine PTC-200 Peltier Thermal Cycler) according to the following steps: initial denaturation for 2 min at 94 ˚C, 35 cycles of denaturation for 1 min at 94 ˚C, annealing for 1 min at 46 ˚C and extension for 1 min 30 s at 72 ˚C. A post-treatment of final extension for 5 min at 72 ˚C was applied.

A 6 µL of each PCR products was run on a 2 % agarose /1× SBA gel, with ethidium bromide (10 mg mL^{-1}) (an intercalating agent commonly used as a fluorescent tag) to stain the DNA, for 20 minutes at 4 V cm^{-1} to confirm amplification. The gel was visualized and photographed under UV light transilluminator.

3.4.4 Standardization of microsatellite markers

Seven sets of primers for amplification of highly polymorphic microsatellite markers were tested at different temperatures to evaluate the effectiveness for our samples of the protocols developed by González-Wangüemert *et al.* (2015) for Balearic population. Standardization of microsatellite markers (SSR standardization) was performed for the 4 samples which showed the best results with COI and 16S PCR (TEL 1, TEL3, TEL4 and MLJ8). Tested primers and their sequence are listed in Table 3.

Polymerase chain reaction (PCR) was performed in 25 µL total volume, which includes 5 - 7.5 µL of DNA, 2.5 µL of Buffer (10×), 0.5 µL dNTPs (200mM), 0.5 µL of each primer (10µM), 1.8 - 2 µL $MgCl_2$ (25mM), 0.5 µL Taq DNA Polymerase SIGMA - Aldrich (5UμL^{-1}), 8.3 - 10.8 µL of Nuclease-free water and 3 µL of BSA (5 µg μL^{-1}). Cycling conditions were same as mentioned above for COI and 16S samples, except annealing temperature was set between 52 - 62 °C (see Table 4 for details).

A 6 µL of each PCR products were run on a 2 % agarose /1× SBA gel, stained with ethidium bromide (10 mg mL^{-1}) to dye the DNA, for 20 minutes at 4 V cm^{-1} to confirm amplification. The gel was visualized and photographed under UV light.

Table 3. The following conditions provided by Gonzalez-Wanguemert et al. (2015) for Balearic populations were used to test the SSR primers in Adriatic populations.

Code	Repeat motif	Primer sequences and fluorescent dyes	Ta (°C)	$MgCl_2$ (mM)	N Alleles	Size range (bp)
P.n 2.1	(GT)14	F: FAM-GCACCTTTTCTTGGACGG R: GGAACTGCACTCGATGACG	57	1.5	12	201-231
P.n 2.2	(AC)10	F: FAM-GGCCATAAGTGCCGAACAC R: ACAGGAAAATTAGAACTTAGGAACG	55	2	4	230-236
P.n 3.2	(AAC)12	F: FAM-CCGAGGTCCCGTATCACAG R: TGCCCTTTGTGTCATTATTTCG	63	1.5	15	194-228

P.n 3.3	(ATT)13	F: HEX-CGAGACGGAGTTCCAAAGC R: TGGCCCTGAACAGTAGGTG	56	2	8	226-242
P.n 4.2	(AGAT)10	F: FAM-TCCTTTAATTCAGTGGGTCGC R: ATTCCCGCAAATCCATCGC	62	1.5	15	163-211
P.n 4.3	(ATTT)17	F: HEX- GATCTAGACTCTTTGTTTGTCTTC R: ACAGTGCCATGCTATGTTGC	56	2	24	238-350
P.n 5.2	(ATAGT)11	F: HEX-TTGCATGTGCCACCATAATC R: TTCATACCGATGAGCCAAATG	61	2	13	181-223

For all microsatellite primers, the annealing temperature was modified, and in some primers concentrations of DNA and $MgCl_2$ was recalculated regarding González-Wangüemert *et al.* (2015) with Balearic population. Since PCR for each sample was performed in 25 µL total volume, volume of nuclease-free water was variable and changed as its volume had to fill in the gap up to total PCR volume. The success rate protocol for amplification of 7 microsatellite markers is shown in Table 4. The trial PCR testing 3 primers pairs is shown in Figure 6.

The samples of microsatellite markers were not sequenced due to not using its data in further genetical analysis.

Table 4. Modifications of protocol for SSR amplifications for Adriatic populations

	Primer 2.1.	**Primer 2.2.**	**Primer 3.2.**	**Primer 3.3.**	**Primer 4.2.**	**Primer 4.3.**	**Primer 5.2.**
DNA volume (µL)	6.0	6.0	5.0	6.0	7.5	6.0	6.0
$MgCl_2$ volume (µL)	1.8	1.8	2.0	1.8	2.0	1.8	1.8
Nuclease-free water volume (µL)	10.0	10.0	10.8	10.0	8.3	10.0	10.0
Annealing temperature (°C)	62	53	53	55	55	55	62

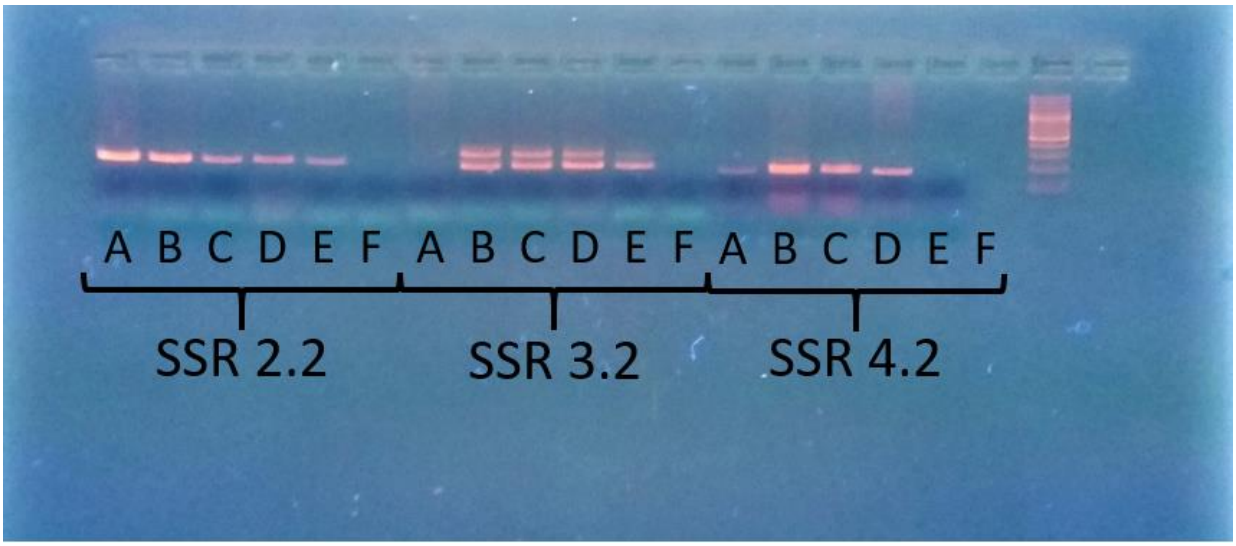


Figure 6. Trial PCR testing 3 primers pairs (2.2., 3.2., 4.2.) on 4 different specimens; A=PTEL1, B=PTEL3, C=TEL4, D=PMLJ8 with positive control (E) and negative control (F) for each primer pair at 53°C annealing temperature with molecular-weight size marker at the right end. Successful PCR is denoted by a single white band on the gel. Based on results from the gel below, this PCR reaction was successful for SSR 2.2. only. In SSR 3.2 pseudogenes were amplified, while in primer SSR 4.2. the positive control is not visible.

3.4.5 Purification and sequencing

The PCR products of COI and 16S were purified using ExoSAP-IT (USB Corporation) according to the manufacturer's protocol for enzymatic cleanup of amplified PCR product. PCR purification hydrolyses excess primers and nucleotides in two steps. The first incubation digests excess primer and dephosphorylates nucleotides. The second, high-temperature incubation inactivates the enzymes. A 6,5 µL of PCR product were used for each sample. To purity the PCR product, 1 µL of Buffer SAP was added to each PCR reaction, as well as 0.5 µL of EXO enzyme and 2 µL of SAP enzyme. All process was kept on ice throughout this procedure. The samples were incubated at 37 °C for 15 minutes to degrade remaining primers and nucleotides, and then incubated at 80 °C for 15 minutes to inactivate added enzymes reagents. These purified samples were stored at -20 °C until they were sent to Macrogen for sequencing.

Purified PCR products were sent to external service Macrogen Europe (Amsterdam, The Netherlands) for sequencing. Each sample was sequenced in both forward and reverse direction. In DNA sequencers, which are automated instruments, PCR products are dissociated and single stranded DNA is used as a template for sequencing a complementary strand. This method evolved from Sanger sequencing method. Along with dNTPs, low concentrations of dideoxynucleotides (ddNTPs) are added to each of four separate reactions (ddATP, ddTTP, ddGTP, ddCTP), each using the same template DNA. Each time a ddNTP is added, the reaction is terminated, resulting in a series of DNA fragments of varying lengths. These fragments are separated by gel electrophoresis and the final nucleotide is detected for each fragment length. Modern automated DNA analysers use dye-terminators, where

ddNTPS are marked with a fluorescent dye and which flashes when integrated into the DNA sequence. A light detector registers these light peaks and thus records the DNA sequence (http://www.macrogen.com/eng/).

3.4.6 Statistical Analysis

The obtained sequences were checked by eye, and if necessary, corrected manually. The low-quality sections at the ends of each sequence were removed, and the final sequence were cut to the length of the shortest sequence so they could be compared. Sequences were aligned using BioEdit 7.1.3.0 software (Hall, 1999). Levels of mtDNA diversity were investigated by comparing the number of polymorphic sites (S), number of haplotypes (H), haplotype diversity (h), and nucleotide diversity (π) using the DnaSP v. 5.10 software (Librado and Rozas, 2009). Gaps were considered for the analysis. The analysis of 109 individuals from six Mediterranean populations (see Table 5 for details) by means of two molecular markers (COI and 16S) allowed us to obtain three different datasets of sequences (COI, 16S, COI-16S). Indeed, the sequences from COI (338 bp long) and 16S (427 bp long) genes were merged together to obtain a larger and more variable mitochondrial DNA fragment 765 bp long (COI-16S) to be analyzed (see Table 6 for details on the genetic divergence of the genes). COI and 16S genes were concatenated, after they were tested for heterogeneity between data partitions (Farris *et al.*, 1995) which measures the significance of incongruence among data sets (Bull *et al.*, 1993; Chippindale and Wiens, 1994; Sullivan, 1996; Cunningham, 1997) by the partition-homogeneity test implemented in PAUP*4.0b10 (Swofford, 2003). The 99 % confidence interval was used to determine the level of significance, following Farias, Orti and Meyer (2000). Since the partition-homogeneity test did not show significant heterogeneity ($P > 0.01$) among the two mitochondrial fragments analysed, they were pooled in the resulting COI-16S dataset. Population differentiation analyses were conducted using Arlequin 3.5.2.2. (Excoffier and Lischer, 2010) with a Tamura and Nei (1993) matrix of genetic distances with a gamma correction, according to the best-fitting model of sequence evolution selected by JModeltest (Posada, 2008) using the Akaike Information Criterion (AIC). The presence of population genetic structure was determined by the Bayesian model-based clustering algorithm implemented in Baps 6.0 (Corander et al., 2013). Clustering among populations was performed using the codon linkage model available in the module for linked molecular data, which is appropriate for sequences. Each analysis was performed ten times with a vector of K values ranging from 1 to 10, each with six replicates, where K is the number of assumed genetic clusters. Genetic relationships among haplotypes were investigated by the Median-Joining network using the software package Network 4.5.0.1 (www.fluxus-engineering.com).

Table 5. Origin of the Pinna nobilis individuals used in this study. Sampling localities and sample sizes (N) of the specimens are indicated for each sample.

Geographic area	**Locality**	**Code**	**N**	**Study**
Sardinia – Italy	Alghero (Ospedale Marino)	OSM	21	Sanna et al. (2013)
Sardinia – Italy	island La Maddalena (Cala Camiciotto)	MAD	17	Sanna et al. (2013)
Veneto – Italy	Venice (Ottagono Alberoni and Santa Maria del Lago)	VEN	15	Sanna et al. (2013)
Thessaloniki – Greece	Epanomi and Aggeloyesori	EPAG	18	Katsares et al. (2008)
Telašćica – Croatia	island Buč, Nature Park Telašćica	TEL	14	Present
Mljet – Croatia	lake Malo Jezero, National Park Mljet	MJL	18	Present

4 Results

4.1 Population density

Along the surveyed circular transects in Nature park Telašćica, we found *Pinna nobilis* on sandy substrate in *P. oceanica* meadows between 8 and 24 meters depth. The rocky substrate is found along shoreline, followed by *P. oceanica* meadows from 3 to approximately 27 m. In National Park Mljet we found *P. nobilis* on muddy sand substrate overgrown with *C. nodosa* from 3 to 10 m depth. In marine lake Malo jezero *C. nodosa* meadow occurs from 3 to 10 m, while bare muddy substrate extends from 10 to 16 m. In Nature Park Telašćica a total of 361 live individual was recorded, corresponding to average 2.39 ± 2.08 SD ind 100 m^{-2} population density (Figure 9), although density varied across transects (Figure 7). The highest density in Telašćica was found on transect 5 of 11.14 ind 100 m^{-2}, while on transect 32 no live individuals were recorded. In two more transects in Telašćica, 41 and 42, the recorded density was clearly increased: 8.28 and 8.91 ind 100 m^{-2}.

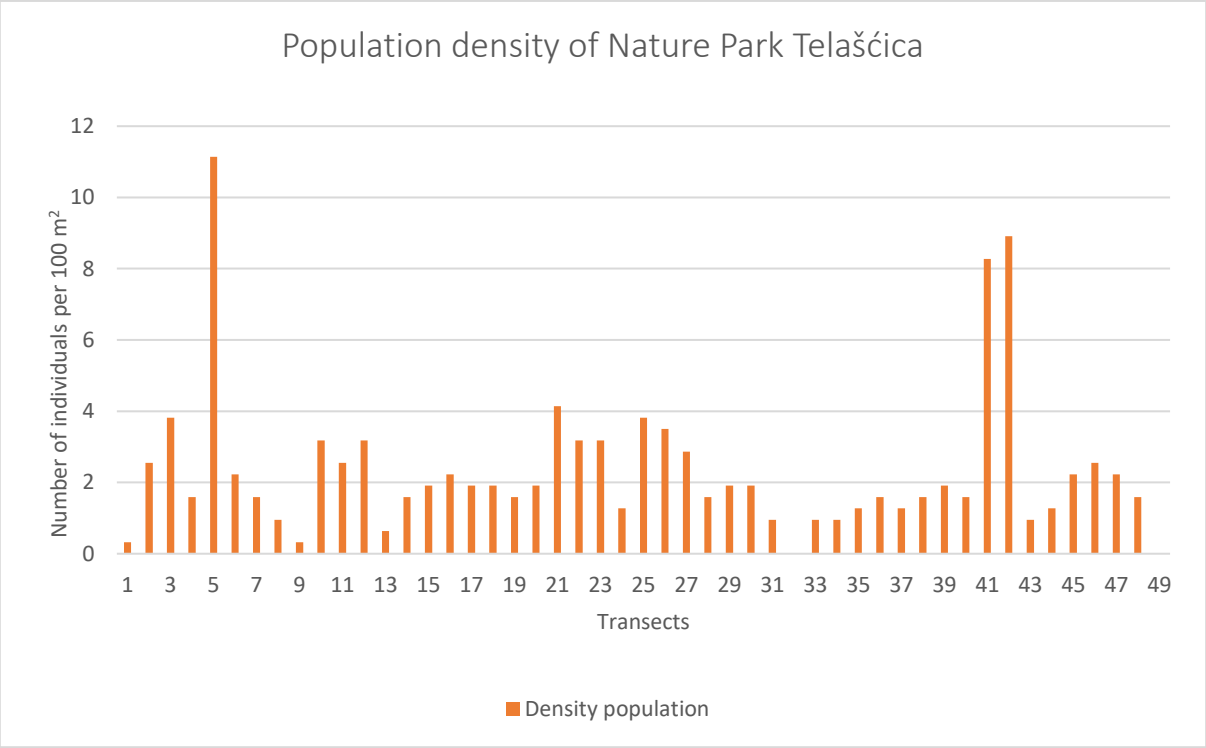


Figure 7: Density of Pinna nobilis in Nature Park Telašćica among transects

In National Park Mljet a total of 427 live individual was counted and measured, corresponding to average 7.55 ± 6.25 SD ind 100 m^{-2} population density (Figure 9), although density varied across transects (Figure 8). The highest density was recorded on transect 10 of 24.19 ind 100 m^{-2}, meanwhile the lowest density was recorded on transect 15 of 1.59 ind 100 m^{-2}. Additionally, on two more transects (17 and 19), density was near minimum and equally recorded of 1.91 ind 100 m^{-2}. There was one more

transect with density greater than 15 ind 100 m^{-2}, additionally three more transects with density greater than 10 ind 100 m^{-2}, and 5 more transects with density above value of 5 ind 100 m^{-2}.

In both results for population density of populations were included 23 individuals from Telašćica and 2 individuals from Mljet found unburied lying on the sea bottom as well. These individuals were pound in sediment.

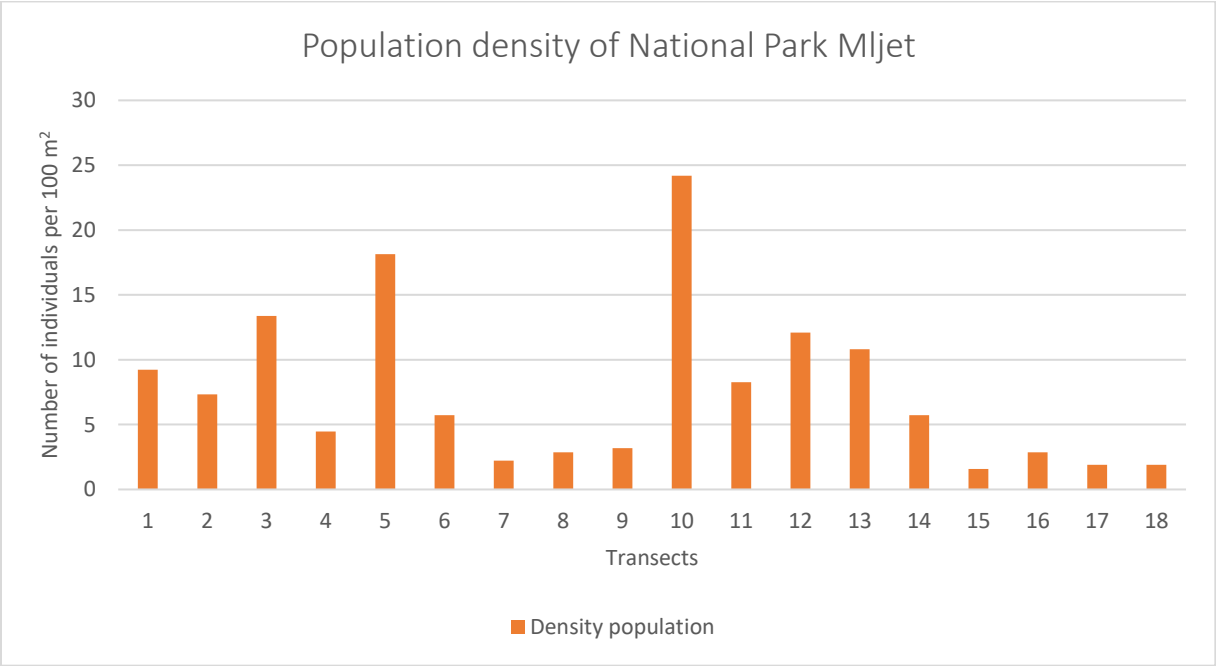


Figure 8: Density of Pinna nobilis in National Park Mljet among transects

Populations density varied significantly between observed transects in both populations; from 0 to maximum 11.14 ind 100 m^{-2} in Telašćica, and from 1.59 to maximum 24.19 ind 100 m^{-2} in Mljet (Figure 9). On all surveyed transects, *P. nobilis* appeared to have much more density in National Park Mljet than in Nature Park Telašćica. There was a statistically significant difference between population density calculated for Nature Park Telašćica and National Park Mljet (Mann-Whitney U = 141.0, p = 0.000029).

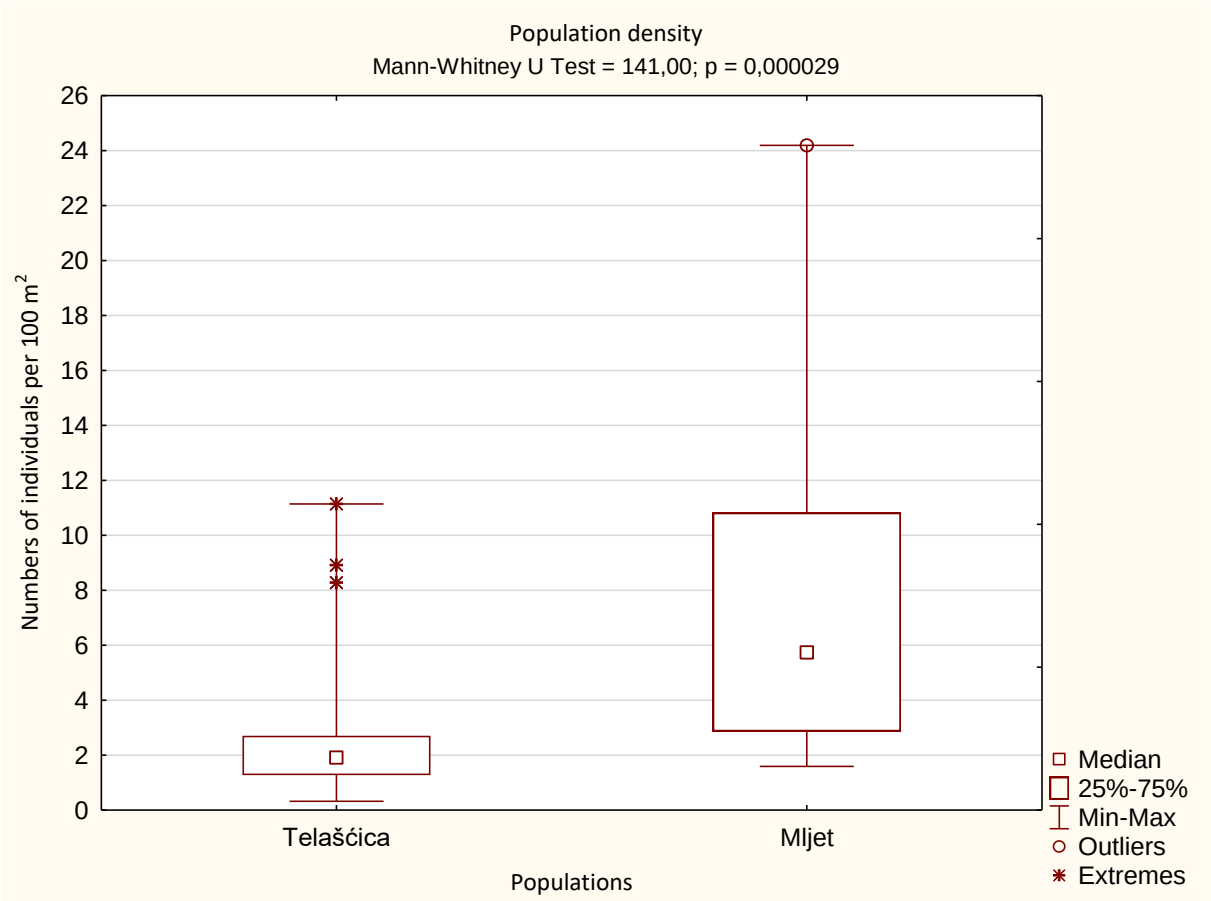


Figure 9: Box-and-Whiskers plot of population density comparing Telašćica and Mljet

Moreover, 91 dead *P. nobilis* individuals were recorded along transects in Telašćica, corresponding to 20.43 % of the total sampled. In Mljet was found 26 dead individuals, corresponding to 5.74 % of total sampled. In Telašćica there were 25 % of transects with no dead individuals found while in NP Mljet that percentage was 56 %. Detailed distribution of recorded dead shell along transects is shown in Figure 10 for Telašćica and in Figure 11 for Mljet.

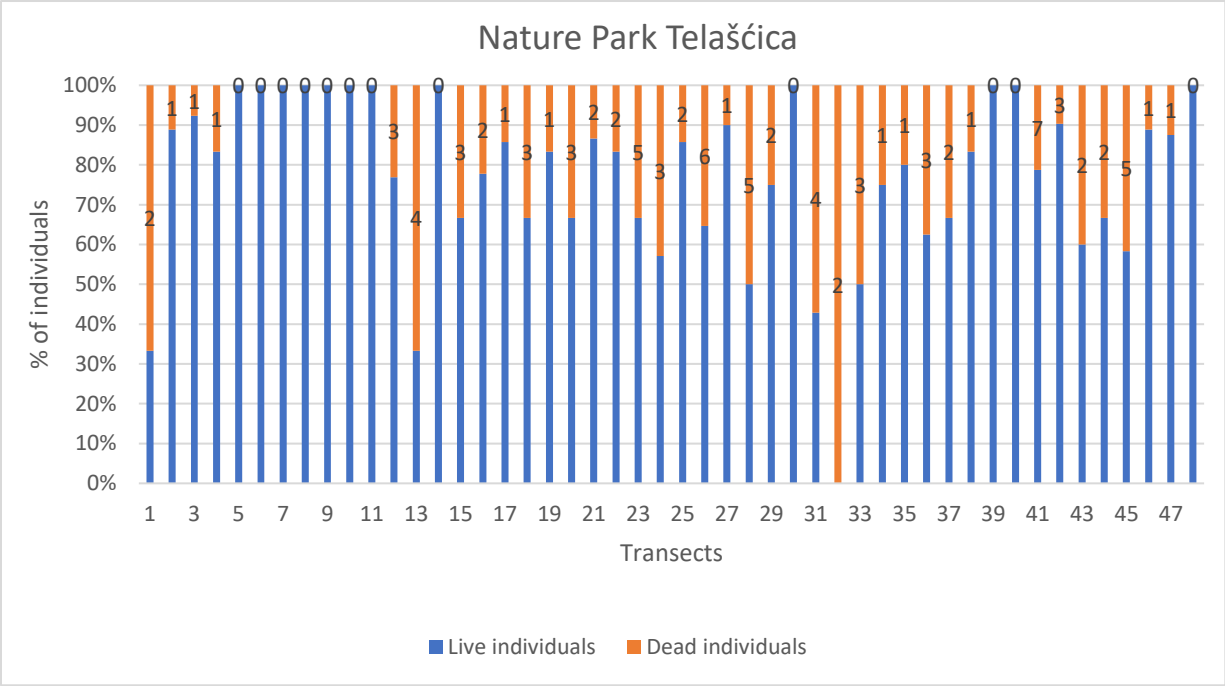


Figure 10: Number of live and dead individuals in Nature Park Telašćica according to transect.

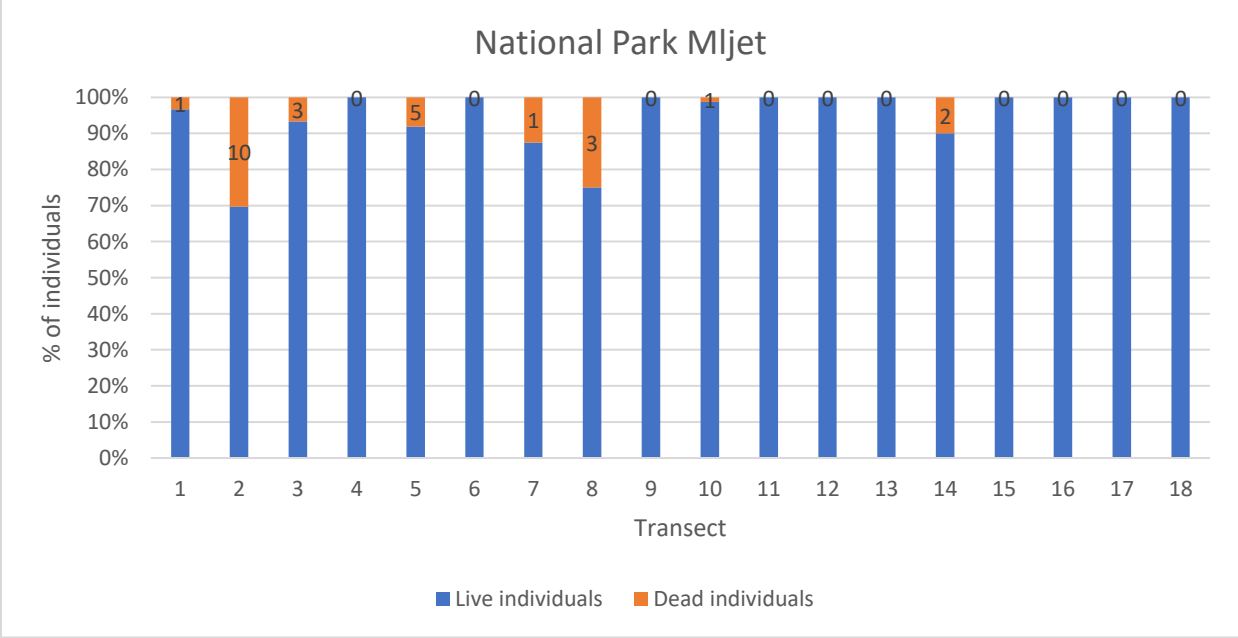


Figure 11: Number of live and dead individuals in National Park Mljet according to transect.

4.2 Morphological analysis

A result in the observed differences in total shell height between transects and between populations was noted. The total shell height (HT) distribution among Telašćica transects is shown in Figure 12. The average total shell height among transects were recorded between 19.45 (at transect 1) and 70.69 centimetres (transect 13). On transect 32, HT is zero since there was no live individuals recorded among that transect. Transect comparison for Telašćica showed that there was a significant difference in total shell height of individuals between transects (Kruskal-Wallis DF = 46, N = 330, H=117.3239, p = 0.0000)

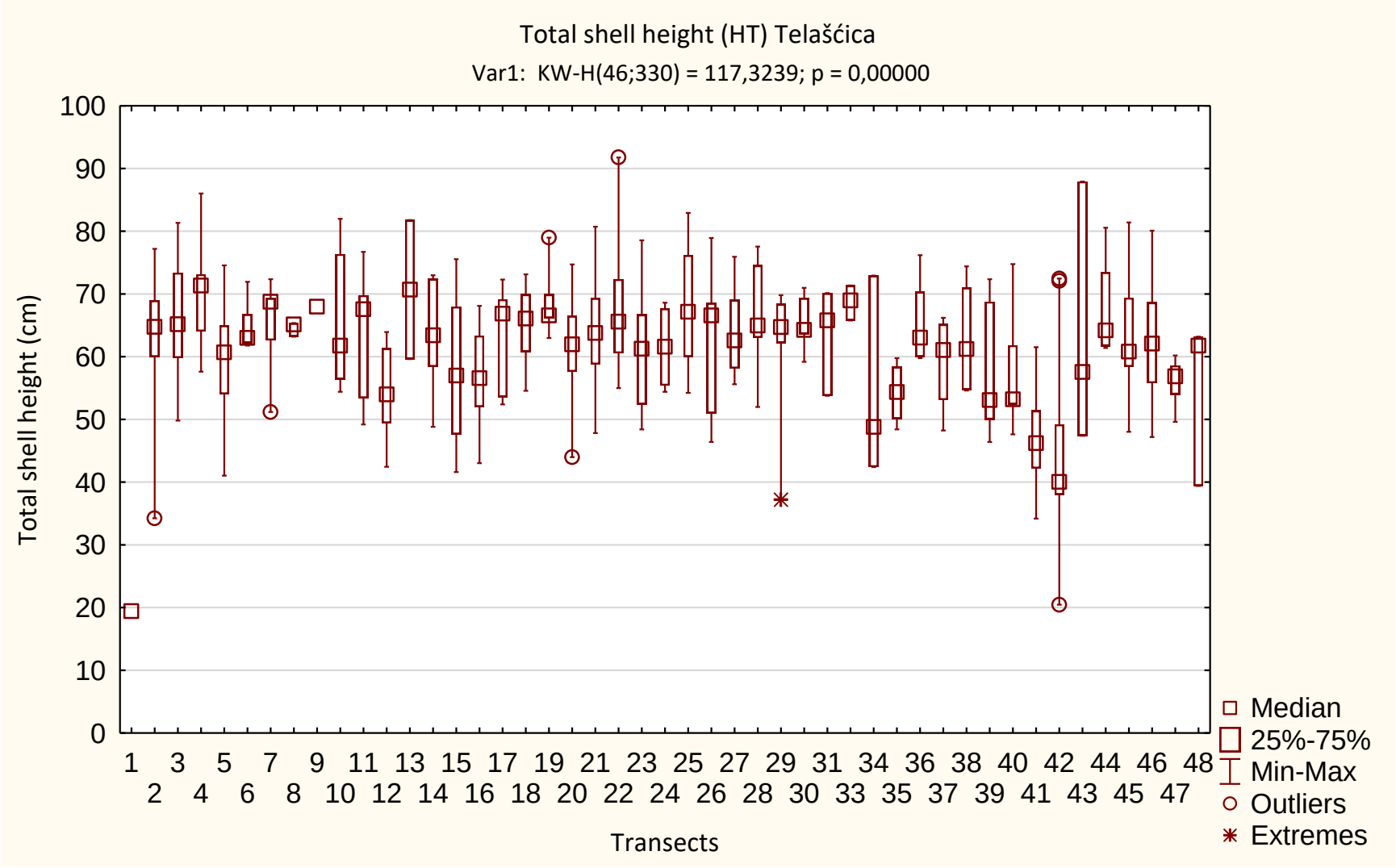


Figure 12: Box-Whiskers plot of total shell height (=length) (HT) distribution of Pinna nobilis in Nature Park Telašćica among 48 transect

The total shell heights (HT) according to observations in National Park Mljet are shown in Figure 13. The maximum recorded average value for HT in Mljet was found on transect 7 with HT of 62.51 centimetres, while minimum mean value was observed on transect 17 with HT of 51.67 centimetres. Transect comparison for Mljet showed that there was a significant difference in total shell height of individuals between transects (Kruskal-Wallis DF = 17, N = 419, H = 40.98, p = 0.0009)

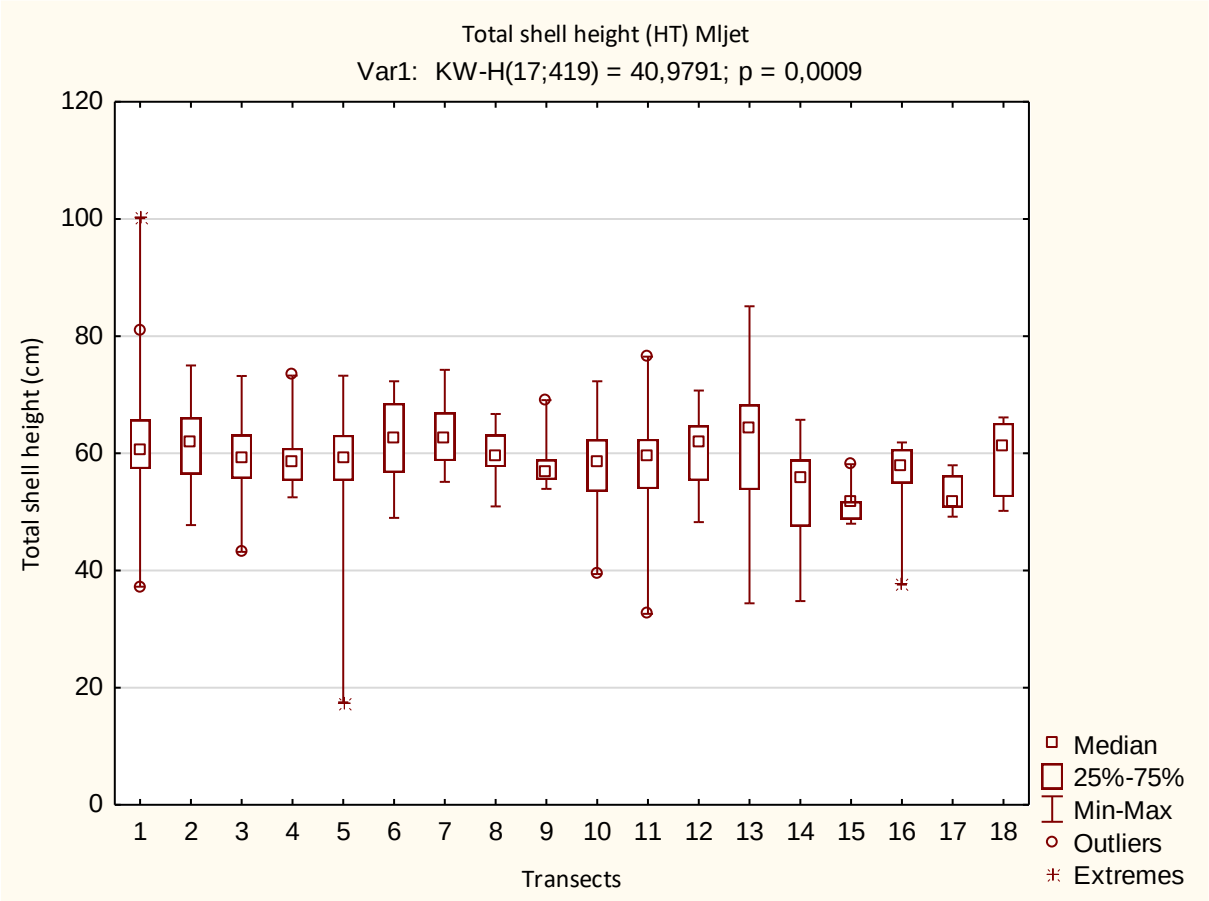


Figure 13: Box-Whiskers plot of total shell height (=length) (HT) distribution of Pinna nobilis in National Park Mljet within 18 transects

The average total shell height (HT) of the *P. nobilis* in Telašćica population calculated for all transects was 60.08 cm ± 11.71 cm (SD), while average HT of Mljet population calculated for all transect was 59.14 cm ± 8.01 cm (SD). The total shell height distribution is shown in Figure 14. There was statistically significant difference between total shell height calculated for Nature Park Telašćica and National Park Mljet (Mann-Whitney U = 62699.0, p = 0.038).

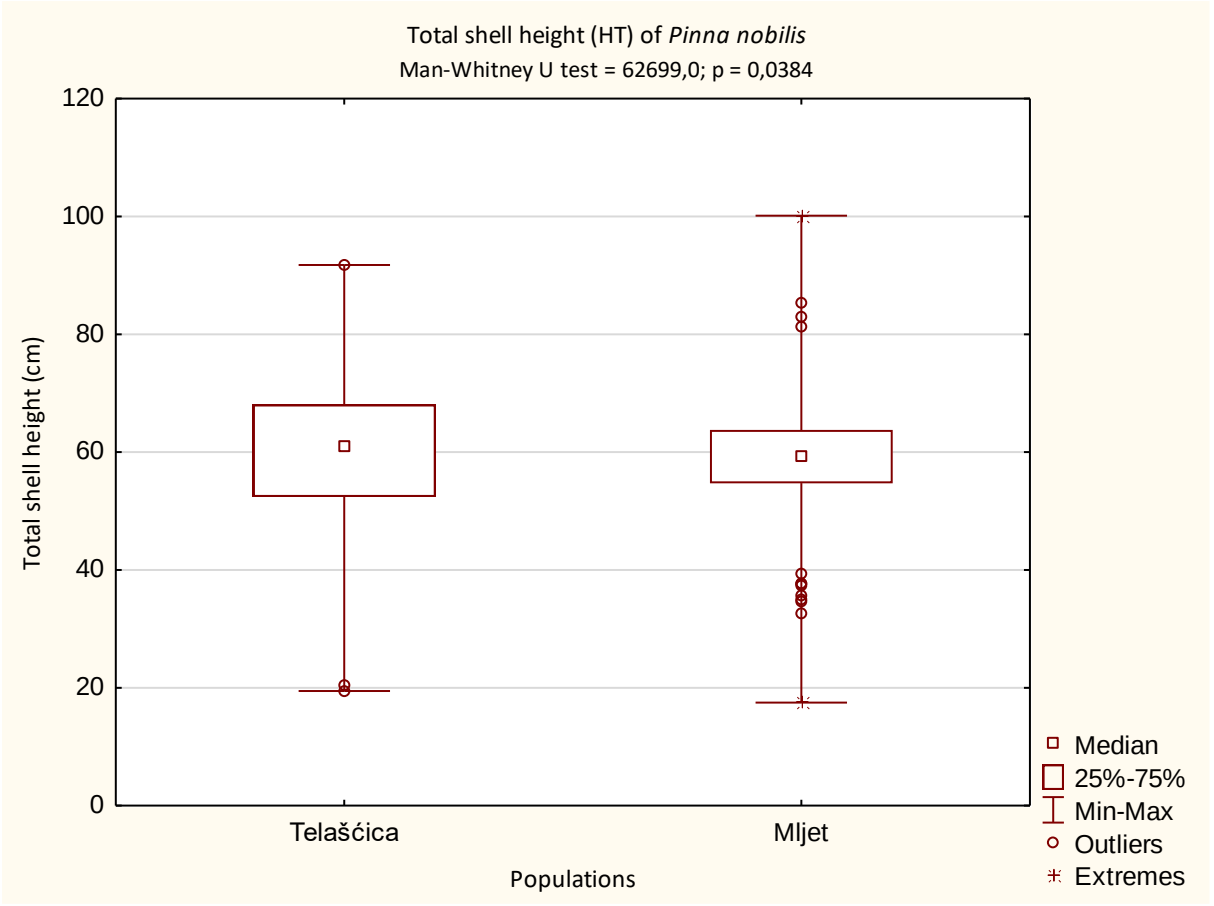


Figure 14: Box-and-Whiskers plot of total shell height (HT) comparing Telašćica and Mljet

According to literature, 20 cm of HT is the threshold under which individuals should be considered juveniles (Butler, Vicente and de Gaulejac, 1993; Richardson *et al.*, 1999). Classes are divided into 0 - 20 cm (juveniles), 20 - 40 cm (adult juveniles), 40 - 60 cm (adults) and more than 60 cm (old individuals) (Tempesta, Del Piero and Ciriaco, 2013). The total shell height distribution for all populations is shown in Figure 14. It is evident that in both Telašćica and Mljet populations, only one juvenile was found. In Telašćica, the abundance of old individuals prevails over adult, while in Mljet population situation is vice versa. In both populations, there is small number of adult juveniles as well as very old, but its abundance is greater in Telašćica population. There was only one individual with total shell height over 1 meter (100.15 cm) recorded in Mljet. In Figure 15 is visible left-skewed data distribution for Telašćica population, meanwhile Mljet population has symmetrical data distribution.

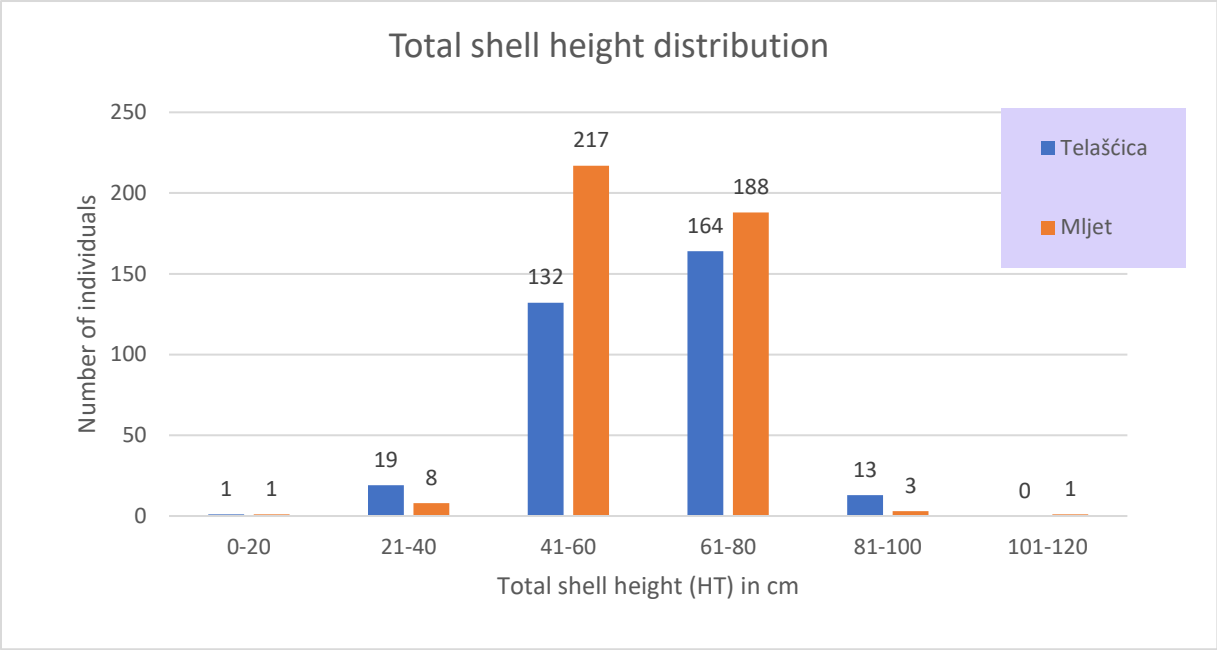


Figure 15: Total shell height distribution (HT) of Pinna nobilis calculated using equation of García-March and Ferrer Ferrer (1995) for both populations

Data comparison among populations, where are confronted results of HT for each individual calculated with all four literature available equations, are shown in Figure 16 for Telašćica population and in Figure 17 for Mljet population. In both populations is clearly visible that equation from de Gaulejac and Vicente (1990) is giving much vary results from other three equations: evidently the most abundant group is adults, with another conspicuous group of juvenile adults and data distribution right-skewed.

Data distribution of HT from equation of Tempesta, Del Piero and Ciriaco (2013), García-March and Ferrer Ferrer (1995) and from García-March and Vicente (2006) are giving very related results within populations. The equation from García-March and Ferrer Ferrer (1995) and from García-March and Vicente (2006) are giving alike results and left-skewed data distribution, indicated there are more old individuals than adults. However, the most recent modified equation from Tempesta, Del Piero and Ciriaco (2013) is giving contrary results: the most abundant group is adults, followed by old individuals, and data distribution is symmetrical. All equations are indicating very small number of juveniles (≤ 3), especially in Mljet population, and small number of extremely old individuals.

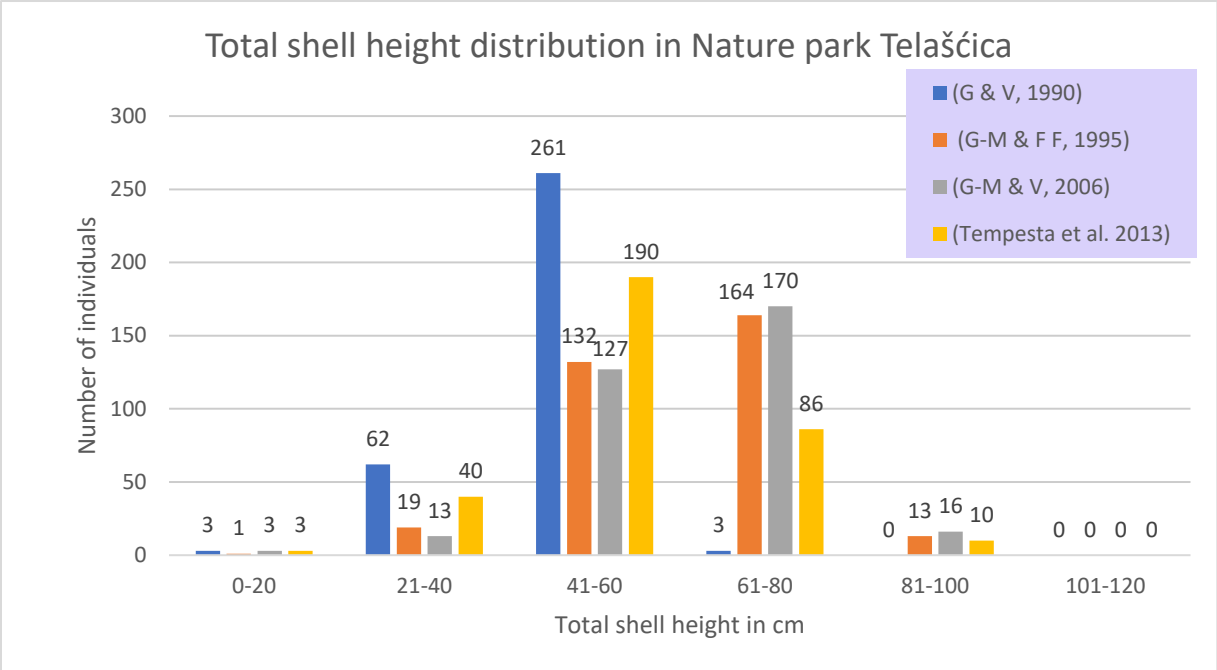


Figure 16: The total shell height (HT) distribution of Pinna nobilis in Nature Park Telašćica. Each of 4 different method for HT calculation is presented with distinct colour.

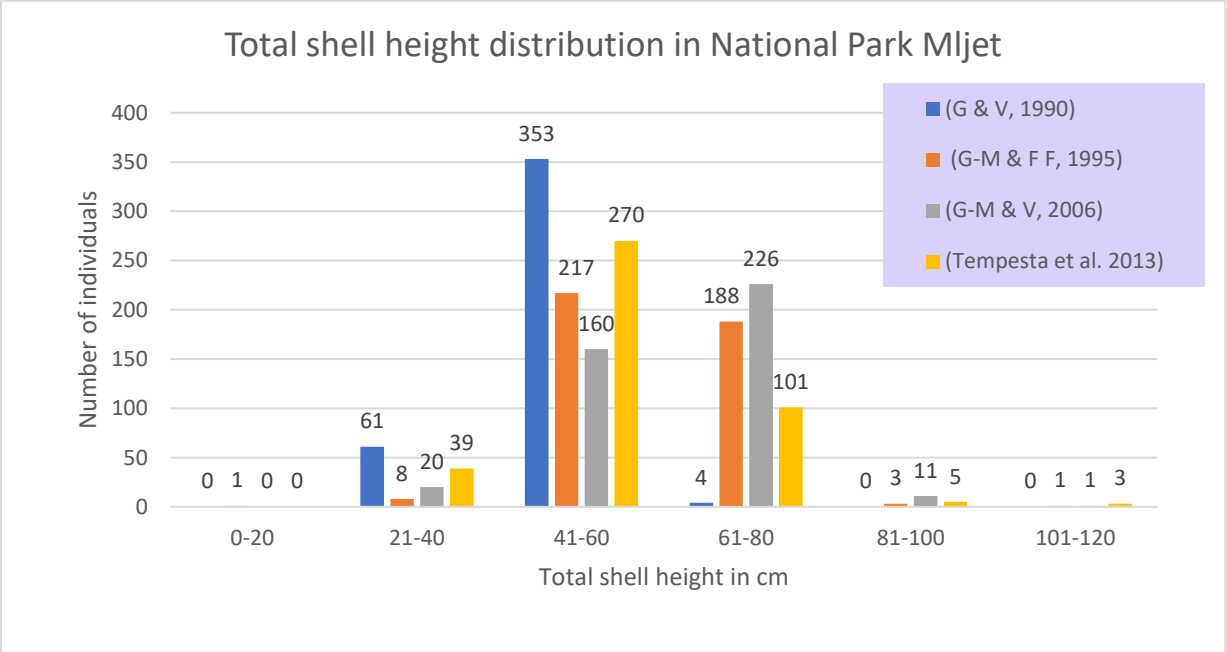


Figure 17: The total shell height (HT) distribution of Pinna nobilis in National Park Mljet. Each of 4 different method for HT calculation is presented with distinct colour.

4.3 Genetical analysis with COI and 16S markers

Overall, the COI-16S dataset showed to be the most variable among the three analysed. We found 52 polymorphic sites (S), which resulted in 54 different haplotypes. The total mean haplotype and nucleotide diversity were h = 0.954 and π = 0.005, respectively. A general trend of high genetic diversity was found for all the populations analysed with the lowest values (h = 0.797, and π = 0.003) reported for the Greek population from Aegean Sea (see Table 6 for details). Similar patterns of high genetic variability were evidenced for both COI and 16S datasets.

*Table 6: Estimates of genetic diversity obtained for each Pinna nobilis population for the three datasets considered (COI, 16S, COI-16S). Sample sizes (N), number of polymorphic sites (S), number of haplotypes (H), haplotype diversity (h), and nucleotide diversity (π). Populations are labelled in Table 5**Pogreška! Izvor reference nije pronađen.**.*

Geographic area	Code	N	S			H			*h*			*π*		
			COI	16S	COI-16S	COI	16S	COI-16S	COI	16S	COI-16S	COI	16S	COI-16S
Sardinia Italy	OSM	21	9	3	12	8	4	10	0.829	0.271	0.871	0.006	0.001	0.003
	MAD	17	11	6	17	10	5	14	0.895	0.549	0.961	0.007	0.002	0.004
Veneto Italy	VEN	15	10	5	15	10	5	12	0.895	0.368	0.921	0.006	0.002	0.004
Thessaloniki Greece	EPAG	18	10	0	13	7	1	8	0.752	0.000	0.797	0.005	0.000	0.003
Telašćica Croatia	TEL	14	9	4	12	8	5	10	0.890	0.505	0.934	0.006	0.002	0.003
Mljet Croatia	MJL	18	5	11	16	5	7	11	0.752	0.608	0.908	0.005	0.004	0.005
TOTAL		109	28	24	52	32	20	54	0.904	0.729	0.954	0.008	0.003	0.005

Hereafter, in order to make inferences on the most variable fragment, we choose to use the COI-16S dataset only to carry out further statistical analysis.

Pairwise Φ_{ST} values (Table 7) were significantly different from zero ($P < 0.05$) in 9 out of 15 comparisons. In particular, both the population of the Venetian Lagoon and the population from Aegean Sea significantly diverged from the other populations in 100% of the comparisons. The remaining populations from Sardinia and Croatia did not diverge among each other showing a general trend of genetic homogeneity.

Table 7: COI-16S dataset. Pairwise ΦST values between Pinna nobilis populations. Significance was assessed by a permutation test with 10,000 replicates. Probability values are reported in bold. Populations are labelled in Table 6.

	OSM	MAD	VEN	EPAG	TEL	MLJ
OSM	-	**0.35135±0.0394**	**0.00000±0.0000**	**0.00000±0.0000**	**0.06306±0.0237**	**0.12613±0.0337**
MAD	0.00139	-	**0.00000±0.0000**	**0.00000±0.0000**	**0.45946±0.0515**	**0.54955±0.0286**
VEN	0.33883	0.35333	-	**0.00000±0.0000**	**0.00000±0.0000**	**0.00000±0.0000**
EPAG	0.49178	0.42671	0.62635	-	**0.00000±0.0000**	**0.00000±0.0000**
TEL	0.05427	-0.00473	0.43904	0.45368	-	**0.18018±0.0429**
MLJ	0.02967	-0.00627	0.34076	0.41266	0.03053	-

The Bayesian analysis identified four distinct groups of haplotypes (see Figure 18). Overall, the two most widespread groups (red and blue bands in the Figure 18), spanning all the populations considered in this study except the Greek population from Aegean Sea, did not display a clear geographic structuring. The third most common group was exclusive to Aegean Sea individuals (green bands in the Figure 18). Finally, the group showing the lowest average frequency (yellow bands in the Figure 18) of distribution was restricted to only few individuals from the Croatian population of Mljet.

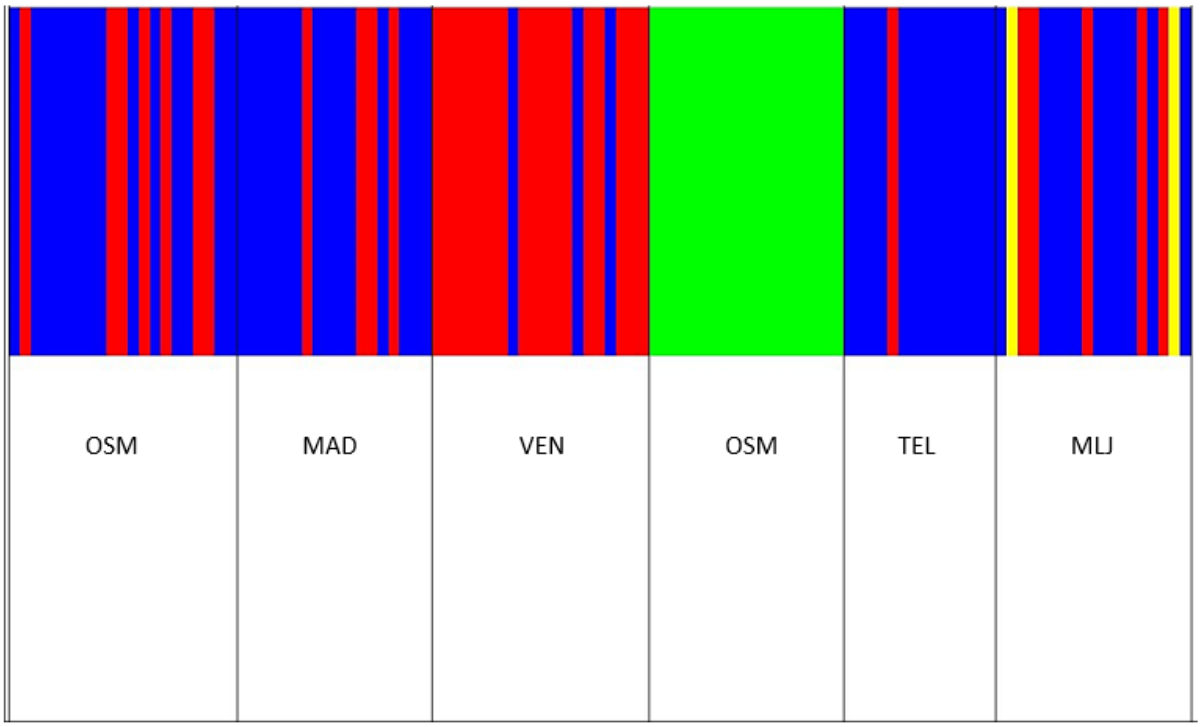


Figure 18: COI-16S dataset. Bayesian model-based clustering analysis of genetic structure among Pinna nobilis populations. Each individual is represented in the bar plot by a thin vertical line; the width of each coloured segment is proportional to the number of individuals. Populations are labelled as in Table 5.

The Median-Joining network analysis revealed a diffused star-shaped phylogeny (Figure 19) with three central most common haplotypes for populations from Sardinia and Croatia, two for the Venetian population and two for the Aegean population. Many derived haplotypes generally diverged from the most common ones by one or two point mutations. The only exceptions were represented by three individuals from Croatia (Mljet population) which diverged from the central haplotype by five and eight points mutations respectively. Overall, a high level of haplotype sharing among the Sardinian and Croatian individuals was pointed out, without strong evidence of geographic structuring among regions. On the contrary, either individuals from the Aegean Sea and individuals from Venetian lagoon presented only private owned haplotypes and did not share sequences with other populations. Interestingly, one specimen from the Greek population highly diverged from the other individuals from Aegean Sea being nested five point mutations apart from a Croatian specimen (Telašćica).

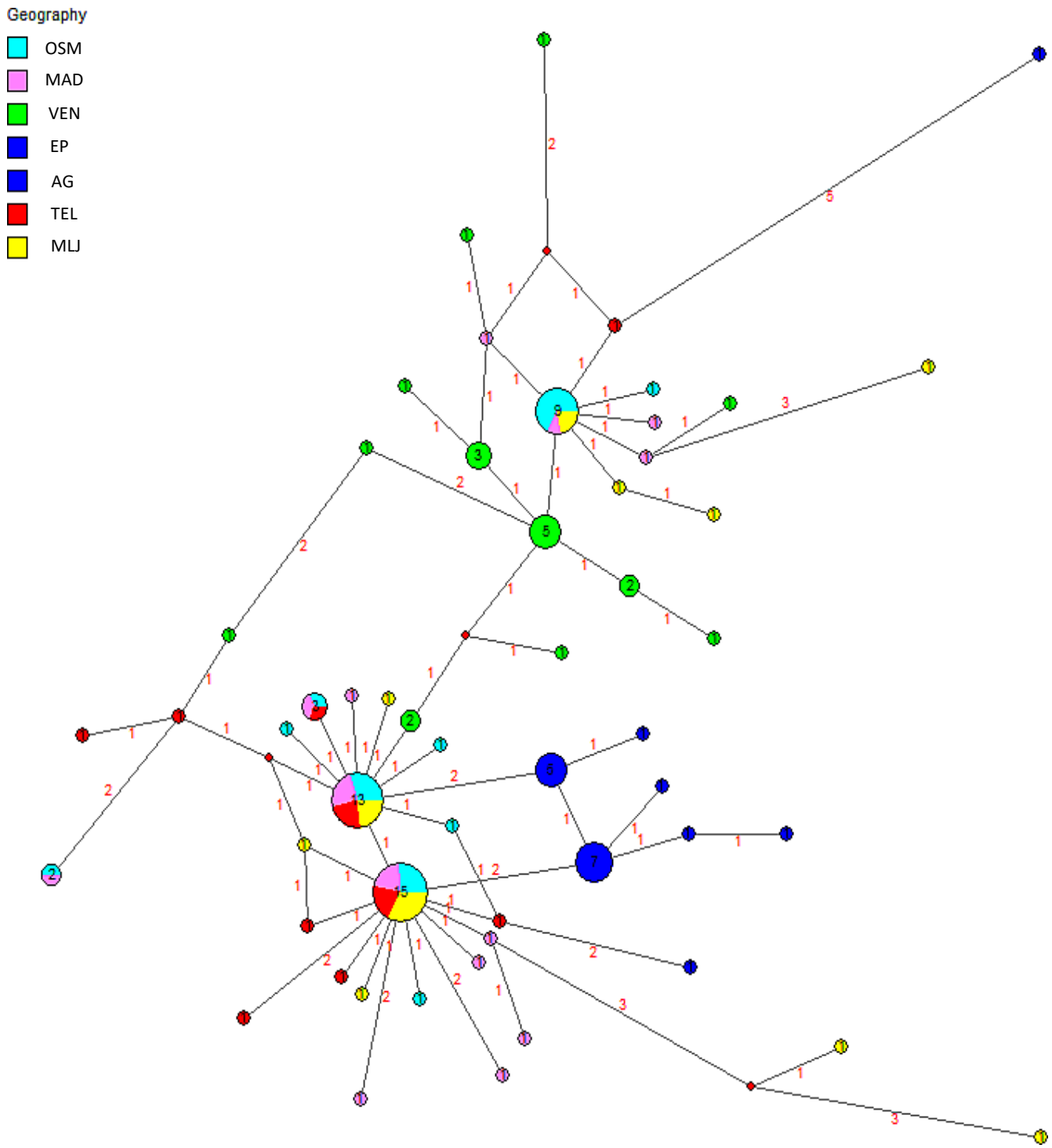


Figure 19: COI-16S dataset. Median-Joining network showing the haplotypes relationships among Pinna nobilis populations. Numbers on the branches of the graphic represent the number of point mutations occurring between two haplotypes. The numbers inside the spot represent the number of individuals sharing the same haplotypes. Little black spots on the graphic represent the median joining vectors. Populations are labelled as in Table 5.

5 Discussion

5.1 Population density

The mean observed density of *P. nobilis* in Adriatic Sea are recorded lower when compared to those referenced in literature. Recorded mean density of Telašćica population (2.39 ± 2.08 ind 100 m^{-2}) and Mljet population (7.55 ± 6.25 ind 100 m^{-2}) are lower compared to studies conducted in Adriatic Sea, where typical population density is about 11.30 ± 2.17 ind 100 m^{-2} (Basso *et al.*, 2015), or 10 ind 100 m^{-2} (De Gaulejac and Vicente, 1990; Butler, Vicente and de Gaulejac, 1993) whilst the typical densities of *P. nobilis* populations in other Mediterranean areas is 1 ind 100 m^{-2} (Centoducati *et al.*, 2007). However the highest mean density on transects 10 in Mljet (24.19 ind 100 m^{-2}) is the highest density recorded in Adriatic Sea so far (except density of 1200 ind 100 m^{-2} noted by Russo (2012), but where measurement method was not reported).

Since there are no literature data of population structure of *P. nobilis* in Nature Park Telašćica, our results suggest that population density in Telašćica is higher than stated for Mediterranean, but lower in comparison of populations found in same substratum (mean density of *P. nobilis* observed in Posidonia oceanica bed among Mediterranean is 8.06 ± 2.35 ind 100 m^{-2} (Basso *et al.*, 2015)).

In Malo Jezero in National Park Mljet, the mean density of *P. nobilis* in the *Cymodocea nodosa* meadows appears to be lower comparing the result of 20 ind 100 m^{-2} from Siletic and Peharda (2003), but similar to average density recorded of 11.06 ± 1.82 ind 100 m^{-2} for *C. nodosa* meadows among Mediterranean (Basso *et al.*, 2015).

Comparison of noble pen mussel densities among different areas in the Mediterranean is problematical, because very different sampling designs and field protocols have been applied. Some of the reported densities do not seem to be representative of a wider region, as they are based on a small number of plots that were not placed randomly in a wider study area, but instead high-density patches were selected for sampling (e.g. Siletic and Peharda, 2003; Galinou-Mitsoudi, Vlahavas and Papoutsi, 2006) (Rabaoui, Tlig-Zouari, *et al.*, 2011). If a comparison is made with other studies using circle sampling, it can be noted that in Malta average density in the sampled stations ranges from 0 to 5.1 ind 100 m^{-2} (García-March and Vicente, 2006) while in Miramare MPA (Trieste, Italy) in 2011 was recorded 13.57 ind 100 m^{-2} (Tempesta, Del Piero and Ciriaco, 2013).

It is not possible to clearly explain the causes of such significant differences in *P. nobilis* densities between Telašćica and Mljet. Since the first record of the substantial population of *P. nobilis* in Malo jezero in Mljet (Orepić *et al.*, 1997), until today has not been found other area as abundant as stated on east coast of Adriatic Sea. It is known that the ecological characteristics of the lake Malo Jezero in Mljet are strongly influenced by the surrounding area and by restricted communication with

open sea (Siletic and Peharda, 2003). Benović *et al.* (2000) reported very high zooplankton densities in Malo Jezero, especially during spring and summer. The factors previously mentioned indicate to potentially more food sources for *P. nobilis* in Malo Jezero, as well as restricted communication with open sea over lake Veliko Jezero act upon notably abundant population of *P. nobilis* in Malo Jezero in Mljet.

Although the population size of *P. nobilis* in Telašćica is currently sizable, there are no data to compare with. Regarding Mljet population, it is noticeable decrease in density along transects reported in Siletic and Peharda (2003) (from 2 to 6 ind 100 m^{-2} in two years depending on transects), likewise decrease average density reported in 2003 and in our study. Reduction in average density of *P. nobilis* is also found in Miramare MPA in north Adriatic Sea; Tempesta, Del Piero and Ciriaco (2013) recorded in 2008 20.84 ind 100 m^{-2}, in 2009 18.88 ind 100 m^{-2}, in 2010 15.69 ind 100 m^{-2} and in 2011 13.57 ind 100 m^{-2}.

Percentage of recorded dead individuals are lower than 78.46 % found in Mar Grande (Ionian sea) (Centoducati *et al.*, 2007) or 44.4 % in Moraira (Spain) (García-March *et al.*, 2007), but mortality in Telašćica is higher than reported 14 % in Gulf of Oristano (Coppa *et al.*, 2010). Even though mortality data in our study shows marked differences in dead individuals between Telašćica and Mljet population, we suppose that factors like natural mortality caused by aging, predation or disease are considered as a main cause for the pinnids distinction in our study. Higher abundance of dead individuals in Telašćica population can be explained with several factors: transects in Telašćica were on greater depth in open sea where possibility of predator attack (*Octopus vulgaris* and *Sparus aurata*) or transmission of disease are tremendous than in isolated marine lake as Malo Jezero. Further on, there was no sign of broken pinnids shells on the transects that would result from other human activities such as anchor damage. Considering that our study did not include neither total shell height of dead individual, not its depth, we have no assumption how hydrodynamic stress has influence on mortality rate in Telašćica and Mljet. Furthermore, illegal removal is noticeable in repeated studies of the same area (confirming decline of population without discovery of dead empty shell left behind by natural mortality), thus we cannot confirm illegal removal.

5.2 Morphology

Size population structure showed a unimodal distribution which is more often in the Mediterranean Sea (Siletic and Peharda, 2003; García-March *et al.*, 2007) than bimodal (Coppa *et al.*, 2010) or plurimodal one (Katsanevakis, 2007b). Hendriks *et al.* (2012) reported that the average growth rate for pinna juveniles is of 0.28 mm / day. Richardson *et al.* (1999) noted that *P. nobilis* growth rates vary by location: the average size of noble pen shells seems to vary eventually according to the availability of zooplankton. Growth analyses conducted in Spanish Mediterranean areas showed, for

example, at Carboneras the height of 30 cm corresponds to an age of two years, while at Aguamarga and Rodalquilar a two-years old *P. nobilis* has a total shell height of 20 cm (Richardson *et al.*, 2004). Another examples from literature are pinnids from Mali Ston bay, exhibited the faster growth reaching a length of 60 cm and an age of 9 years, whereas those from Malo jezero that grew slower, were older (12 years) and attained a length of 50 cm (Richardson *et al.*, 2004). According to literature, 20 cm of HT is the threshold under which individuals should be considered juveniles (Butler, Vicente and de Gaulejac, 1993; Richardson *et al.*, 1999; Siletic and Peharda, 2003), and annual growth reaches an average of 10 cm $year^{-1}$ (Richardson *et al.*, 1999; Siletic and Peharda, 2003). Classes are divided as mentioned above in results. According to the frequency of height distribution, the noble pen shell population in Telašćica mainly consists of old individuals and adults while Mljet population was characterized by more adults than old individuals. Comparing data of Mljet population to previous research done by Siletic and Peharda (2003), height frequency distribution data are changed, since previous survey mainly recorded juveniles and adult juveniles. In both surveyed populations, there were only one juvenile by population, corresponding to 1-2 years old. The investigated areas were characterized by mature population mainly composed of large size specimens that could be from 8 years (Siletic and Peharda, 2003) to 12 (Richardson *et al.*, 2004) or 15 (Siletic and Peharda, 2003) years old. Moreover, the dimension class 20 – 40 cm, which is characterized by 2-8 years old individuals (Richardson *et al.*, 1999) in both populations is represent with relative small number (<20). Even smaller abundance is found in group of extremely old individuals (> 80 cm) which could have more than 20 years old.

Since there are four different equations available in literature for HT calculation and there is no comparison of differences among their results, we tested them all on both populations (Figure 16 and Figure 17). Equation developed by De Gaulejac and Vicente (1990) is giving much vary and questionable results. This could be since it was base for correction and development of new one by García-March and Ferrer Ferrer (1995). Equations from 1995 and 2006, where the same author was involved in development (García-March and Ferrer Ferrer, 1995; García-March and Vicente, 2006), are giving nearly same results. Considering that both are using two biometric data of individual for its HT analysis and differences between results are negligible, it could be the most accurate equations for use in Mediterranean. Equation establish by Tempesta, Del Piero and Ciriaco (2013) is giving dissimilar results in calculated HT, given that it was developed and modified from García-March and Ferrer Ferrer (1995) specifically for population of *Pinna nobilis* in Miramare MPA (Trieste, Italy).

5.3 Genetic data

The population genetic structure of the endangered mollusk *Pinna nobilis* is studied for the first time along eastern shores of Mediterranean Sea (Croatian coast) based on mitochondrial COI and 16S markers. A general trend of high genetic variability is evidenced for both the two mitochondrial markers, although 16S gene showed levels of variability slightly lower than what evidenced for COI gene. This could be attributed to the fact that 16S rDNA gene is a relatively conserved gene, used mainly as an interspecific marker at genus and family level (Saavedra and Peña, 2004). The combined use of these two mitochondrial markers allowed to obtain a longer and more variable fragment to be analysed by merging the two datasets.

Genetic diversity for all populations is generally high in all three different datasets of sequences (COI, 16S, COI-16S). Telašćica population shows higher variability than Mljet population from marine lake Malo Jezero, such a finding could be explained by geographical isolation due to the specific geomorphologic and hydrodynamic conditions of this habitat area. Given that water exchange with open sea is restricted in Mljet, few highly divergent haplotypes were found in this population as a possible footprint of an ancient Mediterranean population. However, the present data do not allow us to rule out the influence of human activities: e.g., passive transport of seed by ballast waters - the latter being a common finding in many marine mollusc invasions, can circumvent geographic barriers (see, e.g., Carlton and Hodder (1995), Apte *et al.* (2000), Gollasch (2007), Johnson and Carlton (2010)).

Another possible explanation is hiding in the dispersal potency of pinnids larva. After spawning and external fertilization, developing larvae spend a variable period of time as part of the plankton, passively drifted by water currents, often over considerable distances (Cho *et al.*, 2007) influencing on gene flow between areas and populations. Given the number of sampled and analysed populations in the Mediterranean, there is a possibility that the Mljet population shares the haplotypes with some unanalysed population from the eastern Mediterranean basin. Although the genetics of marine populations with pelagic larval development has often been characterized by low genetic differentiation among populations (Rivera, Kelley and Roderick, 2004), a pattern driven by high dispersal capabilities and large scale maritime mixing could explain moderate genetic variability of *Pinna nobilis* among Mediterranean Sea.

High levels of haplotype sharing and no evidence of genetic structuring between Sardinia and Croatia populations suggests the occurrence of a constant genetic flow between western Mediterranean and Southern Adriatic Sea, likely mediated by an efficient larval dispersal, thus supporting for *Pinna nobilis* the moving eastward of the genetic boundary between west and east Mediterranean basins as it was already proposed by Sanna *et al.* (2013). Hence, hydrodynamic activities that are related to biogeographic boundaries among Mediterranean sectors, can be invoked

to explain the pattern of genetic structuring shown in this study. In the Mediterranean region, several authors have documented population subdivision in relation to physical barriers in other marine species with a high dispersal potential (Magoulas, Tsimenides and Zouros, 1996; Viñas, Alvarado Bremer and Pla, 2004).

Sanna *et al.* (2013), based on a larger number of individuals and sites, pointing out higher levels of variability, showed the occurrence of three well-defined groups of populations, corresponding to three large Mediterranean main biogeographic sectors (western Mediterranean, Adriatic, and eastern Mediterranean). The genetic structuring evidenced between Venetian and Croatian populations within Adriatic Sea in this study, suggests the possible presence of at least two main mitochondrial lineages (groups of haplotypes) consistent with the occurrence in this basin of an ancient genetic background, represented by Venetian populations, which was likely replaced in the southern Adriatic by a more recent geneflow coming from Western Mediterranean. However, considering that the Venetian lagoon population also diverged from all the other populations analysed, it cannot be ruled out that the genetic settlement of this population may represent the consequence of the geographic isolation of the lagoon mainly involved in its peculiar environmental conditions.

The genetic divergence, also evidenced for Aegean populations from all the other Mediterranean populations analysed from Western basin and Adriatic Sea, is consistent with previous findings of Sanna *et al.* (2013) further excluding a possible genetic relationship between Aegean Sea and Southern Adriatic Sea. Such evidence may be explained considering the peculiar environmental condition of the enclosed Aegean Sea basin.

Network analysis suggested the occurrence of at least three main founder effects among Sardinian and Croatian populations, two within Venetian population and two within Greek population. Such evidence may be explained in the light of the sever past overexploitation of the species in the Mediterranean (Sanna et al. 2013) likely followed by a recent population expansion as suggested by the high number of haplotypes private to a single individual diverging from the ancestral ones for few point mutations.

This study indicates that reapplying previously developed molecular analysis on a larger number of populations in no yet surveyed area could provide a deeper insight into the factors acting on population dynamics of this endangered species, allowing the development of effective and proper conservation measures. Results obtained support the occurrence of a large Mediterranean population spanning in western basin and Southern Adriatic Sea. Such evidence pointed out a new insight on this species evidencing a never described before connection between western Mediterranean and Southern Adriatic Sea. In the future, further sampling campaigns in Adriatic and Ionian seas will be

helpful to better understand for *Pinna nobilis*, the connection between Adriatic Sea and the other Mediterranean basins.

6 Conclusion

In both protected areas, in the Nature Park Telašćica and in the National Park Mljet, the mean population densities were higher than average found in Mediterranean but lower compared to past studies conducted in Adriatic Sea. In National Park Mljet we recorded highest density ever recorded among Croatian coast.

We noted statistically significant difference between populations density, as well as statistically significant differences between total shell height calculated for both populations. Mljet population is denser while Telašćica population has higher total shell height.

In Telašćica we found population structure mainly consists of old individuals and adults while Mljet population has more adults than old individuals, possible from 8 to 15 years old. In both surveyed populations, there is lack of juvenile.

In comparison of four different equations for total shell height, we suggested that those provided by García-March and Ferrer Ferrer (1995) and García-March and Vicente (2006) are giving similar results.

Genetic analyses highlight the occurrence of a never described geneflow coming from western Mediterranean toward southern Adriatic Sea and confirm that Mediterranean populations may have experienced sever past overexploitations likely followed by a recent population expansion.

The genetic structuring evidenced between Venetian and Croatian populations within Adriatic Sea is consistent with the spread in the Adriatic of highly divergent mitochondrial great lineages involved in different population dynamics.

As well as, the genetic structuring occurring between southern Adriatic Sea and Aegean Sea provide support to exclude the possible genetic relationship between Aegean and Adriatic population.

However, only future studies on a larger number of samples from a higher number of Mediterranean sampling sites will shed further light of the population genetics of *Pinna nobilis,* thus supporting or improving the results obtained in the present study. Furthermore, a deeper scientific knowledge and a long-term monitoring study will be necessary in order to improve conservation program aiming to preserve and maintain this endangered species.

7 References

Apte, S. *et al.* (2000) 'Jumping ship: A stepping stone event mediating transfer of a non-indigenous species via a potentially unsuitable environment', *Biological Invasions*, 2(1), pp. 75–79. doi: 10.1023/A:1010024818644.

Basso, L. *et al.* (2015) *The Pen Shell, Pinna nobilis: A Review of Population Status and Recommended Research Priorities in the Mediterranean Sea*, *Advances in Marine Biology*. doi: 10.1016/bs.amb.2015.06.002.

Beer, S. (2001) 'Seagrass Ecology', in *Aquatic Botany*. Cambridge: Cambridge University Press., pp. 79–80. doi: 10.1016/S0304-3770(01)00171-1.

Benović, A. *et al.* (2000) 'Ecological characteristics of the Mljet Island seawater lakes (South Adriatic Sea) with special reference to their resident populations of medusae', *Scientia Marina*, 64(S1), pp. 197–206. doi: 10.3989/scimar.2000.64s1197.

Bensasson, D. *et al.* (2001) 'Mitochondrial pseudogenes: Evolution's misplaced witnesses', *Trends in Ecology and Evolution*, 16(6), pp. 314–321. doi: 10.1016/S0169-5347(01)02151-6.

Bull, J. J. *et al.* (1993) 'Partitioning and Combining Data in Phylogenetic Analysis', *Systematic Biology*, 42(3), pp. 384–397. doi: 10.1093/sysbio/42.3.384.

Butler, A., Vicente, N. and de Gaulejac, B. (1993) 'Ecology of the pterioid bivalves *Pinna bicolor* Gmelin and *Pinna nobilis* L.', *Marine Life*, pp. 37–45.

Canapa, A. *et al.* (1996) 'Phylogenetic analysis of Veneridae (Bivalvia): comparison of molecular and palaeontological data.', *Journal of molecular evolution*, 43(5), pp. 517–522. doi: 10.1007/BF02337522.

Canapa, A. *et al.* (2000) 'Molecular Data from the 16S rRNA Gene for the Phylogeny of Pectinidae (Mollusca: Bivalvia)', *Journal of Molecular Evolution*, 50(1), pp. 93–97. doi: 10.1007/s002399910010.

Carlton, T. J. and Hodder., J. (1995) 'Biogeography and dispersal of coastal marine organisms: experimental studies on a replica of a 16th-century sailing vessel. Marine Biology 121(4):721-730. [*Archidoris montereyensis*, *Cuthona albocrusta*, *Dendronotus frondosus*, *Flabellina trilineata*, Onchido', *Marine Biology*, 121, pp. 721–730. Available at: http://www.springerlink.com/content/r4184477053x84g2/fulltext.pdf.

Catsiki, V. A. and Katsilieri, C. H. (1992) 'Presence of chromium in *P. nobilis* collected from a polluted area', *Fresenius Environmental Bulletin*, 1(10), pp. 644–649.

Centoducati, G. *et al.* (2007) 'Monitoring of the endangered *Pinna nobilis* Linné, 1758 in the Mar Grande of Taranto (Ionian Sea, Italy)', *Environmental Monitoring and Assessment*, 131(1–3), pp. 339–347. doi: 10.1007/s10661-006-9479-z.

Cerruti, A. (1938) 'Primi esperimenti di allevamento della Pinna "*Pinna nobilis* L." nel Mar Piccolo di Taranto', *La Ricerca Scientifica*, I(XVI), pp. 339–347.

Chippindale, P. T. and Wiens, J. (1994) 'Weighting, partitioning, and combinig characters in phylogenetic analysis', *Systematic Biology*, 43(2), pp. 278–287.

Cho, E. S. *et al.* (2007) 'Population genetic structure of the ark shell *Scapharca broughtonii* Schrenck from Korea, China, and Russia based on COI gene sequences', *Marine Biotechnology*, 9(2), pp. 203–216. doi: 10.1007/s10126-006-6057-x.

Coppa, S. *et al.* (2010) 'Density and distribution patterns of the endangered species *Pinna nobilis* within a *Posidonia oceanica* meadow in the Gulf of Oristano (Italy)', *Journal of the Marine Biological Association of the United Kingdom*, 90(5), pp. 885–894. doi: 10.1017/S002531540999141X.

Corander, J. *et al.* (2013) 'BAPS: Bayesian analysis of population structure'. Department of Mathematics and statistics. University of Helsinki, Finland. Available at: http://www.helsinki.fi/bsg/software/BAPS/macSnow/BAPS6manual.pdf.

Cunningham, C. W. (1997) 'Is Congruence between Data Partitions a Reliable Predictor of Phylogenetic Accuracy? Empirically Testing an Iterative Procedure for Choosing among Phylogenetic Methods', *Systematic Biology*, 46(3), pp. 464–478. doi: 10.1093/sysbio/46.3.464.

Czihag, G. and Dierl, W. (1961) '*Pinna nobilis* L eine prëparationsanleitung', *Gustav Fisher Verlag, Stuttgart*, p. 44.

Draganović, E. (1980) *Litoralne biocenoze Mljetskih jezera i problemi njihove zaštite*. University of Zagreb.

Escudero, A., Iriondo, J. M. and Torres, M. E. (2003) 'Spatial analysis of genetic diversity as a tool for plant conservation', *Plant Conservation Biology: Emerging Tools and Strategies*, 113(3), pp. 351–365. doi: DOI: 10.1016/S0006-3207(03)00122-8.

Excoffier, L. and Lischer, H. E. L. (2010) 'A new sereis of programs to perform population genetic analyses under Linux and Windows', *Molecular Ecology Resources*, 10(3), pp. 564–567. doi: 10. 1111/j. 1755-0998. 2010. 02847. x.

Farias, I. P., Orti, G. and Meyer, A. (2000) 'Total evidence: molecules, morphology, and the

phylogenetoics of Cichlid fishes', *Jurnal of Experimental Zoology (Mol.Dev.Evol.)*, 288, pp. 76–92.

Farris, J. S. *et al.* (1995) 'Constructing a significant test for incongruence', *Systematic Biology*, 44(4), pp. 570–572.

Fiorito, G. and Gherardi, F. (1999) 'Prey-handling behaviour of *Octopus vulgaris* (Mollusca, Cephalopoda) on Bivalve preys', *Behavioural Processes*, 46(1), pp. 75–88. doi: 10.1016/S0376-6357(99)00020-0.

Frankham, R. (2007) 'Efective population size/adult population size ratios in wildlife: a review', *Genetical Research*, 89(5–6), p. 491. doi: 10.1017/S0016672308009695.

Galinou-Mitsoudi, S., Vlahavas, G. and Papoutsi, O. (2006) 'Population study of the protected bivalve *Pinna nobilis* (Linnaeus, 1758) in Thermaikos Gulf (north Aegean Sea)', *Journal of Biological Research (Thessaloniki)*, 5(May 2004), pp. 47–53.

García-March, J. R. (2003) 'Contribution to the knowledge of the status of *Pinna nobilis* (L.) 1758 in Spanish coasts', *First International Meeting on the Mediterranean Fan-shell Pinna nobilis, 10-12 October 2002, Ile des Embiez, Var, France.*, (January), pp. 29–41.

García-March, J. R. (2005) 'Aportaciones al conocimiento de la biología de *Pinna Nobilis* Linneo, 1758 (Mollusca bivalvia) en el litoral mediterráneo ibérico.', 1758, p. 303. Available at: http://dialnet.unirioja.es/servlet/tesis?codigo=7131&orden=0&info=link.

García-March, J. R. *et al.* (2007) 'Population structure, mortality and growth of *Pinna nobilis* Linnaeus, 1758 (Mollusca, Bivalvia) at different depths in Moraira bay (Alicante, Western Mediterranean)', *Marine Biology*, 150(5), pp. 861–871. doi: 10.1007/s00227-006-0386-1.

García-March, J. R. and Ferrer Ferrer, J. (1995) 'Biometría de *Pinna nobilis* L., 1758: una revisión de la ecuación de De Gaulejac y Vicente (1990)', *Bol. Inst. Esp. Oceanogr*, 11(June), pp. 175–181.

García-March, J. R., García-Carrascosa, A. M. and Pena, A. L. (2002) 'In Situ Measurement of *Pinna nobilis* Shells for Age and Growth Studies : A New Device', *Marine Ecology*, 23(3), pp. 207–218. doi: 10.1046/j.1439-0485.2002.02781.x.

García-March, J. R. and Márquez-Aliaga, A. (2006) 'Polymorphism and shell reshaping in *Pinna nobilis* L., 1758: the reliability of shell dimensions for ontogenetic age and population growth rate estimates [abst.]. In N. Malchus and J. M. Pons, eds., Bivalvia', *Organisms Diversity & Evolution*, 6.

García-March, J. R. and Vicente, N. (2006) *Protocol to study and monitor Pinna nobilis populations*.

De Gaulejac, B. (1993) 'Ecophysiological study of the Mediterranean bivalve mollusc *Pinna nobilis* L.

Reproduction, Growth, Respiration', *France Univ. Aix*.

De Gaulejac, B., Henry, M. and Vicente, N. (1995) 'An ultrastructural study of gametogenesis of the marine bivalve *Pinna nobilis* (Linnaeus 1758)II. Spermatogenesis', *Journal of Molluscan Studies*, 61(3), pp. 393–403. doi: 10.1093/mollus/61.3.393.

De Gaulejac, B. and Vicente, N. (1990) 'Ecologie de *Pinna nobilis*(L.) mollusque bivalve sur les côtes de Corse. Essais de transplantation et expériences en milieu contrôlé. Grand forum de la malacologie et Symposium int. d'écophysiologie des mollusques. Société française de Malacologie. Île des', *Haliotis*, 20, p. 83.

Gollasch, S. (2007) 'Is Ballast Water a Major Dispersal Mechanism for Marine Organisms ?', *Biological Invasions - Ecological Studies Vol. 193*, 193, pp. 49–57. doi: 10.1007/978-3-540-36920-2.

González-Wangüemert, M. *et al.* (2015) 'Highly polymorphic microsatellite markers for the Mediterranean endemic fan mussel *Pinna nobilis*', *Mediterranean Marine Science*, 16(1), pp. 31–35. doi: 10.12681/mms.949.

Greenwald, M. (1996) *The cruising chef cookbook*, *Paradise Cay publication*.

Habdija, I. *et al.* (2011) *Protista-Protozoa i Metazoa-Invertebrata: Strukture i funkcije*. Alfa d.d.

Hall, T. (1999) 'BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT', *Nucleic Acids Symposium Series*, 41, pp. 95–98. doi: citeulike-article-id:691774.

Hazkani-Covo, E. and Graur, D. (2007) 'A comparative analysis of numt evolution in human and chimpanzee', *Molecular Biology and Evolution*, 24(1), pp. 13–18. doi: 10.1093/molbev/msl149.

Hendriks, I. E. *et al.* (2012) 'Relative Growth Rates of the Noble Pen Shell *Pinna nobilis* Throughout Ontogeny Around the Balearic Islands (Western Mediterranean, Spain)', *Journal of Shellfish Research*, 31(3), pp. 749–756. doi: 10.2983/035.031.0319.

Johnson, L. E. and Carlton, J. T. (2010) 'Post-Establishment Spread in Large-Scale Invasions : Dispersal Mechanisms of the Zebra Mussel *Dreissena Polymorpha* Published by : Ecological Society of America POST-ESTABLISHMENT SPREAD IN LARGE-SCALE INVASIONS : DISPERSAL MECHANISMS OF THE ZEBRA MUSSEL', *Ecology*, 77(6), pp. 1686–1690.

Juračić, M. *et al.* (1995) 'Sedimenti i sedimentacija u mljetskim jezerima. In: Durbešić P, Benović A (eds) Prirodne značajke i društvena valorizacija otoka Mljeta, Hrvatsko ekološko društvo etc.', *Ekološke monografije*, 6, pp. 107–116.

Katsanevakis, S. (2005) 'Population ecology of the endangered fan mussel *Pinna nobilis* in a marine

lake', *Endangered species research*, 1, pp. 1–9.

Katsanevakis, S. (2007a) 'Density surface modelling with line transect sampling as a tool for abundance estimation of marine benthic species: The *Pinna nobilis* example in a marine lake', *Marine Biology*, 152(1), pp. 77–85. doi: 10.1007/s00227-007-0659-3.

Katsanevakis, S. (2007b) 'Growth and mortality rates of the fan mussel *Pinna nobilis* in Lake Vouliagmeni (Korinthiakos Gulf, Greece): A generalized additive modelling approach', *Marine Biology*, 152(6), pp. 1319–1331. doi: 10.1007/s00227-007-0781-2.

Katsanevakis, S. *et al.* (2011) '"Protected" marine shelled molluscs: Thriving in Greek seafood restaurants', *Mediterranean Marine Science*, 12(2), pp. 429–438. doi: 10.12681/mms.42.

Katsares, V. *et al.* (2008) 'Genetic structure of the endangered species *Pinna nobilis* (Mollusca: Bivalvia) inferred from mtDNA sequences', *Biologia*, 63(3), pp. 412–417. doi: 10.2478/s11756-008-0061-8.

Knutsen, H. *et al.* (2003) 'Fine-scaled geographical population stucturing in a highly mobile marine species: the Atlantic cod', *Molecular Ecology*, 12(2), pp. 385–394. doi: Doi 10.1046/J.1365-294x.2003.01750.X.

Kojima, S. *et al.* (1995) 'Phylogenetic relationships among species of *Calyptogena* (Bivalvia, Vesicomyidae) collected around Japan revealed by nucleotide sequences of mitochondrial genes', *Marine Biology*, 122(3), pp. 401–407.

Kojima, S. *et al.* (1997) 'Molecular phylogeny of vestimentiferans collected around Japan,revealed by the nucleotide sequences of mitochondrial DNA', *Marine Biology*, 127, pp. 1–7.

Kružić, P. (2007) 'Anthozoan Fauna of Telascica Nature Park (Adriatic Sea , Croatia)', *Nat. Croat.*, 16(4), pp. 233–266.

Librado, P. and Rozas, J. (2009) 'DnaSP v5: A software for comprehensive analysis of DNA polymorphism data', *Bioinformatics*, 25(11), pp. 1451–1452. doi: 10.1093/bioinformatics/btp187.

MacDonald, D. and Barrett, P. (2008) *Guía de campo de los mamíferos de España y de Europa*, *Ediciones Omega S. A., Barcelona*. Available at: http://cataleg.udl.cat/record=b1248727~S11*cat.

Magaš, D. (1998) 'Nature Park "Telašćica" on Dugi Otok (Croatia) - some Aspects of Environmental Planning', *Geoadria*, 3(3), pp. 81–91. Available at: http://hrcak.srce.hr/file/14998.

Magoulas, A., Tsimenides, N. and Zouros, E. (1996) 'Mitochondrial DNA phylogeny and the reconstruction of the population history of a species: the case of the European anchovy (*Engraulis*

encrasicolus)', *Molecular Biology and Evolution*, 13(1), pp. 178–190. doi: 10.1093/oxfordjournals.molbev.a025554.

Marba, N., Díaz-Almela, E. and Duarte, C. M. (2014) 'Mediterranean seagrass (*Posidonia oceanica*) loss between 1842 and 2009', *Biological Conservation*, 176, pp. 183–190. doi: 10.1016/j.biocon.2014.05.024.

Matsumoto, M. (2003) 'Phylogenetic analysis of the subclass Pteriomorphia (Bivalvia) from mtDNA COI sequences', *Molecular Phylogenetics and Evolution*, 27(3), pp. 429–440. doi: 10.1016/S1055-7903(03)00013-7.

Mihailinović, M. (1955) 'Orijentacioni podaci o brzini rastenja losture', *Morsko ribarstvo*, 7(9), p. 238.

Orepić, N. *et al.* (1997) 'A marine benthos survey in the lakes of the National park Mljet (Adriatic Sea)', *Periodicum Biologorum*, 99(2), pp. 229–245.

Passamonti, M. (2007) 'An unusual case of gender-associated mitochondrial DNA heteroplasmy: the mytilid *Musculista senhousia* (Mollusca Bivalvia)', *BMC Evolutionary Biology*, 7(Suppl 2), p. S7. doi: 10.1186/1471-2148-7-S2-S7.

Peharda, M. (2000) *Školjkaši (Bivalvia, Mollusca) Malog jezera otoka Mljeta*. University of Zagreb.

Peharda, M. *et al.* (2002) 'Spatial distribution of live and dead bivalves in saltwater lake Malo Jezero (Mljet National Park)', *Periodicum Biologorum*, 104(2), pp. 115–122.

Phelger F.B. (1969) 'Some general Features of Coastal lagoons', *Coastal Lagoon A symposium*, pp. 5–25.

Posada, D. (2008) 'jModelTest: Phylogenetic model averaging', *Molecular Biology and Evolution*, 25(7), pp. 1253–1256. doi: 10.1093/molbev/msn083.

Rabaoui, L., Tlig-Zouari, S., *et al.* (2011) 'Differences in absolute and relative growth between two shell forms of *Pinna nobilis* (Mollusca: Bivalvia) along the Tunisian coastline', *Journal of Sea Research*, 66(2), pp. 95–103. doi: 10.1016/j.seares.2011.05.002.

Rabaoui, L., Mejri, R., *et al.* (2011) 'Genetic variation among populations of the endangered fan mussel *Pinna nobilis* (Mollusca: Bivalvia) along the Tunisian coastline', *Hydrobiologia*, 678(1), pp. 99–111. doi: 10.1007/s10750-011-0827-9.

Ramos, M.-A. (1998) 'Implementing the Habitats Directive for Mollusca in Spain', *Journal of Conchology*, (2), pp. 125–132.

Rawson, P. D. and Hilbish, T. J. (1995) 'Evolutionary relationships among the male and female mitochondrial DNA lineages in the *Mytilus edulis* species complex.', *Molecular biology and evolution*, 12(5), pp. 893–901.

Reid, E. (1998) *Emery's Elements of Medical Genetics*, *Journal of medical genetics*. Elsevier Health Sciences. doi: 10.1136/jmg.35.9.792-a.

Richardson, C. A. *et al.* (1997) 'The occurrence of *Pontonia pinnophylax* (Decapoda: Natantia: Pontoniinae) in *Pinna nobilis* (Mollusca: Bivalvia:Pinnidae) from the Mediterranean', *Joural of the Marine Biological Association*, 77, pp. 1227–1230. doi: 10.1017/S0025315400038741.

Richardson, C. A. *et al.* (1999) 'Age and growth of the fan mussel *Pinna nobilis* from south-east Spanish Mediterranean seagrass (*Posidonia oceanica*) meadows', *Marine Biology*, 133(2), pp. 205–212. doi: 10.1007/s002270050459.

Richardson, C. A. *et al.* (2004) 'Age, growth rate and season of recruitment of *Pinna nobilis* (L) in the Croatian Adriatic determined from Mg:Ca and Sr:Ca shell profiles', *Journal of Experimental Marine Biology and Ecology*, 299(1), pp. 1–16. doi: 10.1016/j.jembe.2003.08.012.

Rivera, M. A. J., Kelley, C. D. and Roderick, G. K. (2004) 'Subtle population genetic structure in the Hawaiian grouper, *Epinephelus quernus* (Serranidae) as revealed by mitochondrial DNA analyses.', *Biological Journal of the Linnean Society*, 81(3), pp. 449–468. doi: 10.1111/j.1095-8312.2003.00304.x.

Russo, P. (2012) 'Segnalazione di una grande colonia di *Pinna nobilis* (Linnaeus, 1758) nella Laguna di Venezia', *Notiziario S.I.M.*, 30(1), pp. 31–34.

Saavedra, C. and Peña, J. B. (2004) 'Phylogenetic relationships of commercial European and Australasian king scallops (*Pecten spp.*) based on partial 16S ribosomal RNA gene sequences', *Aquaculture*, 235(1–4), pp. 153–166. doi: http://dx.doi.org/10.1016/S0044-8486(03)00442-3.

Saccone, C. *et al.* (1999) 'Evolutionary genomcis in Metazoa: the mitochondrial DNA as a model system', *Gene*, 238, pp. 195–209.

Sanna, D. *et al.* (2013) 'Mitochondrial DNA Reveals Genetic Structuring of *Pinna nobilis* across the Mediterranean Sea', *PLoS ONE*, 8(6), pp. 1–16. doi: 10.1371/journal.pone.0067372.

Sanna, D. *et al.* (2014) 'New mitochondrial and nuclear primers for the Mediterranean marine bivalve *Pinna nobilis*', *Mediterranean Marine Science*, 15(2), pp. 416–422. doi: 10.12681/mms.459.

Siletic, T. and Peharda, M. (2003) 'Population study of the fan shell *Pinna nobilis* L. in Malo and Veliko Jezero of the Mljet National Park (Adriatic Sea)', *Scientia Marina*, 67(1), pp. 91–98. doi:

10.3989/scimar.2003.67n191.

Sullivan, J. (1996) 'Combining data with different distributions of among-site rate variation', *Systematic Biology*, 45(3), pp. 375–380. doi: 10.2307/2413571.

Swofford, D. (2003) 'Phylogenetic analysis using parsimony', *Computer Program Distributed by Illinois National*. Sunderland, MA: Sinauer Associates. Available at: http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Phylogenetic+Analysis+Using+Parsimony+*#8.

Tempesta, M., Del Piero, D. and Ciriaco, S. (2013) 'Definition of a New Formula for the Calculation of the Total Height of the Fan Shell *Pinna Nobilis* in the Miramare Marine Protected Area (Trieste, Italy)', *Annales Series Historia Naturalis*, 23(1), pp. 17–24.

Templado, J. *et al.* (2004) *Guía de invertebrados y peces marinos protegidos por la legislación nacional e internacional*, *Oapn. Naturaleza Y Parques Nacionales. Serie Técnica*.

Vicente, N. (1990) 'Estudio ecológico y protección del molusco lamelibranquio *Pinna nobilis* L. 1758 en la costa mediterránea', *Iberus*, 9(1–2), pp. 269–279.

Viñas, J., Alvarado Bremer, J. and Pla, C. (2004) 'Phylogeography of the Atlantic bonito (*Sarda sarda*) in the northern Mediterranean: The combined effects of historical vicariance, population expansion, secondary invasion, and isolation by distance', *Molecular Phylogenetics and Evolution*, 33(1), pp. 32–42. doi: 10.1016/j.ympev.2004.04.009.

Wunsam, S., Schmidt, R. and Müller, J. (1999) 'Holocene lake development of two dalmatian lagoons (Malo and Veliko Jezero, Isle of Mljet) in respect to changes in Adriatic sea level and climate', *Palaeogeography, Palaeoclimatology, Palaeoecology*, 146(1–4), pp. 251–281. doi: 10.1016/S0031-0182(98)00147-3.

Yonge, C. M. (1953) 'Form and Habit in *Pinna carnea* Gmelin', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 237(648), pp. 335–374. doi: 10.1098/rstb.1953.0006.

Zavodnik, D. (1967) 'Contribution to the ecology of *Pinna nobilis* L. (Moll., Bivalvia) in the northern Adriatic.', *Thalassia Jugoslavica*, 3(1–6), pp. 93–103.

Zavodnik, D., Hrs-Brenko, M. and Legac, M. (1991) 'Synopsis on the fan shell *Pinna nobilis* L. in the eastern Adriatic Sea', *Les espèces marines à protéger en Méditerranée*, pp. 169–178.

Žerlić, T. (1999) *Školjkaši litoralnog područja Nacionalnog parka 'Mljet'*. University of Zagreb.

7.1 Web source

http://www.fluxus-engineering.com/, accessed 31.5.2017

http://www.macrogen.com/eng/, accessed 02.05.2017

Narodne novine. 1988. Zakon o proglašenju Parka prirode Telašćica. Narodne novine d.d. 14/88 (http://digured.srce.hr/arhiva/18/18255/www.hidra.hr/hidrarad/pobirac-upload/CD-1947-2000/024136.pdf, accessed 17.06.2017)

Narodne novine. 2013. Pravilnik o strogo zaštićenim vrstama. Narodne novine d.d. 144/13 (http://digured.srce.hr/arhiva/263/111883/narodne-novine.nn.hr/clanci/sluzbeni/2013_12_144_3086.html, accessed 28.04.2017.)

Narodne novine. 2016. Pravilnik o izmjenama i dopunama pravilnika o strogo zaštićenim vrstama. Narodne novine d.d. 144/13 (http://digured.srce.hr/arhiva/263/155241/narodne-novine.nn.hr/clanci/sluzbeni/2016_08_73_1745.html, accessed 28.04.2017.)

http://pp-telascica.hr/o-parku/?lang=en, accessed 1.6.2017

http://np-mljet.hr/en/about-the-park/, accessed 1.6. 2017

8 Curriculum Vitae

PERSONAL INFORMATION

Pavel Ankon

Križnog puta 69, 10040 Zagreb (Croatia)

+385 989778665

pavel@ankon.hr

Skype pavel.ankon

Sex Male | Date of birth 01/11/1991 | Nationality Croatian

WORK EXPERIENCE

2016 — Member of Management Board

Marine Explorers Society 20000 League

11/2016–12/2016 — Professional training

University of Sassari, Department of Sciences for Nature and Environmental Resources, Sassari (Croatia)

2015–2016 — Member of Supervisory Board

Biology students association BIUS, Zagreb (Croatia)

2015 — Leader of Section for Marine biology

Biology students association BIUS, Zagreb (Croatia)

2014–2015 — Secretary of Management Board

Biology students association BIUS, Zagreb (Croatia)

2012 — Secretary of Management Board

Campus Lions Cluba Universitas Zagrabiensis, Zagreb (Croatia)

06/2010–09/2012 — Diving Instructor

Diving center Punta Skala, Falkeinsteiner resort Punta Skala, Petrčane, Zadar (Croatia)

EDUCATION AND TRAINING

2014–2017 — Masters in Ecology and Nature Preservation, Module Marine Biology — EQF level 7

Faculty of science, Zagreb (Croatia)

Master's thesis conducted at the University of Sassari, Department of Sciences for Nature and Environmental Resources in prof. Casu group with title "Genetic and morphological variability of the noble pen shell (*Pinna nobilis* Linnaeus, 1758) in Nature Park Telašćica and National Park Mljet"

02/2015–07/2015 **Erasmus Student Exchange** EQF level 7

University of Sassari, Department of Sciences for Nature and Environmental Resources, Sassari (Italy)

2010–2014 **Bachelor Degree in Biology** EQF level 6

Faculty of Science, Zagreb (Croatia)

2009–2011 **NAUI Instructor course (#53423)** EQF level 2

NAUI EUROPE (Croatia)

03/2009–06/2009 **Speleologist trainee** EQF level 2

PDS Velebit, Zagreb (Croatia)

2006–2010 **High school graduate** EQF level 4.1

XV. Gimnazija, Zagreb (Croatia)

2001–2009 **NAUI open water, advanced, rescue, advanced rescue, CPR/First aid, dive master, NITROX**

NAUI EUROPE (Croatia)

PERSONAL SKILLS

Mother tongue(s) Croatian

Other language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	B2	B2	B2	B2	B2
	Language school certificate B2				
Italian	C1	C1	C1	C1	C1
	University of Siena, CILS exam				
German	A1	A1	A1	A1	A1

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages

Communication skills

- skilled in knowledge transfer and outreach
  - Diving instructor
  - multiple educational projects
- able project/problem presenter
- certain experience with PR and fundraising as secretary of student association and project organiser

- Inventory of macrobenthos, mapping of Posidonia oceanica habitat and recording a zero-state of Komiža gulf, 2014, BIUS
- "Grabovača, 2014.", BIUS
- "Papuk 2015.", BIUS
- "Mura – Drava 2016.", BIUS

Organisational / managerial skills

- Leader of Marine Biology Section, BIUS, 2015 - 2017
- Successful project organisation
  - student projects, seminar, lectures, conferences
- Effective managing of the organisations
  - Biology student association BIUS
  - Marine Explorers Society 20000 League
- Student engagement
  - Student representative in Faculty Council, PMF (01.10.2013 – 30.06.2017)
  - Student representative in Council of Department of Biology, PMF (01.10.2013 – 30.06.2017)

Digital competence

SELF-ASSESSMENT				
Information processing	Communication	Content creation	Safety	Problem solving
Proficient user	Proficient user	Independent user	Independent user	Independent user

- proficient use of Microsoft Office™ (Word, Excel, Powerpoint) and similar packages
- basic use of QGIS, Grapher, R program, Ocean dana view, Statistica, Primer, GeneMapper, Past, SplitsTree, Structure, Convert, Microsat, Phylip, Powermaker, Mendeley

Other skills

- Boat Skipper, B category
- Active ice hockey player (1998 - 2009, KHL Zagreb)

Driving licence

B

ADDITIONAL INFORMATION

Projects

Organiser:

- Inventory of macrobenthos, mapping of Posidonia oceanica habitat and recording a zero-state of Komiža gulf, 2014, BIUS
- Grabovača, 2014, BIUS

Coordinator:

- Papuk, 2015, BIUS
- Mura – Drava, 2016, BIUS

Participant:

- ESF project: “Misli plavo, Otok Ugljan”, 03. - 28.11.2015, 20 000 Leagues
- ESF project “Razvoj studija ekologije, računarstva i matematike uz primjenu HKO-a”, 18. 09.2015 – 18.09.2016, PMF
- FP7 project: CADDY - „Cognitive Autonomous Diving budDY", 2014 - 2016, FER,
- EU projekt: "SeaTizen: the role of citizens in preserving the Mediterranean Sea", 2016, BIUS
- "Zadar za čisti Jadran", 16. - 23.11.2015, 20 000 Leagues
- Research of eastern islands PP Lastovsko otočje,17. - 27.08.2015, 20 000 Leagues
- Mapping of marine habitat and monitoring of Posidonia oceanica in Natura 2000 in Šibensko - kninska županija, 22. - 31.05.2016, 20 000 Leagues
- Establishment of fish stock monitoring in MPA in Adriatic Sea, 2016, 20 000 Leagues

Honours and awards

- Chancellor's Award for the education project "Night of biology" 2013
- Chancellor's Award for the research and educational project "Grabovača 2014."

Memberships

- BIOM (BirdLife Croatia) (2015 - present)
- Marine Explorers Society 20000 League (2013 - present)
- Biology students association BIUS (2012 - present)
- Campus Lions Cluba Universitas Zagrabiensis (2011- present)

Conferences

- International Conference on Evolution and Behaviour ICEB 2014
- International Conference on Evolution and Behaviour ICEB 2015
- Students' Symposium in Biology and Life Sciences 2015
- Students' Symposium in Biology and Life Sciences 2016