UNIVERSITY OF PRIMORSKA

FACULTY OF MATHEMATICS, NATURAL SCIENCES
AND INFORMATION TECHNOLOGIES

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COMPARISON OF MITOCHONDRIAL AND NUCLEAR GENETIC MARKERS OF BARREL JELLYFISH (*Rhizostoma pulmo*) FROM NORTH ADRIATIC AND CENTRAL MEDITERRANEAN BIOGEOGRAPHIC REGION

MASTER THESIS

Supervisor: Assist. Prof. Andreja Ramšak

Co-supervisor: Prof. Dr. Alenka Malej

Koper, 2012

The master thesis is the final work of the second Bologna cycle, master's study programme in Marine biology. The work was conducted at the Marine Biology Station - National Institute of Biology.

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Abstract: The genetic diversity of barrel jellyfish (*Rhizostoma pulmo*) populations was investigated in the Mediterranean Sea. Samples were collected in different biogeographic regions (Northern Adriatic, central and western Mediterranean). The COI and ITS nucleotide sequences were analysed in 65 samples from 8 sampling sites. COI and ITS regions were amplified by PCR and sequences were later used for the phylogenetic analyses. COI haplotypes were grouped to the same phylogenetic group (78 percentage of likelihood). Phylogenetic analyses of nucleotide sequences of ITS 1 region were concordant with COI results. ITS 1 haplotypes grouped to one phylogenetic group (76 percentage of likelihood); meanwhile ITS 2 region sequences were the same and gave no information. Better understanding of the genetic diversity across different species belong to the phylum Cnidaria, will enable us to gain detailed insight in the species evolution and possible explanation for present phylogeographic patterns. Such knowledge might give us useful hints to explain jellyfish blooms in the coastal areas.

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Ključne besede: DNK, genetski markerji, COI, ITS, *Rhizostoma pulmo*, Sredozemsko morje, biogeografska regija

Izvleček: Ugotavljal sem genetsko raznolikost v populacijah velikega klobučnjaka (*Rhizostoma pulmo)*, ki so bili nabrani v Sredozemskem morju. Vzorci so bili nabrani v različnih biogeografskih regijah (severni Jadran, obala severne Afrike, zahodno Sredozemlje). V 65 vzorcih iz 8 vzorčnih mest sem analiziral nukleotidna zaporedja COI in regij ITS. Z reakcijo PCR sem pomnožil zapis za dva genetska markerja, ki sem jih nato uporabil za filogenetske analize. Ugotovil sem, da vsi haplotipi COI tvorijo enotno filogenetsko skupino, populacijo s 78 odstotno verjetnostjo za COI in 76 verjetnostjo za ITS. V boljšim poznavanjem genetske raznolikosti v različnih vrstah klobučnjakov bomo pridobili boljši vpogled v evolucijo vrst in možne vzroke, ki privedejo do množičnih pojavljanj klobučnjakov ob obalah morij.

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SYMBOLS AND ABBREVIATIONS

bp	base pair
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dH_2O	distilled water
dNTP	2'- deoxyribonucleotide 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
F	»forward primer«
ml	mililiters
Mg^+	magnesium ion
ng	nanograms
µ	micro
µg	micrograms
PCR	polymerase chain reaction
pH	a negative decimal logarithm of the hydrogen ion activity in a solution
buffer TAE	a mixture of Tris base, acetic acid and EDTA
R	»reverse primer«

rpm	revolutions per minute
Taq DNA-polymerase	DNA-polymerase, isoleted from *Thermus aquaticus*
w/v	weight per volume

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1 INTRODUCTION

Class Scyphozoa belongs to the evolutionary very old phylum of Cnidaria. Most of representatives have the life cycle consisted of sexual and asexual generation. The free swimming medusa represents the pelagic and sexual stage, while the sessile polyp is the asexual stage. The major advantage is a rapid replication and mass growth, which are the combination of sexual and asexual generations, beside to environmental factors that stimulate their growth and abundance. Mass occurrences of jellyfish have been observed in many marine ecosystems, but the increasing numbers of jellyfish blooms in the world's seas, raised interest and concern among researchers. Some evidences strongly suggests that for the increase of jellyfish occurrences in the recent years, the major cause is the man himself, mostly with the impairment of natural balance [32]. Jellyfish blooms can cause a considerable damage by clogging fishing nets, harming tourism and aquaculture. In the last decades several mass occurrences of certain species had been noticed, such as the moon jellyfish (*Aurelia aurita*), barrel jellyfish (*Rhizostoma pulmo*), mauve stinger (*Pelagia noctiluca)* and compass jellyfish (*Chrysaora hyoscella*), *Cotylorhiza tuberculata*, hydromedusae such as *Aequorea forskalea* etc. Such large jellyfish aggregations have been observed on many Mediterranean coasts, from the coast of Spain to the coasts of the Black Sea.

Currently, there isn't much information available on the population structure of *Rhizostoma pulmo.* The purpose of this thesis is to find out whether large populations of *Rhizostoma pulmo* that inhabit the Mediterranean Sea, are geographically isolated from each other and what is the extent of differentiation at the level of nucleotide sequences of selected genetic markers from mitochondrial and nuclear DNA. Sequence analyses were performed with cytochrome c oxidase subunit I and ITS1 and ITS2 regions from nuclear DNA. I used nucleotide sequences of the mentioned genetic markers, to uncover possible phylogenetic pattern of the barrel jellyfish in the Mediterranean Sea.

2 LITERATURE OVERVIEW

2.1 MASS OCCURRENCES OF JELLYFISH

Increasing frequencies of jellyfish mass occurrences had prompted scientists to begin rising hypotheses about the possible causes for their occurrences. The most frequently exposed causes are climate change, nutrient inputs (eutrophication) and overfishing. Research on jellyfish populations through long-term trends (8-100 years); have shown that their mass occurencies varied in relation to the climate and often in the ten-year cycles [1, 22]. The increasing problems of people with jellyfish and reports about accidents have as result attracted attention of the general public; mainly due to accidents such as: stings (stings can cause allergies, pain, death etc.), interfering with fishing, aquaculture and power plant operations [32].

Mass occurrences are often seen in the eutrophic areas, but also in areas that aren't under the direct influence of human activities [32]. Purcell and colleagues reported about recordings of frequent blooms on the northwest coast of the U.S., Gulf of Mexico, Mediterranean Sea, coast of Red Sea and Arabic Sea and by the coast of the Japan Sea [32]. A conclusion of the research project EcoJel (financed by the EU), was that the jellyfish blooms are associated with a large number of direct human impacts on the environment. Some of the causes are overfishing, fertilizers run-off which causes eutrophication and algal blooms, introduction of invasive jellyfish species by ballast waters, but also global warming and any interference related with the balance of marine habitats. Scientists also believe that the increased number of jellyfish is caused by the ocean acidification as a result of the increased atmospheric carbon amounts [42]. In some cases mass occurrences happened after an extensive habitat change, as was recorded in the lagoon Mar Menor in Spain. The sand bottom was replaced with muddy bottom, which lead to a chain reaction in the ecosystem that was caused by the eutrophication phenomena. Eutrophication is ecosystem response to excessive nutrient input what often leads to higher biomass at several trophic levels; consequently more food [19]. The increase in nutrients often leads to higher biomass on all trophic levels [6], which mean that there is more food available for survival and reproduction for a large number of polyps and medusas [31].

Such situation has been associated with mass occurrences of fried egg jellyfish (*Cotylorhiza tuberculata*) and barrel jellyfish (*Rhizostoma pulmo*) in the lagoon of Mar Menor. The data shows that high nitrogen concentrations in this particular area can lead to jellyfish blooms [29]. Since 1990, reports showed that massive blooms of jellyfish are occurring along the Mediterranean coast [24, 22]. A high density of barrel jellyfish was recorded along the Catalan coast. Most sightings have been concentrated in the central area, especially in July and August [13]. Massive occurrence is characterized by a rapid and seemingly sudden increase in the number of jellyfish [9].

Figure 1: Massive catch of *Nemopilema nomurai* in Japan [41].

2.2 DESCRIPTION OF BARREL JELLYFISH (*RHIZOSTOMA PULMO*)

In addition to *Rhizostoma pulmo* (Macri, 1778), there are two other species *Rhizostom*a *luteum* (Quoy and Gaimard, 1827) and *Rhizostoma octopus* (Linnaeus, 1788). The family Rhizostomatidae includes also genera Rhopilema, Nemopilema and Eupilema [26]. The taxonomic status of *R. luteum* is uncertain and some authors do not recognize this species. *Rhizostoma pulmo* is widespread in the Mediterranean and Black Sea, while the other two inhabit the Atlantic Ocean. The species *R. pulmo* and *R. octopus* are distinguished by few morphological characters such as the number and shape of marginal lappets per velar lobes on the umbrella, length of oral arms and appendices and the distribution area. *R. pulmo* has usually eight marginal lappets, while *R. octopus* has an average of ten marginal lappets [34].

Figure 2: Barrel jellyfish (*Rhizostoma pulmo*) [43**].**

The bell of barrel jellyfish is composed of mesoglea, which is quite solid, and contains few cells and collagen fibres. In the central part is thick and is getting thinner near the edge of bell. Surface of the umbrella is covered with small, firmly compressed nematocysts. The edge of the umbrella is divided into a number of semi-circular lappets, with an intense dark purple-blue pigmentation. Around the edge of the umbrella are eight pairs of small pointed lobes called rhopalar lappets and between them is sense organ (rhopalium). In the middle of umbrella is gastrovascular cavity which forms to the outside of a bell a network of interconnected channels. Several kinds of channels are present in the umbrella [26].

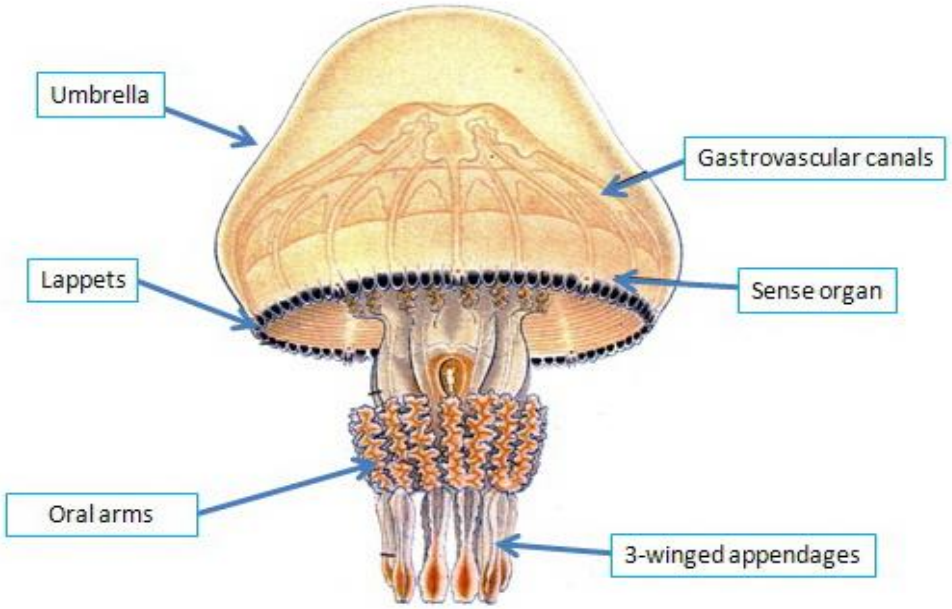

Figure 3: Main morphological characteristics of the barrel jellyfish (*Rhizostoma pulmo*) [25].

At the umbrella margins are muscle fibres that help contract the umbrella. Barrel jellyfish oral cone is composed of short, pillar like structures continued to oral arms. Oral cone and arms are covered by strapped structures named epaulette. Small oral openings are on these structures surrounded by nematocysts. The oral arms end with appendages which are easily to cut off [26].

2.2.1 Life cycle of barrel jellyfish (*Rhizostoma pulmo*)

The life cycle of barrel jellyfish consists of a sessile polyp and a free swimming jellyfish. Polyp can reproduce by budding and strobilation. Medusae are of separate sexes and release gametes into the water [13].

Planula develops from the fertilized egg into a free swimming larva, which soon attach to the substrate and develops into a polyp. New polyps can develop with the formation of lateral buds on the polyp or the secession of the basal part of polyps, which is formed in a stationary formation, surrounded by chitin (podocyst), and is designed to survive adverse conditions. Under favourable conditions, the polyp reproduces asexually by strobilation.

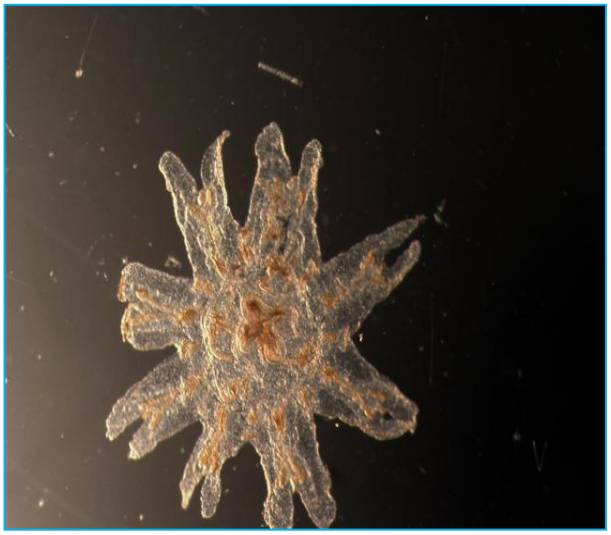

Figure 4: Ephyra of barrel jellyfish (*Rhizostoma pulmo)* [Photo: Turk, MBS].

Ephyrae detach one by one or in group simultaneously. Two of three days after the release, the ephyra begins to develop small formations between lappets that in a period of three weeks reach the size of lappets. In a period of three or four weeks after the ephyra was released it grows in a bell shaped medusa [18].

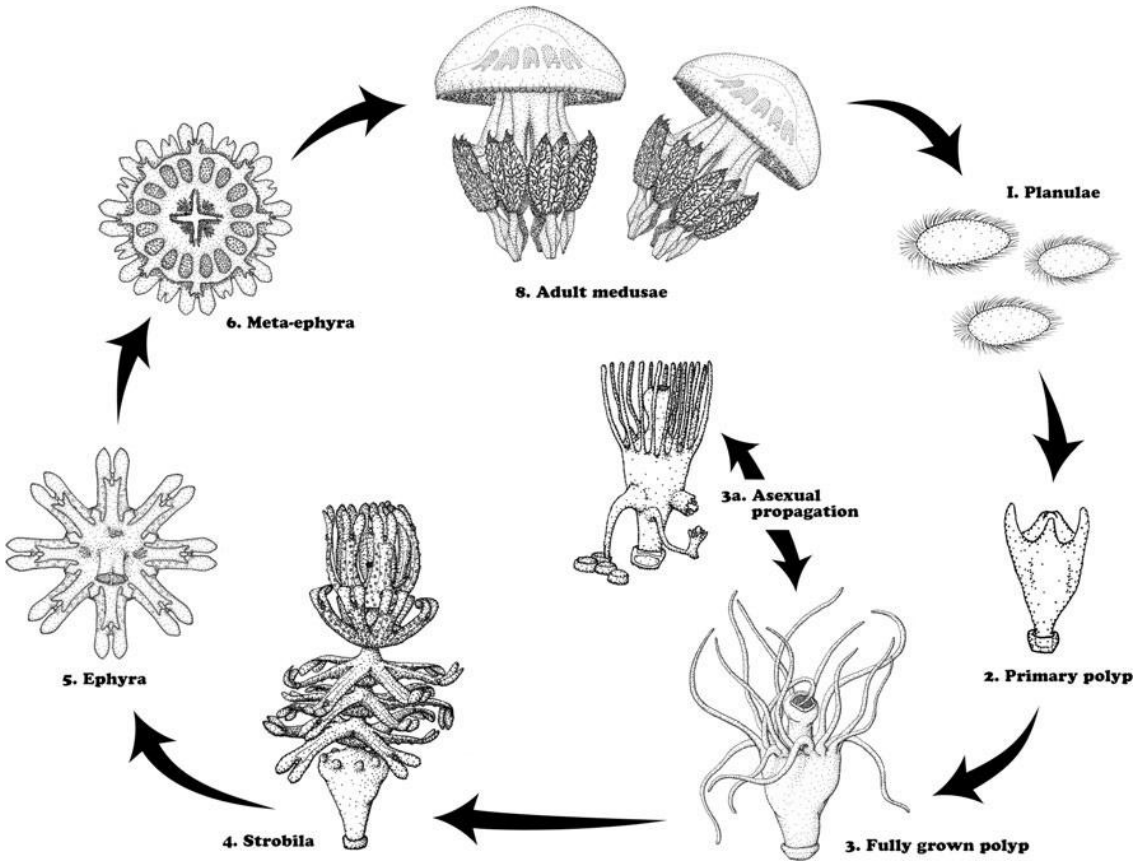

Figure 5: Life cycle of *Rhizostoma pulmo* [13].

2.3 SCYPHOZOA AND PHYLOGENETIC ANALYSES

Phylogeny is the discipline dealing with relationships between species, discovered through different features from molecular data sequencing (proteins and DNA) and morphological data matrices. It tries to show the course of events in the evolution and display them in the form of a system or phylogenetic trees. Evolutionary events that have occurred in the history of a certain species are not possible to observe, for that matter researchers are now making use of living organisms and the reconstruction of phylogenetic relationships using the principles of genetics. The modern concept of species, also include genetic differences. The real progress was made when DNA segments were discovered, containing information

to help us distinguish organisms and sufficiently short to make amplification and sequencing rapid and efficient. Members of the same species have the ribosomal 16S or 18S rRNA sequences similar to 97% [36].

In cnidarians, due to lack of macro-morphological differentiation [26, 7] and the great diversity in life cycles, the question remains, what was the older form in the evolutionary sense, the polyp or medusa. The first phylogenetic studies on cnidarians were made on the basis of ribosomal 16S rDNA and 18S rDNA [2]. Groups that have a medusa stage (Hydrozoa, Scyphozoa and Cubozoa), forming a single group named Medusozoa. Researchers are inclined to the theory that the common ancestor of cnidarians had a polyp stage and the medusa stage is a synapomorphy of the Medusozoa clade. Collins and colleagues [5] have suggested dividing Cnidarians in two groups, Anthozoa (corals and sea anemones) and Medusozoa. They hypothesized that Anthozoa evolved first to and later the groups of Cubozoa and Scyphozoa were formed. The strobilation of polyps and ephyra are synapomorphic characters for all Scyphozoa. In a research, Collins and colleagues [5] confirmed previous hypothesis that the order Rhizostomea originated from ancestors in the Semaeostome order, because of the similarity in the system of radial channels, which is in accordance with earlier morphological studies [26].

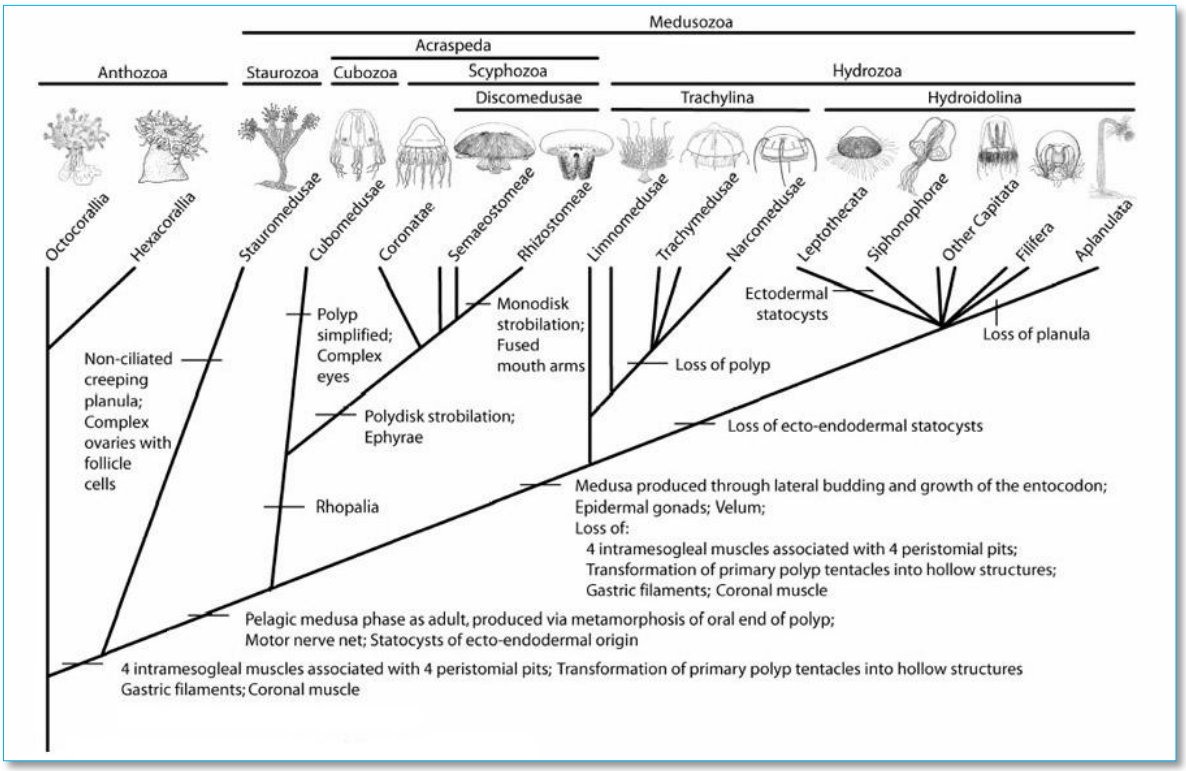

Figure 6: Phylogenetic tree of Cnidaria [5].

2.4 PHYLOGEOGRAPHY OF SCYPHOZOA

The complex processes that affect contemporary patterns of taxa biodiversity in the coastal zone are still poorly researched. Phylogeography, explicitly links micro and macro evolution, linking ecology and evolution of current distribution in relation to historical events, creating a network that can further examine the evolutionary entities and processes that involve a multiple-layer biodiversity [8].

The core of phylogeography is describing the variability of a population and current pattern of genealogical lineages distribution with use of genetic markers, e. g. with widely used mtDNA cytochrome c oxidase subunit I (COI) and nuclear DNA internal transcribed spacer 1 and 2 (ITS 1 and ITS 2 region).

Large quantities of individuals are essential for achieving best results in testing phylogeographic hypotheses and determine the spatial distribution, genetic lineages within

and between close related species. The marine environment is challenging to set up phylogeographic hypothesis, as it lacks the obvious geographical barriers in comparison with terrestrial environment. There are many possible scenarios from panmixia to geographically isolated populations in the marine environment. Researchers managed to detect phylogeographic patterns in many taxa that lead to a discovery of significant barriers in the sea for the gene flow and cases of panmixia [28, 8, 33, 30].

The traditional view, that morphologically cohesive marine invertebrate species have a natural, global distribution, has recently come under investigation. Many species have shown to contain a cryptic complex or a deep phylogeographic structure that is evident at a molecular level. Cryptic species are organisms difficult or impossible to distinguish only by morphological characteristics [17]. In the marine environment; invertebrates generally show weak genetic structuring due to large populations, high dispersal at the larval stage, lack of physical barriers and sometimes interspecies hybridization. Such examples can be found among scyphozoans; among species with global distribution and the only an accurate phylogeographic analysis would show the genetic diversity and species differentiation. The moon jellyfish (*Aurelia aurita*) is a cosmopolitan species and in the last century has attracted the attention of taxonomists and ecologists. Because of its high morphological variability, was divided into 12 species or subspecies of *Aurelia* [26]. However, there are only three species recognized by the taxonomists nowadays (*A. aurita, A. labiata, A. limbata*). In recent time, phylogeographic studies on several scyphozoan species (e. g. *Catostylus mosaicus)* [8], *Cassiopea* spp. [17], *Pelagia noctiluca*, *Rhizostoma pulmo* and *Aurelia aurita* [37, 7, 33] were revealed different phylogeographic patterns among them from admixture to deep separation between lineages in small geographic area. The extreme example is *Aurelia sp*. 8 in Adriatic Sea and *Aurelia sp*. 5 in Mljet Lake in the Adriatic Sea [7, 33].

2.5 GENETIC MARKERS

Parts of DNA are very applicable as genetic markers because of their uniqueness, easy to manage (PCR amplification, sequencing), and reliability. A DNA genetic marker is an easily identifiable piece of genetic material that can be used in the laboratory for identification of cells, individuals, populations or species. Primarily, it is necessary to isolate the genetic marker (DNA isolation from tissues, PCR amplification). With the use of genetic markers we are able to identify genetic diversity [46].

Choosing the most appropriate marker depends on the level of variability, the nature of the information (dominance vs. codominance, ploidy) which must be determined according to the biological question. Several procedures are proposed for drawing up genetic markers suitable to answer particular biological questions: choice of markers regarding to the biological question, level of variability and the nature of information, suitable DNA region and techniques for isolation and amplification of markers [4].

2.5.1 Mitochondrial genetic marker Cytochrome c oxidase (COI)

Mitochondrial gene cytochrome c oxidase subunit I (COI) is one of the most popular markers in molecular systematics and phylogeography. Parts of the gene are often used for identification of higher metazoans. The length of COI nucleotide sequence, amplified by universal invertebrate primers, is approximately of 640 base pairs [12] and it is a unique identification code for many animal species. Its small size, made COI possible to unravel nucleotide sequence in one screening [36].

In phylogenetic studies are mostly used nucleotide sequences from mitochondrial DNA (mtDNA) large (16S) and small (12S) rRNA, cytochrome c oxidase subunit I (COI) and cytochrome b. MtDNA segments are suitable for phylogenetic and phylogeographic studies because within these sequences is possible to locate regions which have different level of variability, that allow comparison at different taxonomic ranks and inference of phylogenetic relationships [10].

There are some important exceptions among cnidarians, in which use of COI is not suitable. Anthozoa are group in which the COI variability was very low and not suitable for comparisons at intra-species and inter-species level [20, 15]. They found that pair wise genetic distances between close interspecific and intraspecific pairs of anthozoan congeneric species are very low (<2%). Meanwhile, the COI diversity among close interspecific and intraspecific species of Scyhozoa is higher and comparable to other multicellular animals (*Aurelia aurita* variability is 13% -24%, *Cyanea* 11.8-15.3% and 10.9-23.4% for *Cassiopea*) [20].

Despite some concerns, recent studies have confirmed the gene suitability in phylogenetic studies of Scyphozoa. In the year 2006 was published first complete mtDNA sequence of moon jellyfish [35]. Mitochondrial DNA of the moon jellyfish is 16.937 bp long and it is not a circular molecule, as in Anthozoa, but it is linear, which is relatively rare among animals [2].

2.5.2 Nuclear genetic markers ITS marker

The non-functional piece of RNA, which is located between the genes for ribosomal rRNA, is called internal transcribed spacer or ITS regions. Those regions are suitable for the inference of phylogenetic relationships at various taxonomic levels in many plant and animal species [27] and are widely used in taxonomy and molecular phylogeny. Mainly due to an easy amplification even with low DNA concentrations and a high degree of variability among closely related species. It was presumed that a high ITS variability was because it has no function, however, recent studies have shown that the ITS regions have a primary role in rRNA processing [21]. Some mechanisms such as concerted evolution can cause less polymorphism in ITS regions [23].

3 MATERIAL AND METHODS

3.1 MATERIALS

3.1.1 Tissue samples

Tissue samples (umbrella margins and gonads) of barrel jellyfish were collected at locations in Mar Menor Lagoon (Spain), Gulf of Tunis and Lake Bizerte (Tunisia), Venetian Lagoon, Varano Lagoon (Italy), in small bays at coast of Slovenia (Bay of Strunjan and Bay of Koper). Tissue samples were stored in the tubes with a 96% ethanol or in mixture of 20% DMSO saturated with NaCl. Tissues were stored for longer time at temperature -80^{o}C until the isolation of DNA. Tubes were marked with the abbreviation of the species, date and sampling location.

Example: 20/08/RH01

20- Day in month,

08- Month of sampling,

RH- *Rhizostoma pulmo* species name

01-serial number of sampled specimen

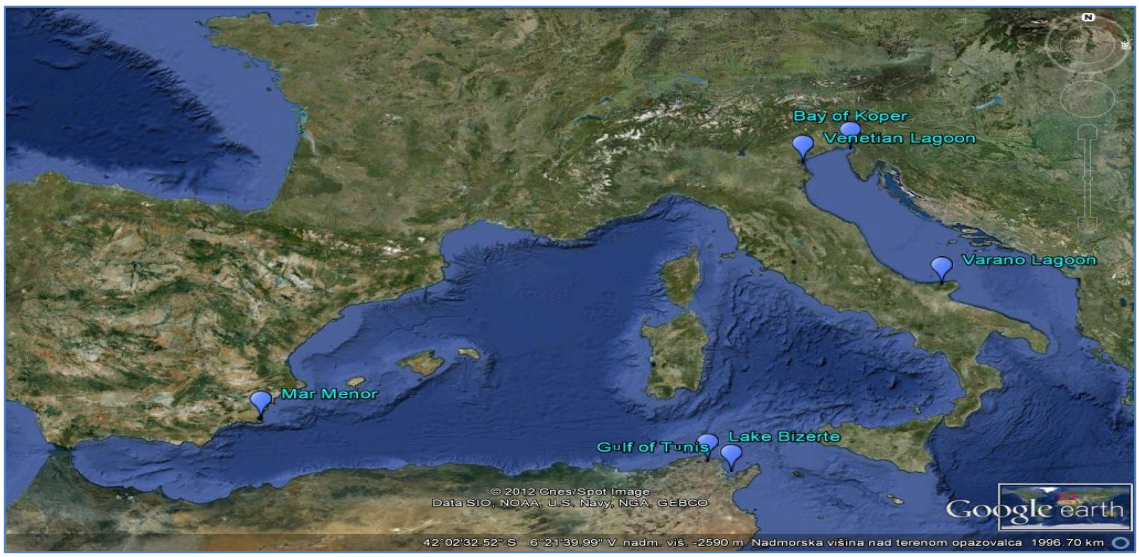

Figure 7: Sampling sites of barrel jellyfish (*Rhizostoma pulmo* [44].

In the following table are listed all the 63 samples used in the experiment, sorted by sampling sites.

Table 1: List of sampling sites of barrel jellyfish (*Rhizostoma pulmo*) used in the experiment.

Sampling site		Sample ID	Sampling date	No. Samples
Spain	Mar Menor Lagoon	**20/08/RH**	20.9.2008	14
Tunisia	Lake Bizerte	**16/07/RH**	16.7.2007	6
	Gulf of Tunis	**15/07/RH**	15.8.2007	6
Italy	Venetian Lagoon	**18/07/RH**	18.8.2008	6
	Varano Lagoon	**17/07/RH**	17.7.2007	10
Slovenia	Bay of Koper	**13/07/RH**	13.7.2007	2
		12/06/RH	15.12.2006	3
		11/06/RH	21.11.2006	6
		9/06/RH	29.9.2006	3
	Bay of Strunjan	**5/05/RH**	17.5.2005	7

3.1.2 Enzymes

Taq polymerases:

- Taq DNA-polymerase (5U/µl, Fermentas, Cat. No. EP0402)

- Taq DNA- polymerase (5U/µl, Roche, Cat. No. 11146173001)

- TopTaq DNA Polymerase (5U/µl, Qiagen, Cat. No. 200203)

- Proteinase K (900U/ml, Fermentas, Cat. No. E00491)

3.1.3 Buffers and reagents

-6x concentrated loading buffer for agarose gel:

- 0,05-percentage bromophenol blue
- 0,05- percentage xylene cyanol in formamid
- 40- percentage sucrose in dH_2O

Buffer 1xTAE composition for electrophoresis:

- 0,04 M Tris-acetate
- 0,001 M EDTA
- pH 8,0

-Stock solution of ethidium bromide 10µg/ml (Sigma, Cat. No. E1385)

3.1.4 DNA molecular weight standards

- Lambda DNA/Hind III Marker, 2 (Fermentas); 23130-125 bp
- GeneRuler™ 100bp DNA Ladder (100-1000 bp, Fermentas)

3.1.5 Instruments and kits

- Centrifuge R1505 (Sigma)

- Cyclic thermostat MJ Research PTC-200 (MJ Research)
- Water bath (Kambič)
- Tanks for agarose gel electrophoresis (Biometra)
- High voltage power supply for electrophoresis (Biometra)
- UV transiluminator (UVSpec)
- Fluorometer (QubitTM, Invitrogen)
- DNeasy Blood & Tissue Kit for DNA purification (QIAGEN)

3.1.6 Reaction mixture for PCR amplification of genetic markers

The following tables represent the recipes for the reaction mixture for COI, ITS1 and ITS2. The presented recipe is for one reaction volume of 50 µl. The mixtures differ in the Taq polymerase used, quantity of used template DNA (from 3-5 µl) and water to reach the total volume of 50 µl.

Table 2 and Table 3 are the recipes for COI, ITS1 and ITS2 reaction mixture for Qiagen Top Taq polymerase, with and without Q-solution.

Table 2: Mixture for PCR with TopTaq DNA Polymerase (Qiagen) without Q-solution.

PCR Reagents	Stock concentration	Added volume (µl)	Working concentration
Taq polymerase	5 U/µl	0.25	1,25 U
PCR Buffer	10x	5	1x
dNTP	10 µM	1	0,8 µM
Primers: Forward +Reverse	10 µM	1+1	0,2 µM F, 0,2 µM R
$MgCl_2$	25 µM	1	0,5 µM

Table 3: Mixture for PCR with TopTaq DNA Polymerase (Qiagen) with Q-solution.

PCR Reagents	Stock concentration	Added volume (µl)	Working concentration
Top Taq polymerase	5 U/µl	0.25	1,25 U
PCR Buffer	10x	5	*1x*
dNTP	10 µM	1	0,8 µM
Primers: Forward +Reverse	10 µM	1+1	0,2 µM F, 0,2 µM R
Q solution	5x	5	2µl
$MgCl_2$	25µM	1	0,5 µM

In Tables 4 and 5 are the recipes for COI, ITS1 and ITS2 reaction mixture for Fermetas Taq polymerase and Roche Taq polymerase.

Table 4: Mixture for PCR with Taq DNA Polymerase (Fermentas) for COI and ITS.

PCR Reagents	Stock concentration	Added volume (µl)	Working concentration
Taq polymerase	5 U/µl	0.25	1,25 U
PCR Buffer	10x	5	2x 1.25 µl
dNTP	2µM	1	0,8 µM
Primers: Forward + Rewerse	10 µM	1+1	0,2 µM F, 0,2 µM R
$MgCl_2$	25 µM	1	0,5 µM

Table 5: Mixture for PCR with Taq DNA Polymerase (Roche) for COI and ITS.

PCR Reagents	Stock concentration	Added volume (µl)	Working concentration
Taq polymeraze	5 U/µl	0.25	1,25 U
PCR Buffer	10x	5	1x z 1,5 mM MgCl
dNTP	2 µM	1	0,8 µM
Primers: Forward + Rewerse	10 µM	1+1	0,2 µM F, 0,2 µM R

3.2 METHODS

3.2.1 DNA isolation

DNA was isolated from the tissue sampled at umbrella margin or from gonads by commercial kit DNeasy Blood & Tissue Kit (Qiagen). The procedure was done according to the manufacturer instructions. For one DNA isolation was necessary:

- approximately 25 mg of tissue sample
- 180 µl of ATL buffer
- 20 µl Proteinase K
- 200 µl of AL buffer
- 200 µl of 96% ethanol
- 500 µl of AW1 buffer
- 500 µl of AW2 buffer
- 100 µl of AE buffer
- 1.5 ml tubes
- 2 ml tubes

Procedure for tissue degradation: tissues stored in the freezer at -80°C were defrosted on ice and transferred with forceps into new tubes, with added 180 µl of ATL buffer, mixed thoroughly and added 20 µl of proteinase K. The tubes were wrapped with parafilm, labelled and placed in the shaking water bath at 56^{o}C for 16 hours.

The next day I took the samples from the water bath and centrifuged them for 15 seconds. Then I added 200 µl of AL buffer and 200 µl of 96% ethanol, mixing for a few seconds and centrifuged at 8000 rpm. The reaction mixture from the tube was transferred into fresh 2 ml tubes, containing a silica membrane for the adsorption of nucleic acids. The reaction mixture was centrifugated at 8000 rpm for one minute and collected liquid below silica membrane was discarded. The membrane was washed with added 500 µl of buffer AW1 and centrifuged for one minute at 8000 rpm. Then I changed the tube, added 500 µl of AW2 buffer on the membrane and centrifuged for three minutes at 14,000 rpm. The eluate

was collected in 1.5ml collection tubes, added 100 µl of AE buffer (0,1 M Tris) on the membrane, waited for a minute and centrifuged for one minute at 8000 rpm. I washed membrane two times, this way I got two fractions of each sample. Each fraction (first and second) was collected separately and stored in a freezer at -20^{o}C until further analysis.

3.2.2 Fluorimetric quantification of isolated DNA from tissue samples

After the DNA isolation, I measured the DNA concentration in all 63 samples. The procedure was performed with Qubit fluorometer, according to the protocol of the manufacturer (Invitrogen, Quant-iT dsDNA BR Assay Kit). During the procedure, the samples and reagents were kept on ice. A labelled 0,5ml tubes were used for the preparation of standards to calibrate the Qubit Fluorimeter, but also is possible to use previously calibrated setting in fluorimeter. Working solution was made by 199µl buffer dsDNA BR and 76µl of dsDNA BR reagent that intercalate between strands of DNA. DNA was diluted with 199 µl of working solution and 1µl of the DNA from tissue sample. Reaction was mixed and shortly centrifuged for 2 to 3 seconds and incubated at room temperature for 2 to 3 minutes and then the fluorescence was measured in samples. Standard curve for calculation of concentrations were prepared with two standards (standard 1 and standard 2) from kit. DNA concentration of sample was calculated according the formula (Equation 1) from measured fluorescence. The measured and calculated fluorescence values are presented in the Appendix 1. Samples in which DNA concentration did not exceed 10 ng/ml were removed from further work.

Figure 8: Preparation of mixture for fluorescent measurements of DNA concentration.

Reagents:

- Quant-iT dsDNA BR reagent (200x concentration in DMSO)
- Quant-iT dsDNA BR buffer
- Quant-iT dsDNA BR Standard 1 (0 ng / µL in TE buffer)
- Quant-iT dsDNA BR Standard 2 (100 ng / µL in TE buffer)

Equation 1: Calculation of DNA concentration in tissue sample:

$$Concentration = Qf\ x\ \left(\frac{200}{x}\right)$$

Qf – measured fluorescence

X – added amount of DNA sample in microliters (1 µl)

3.3 AGAROSE GEL ELECTROPHORESIS

3.3.1 Preparing the agarose gel

Quantity and quality of isolated DNA was verified on agarose gel. I used 1.5% gel, which was prepared by following recipe: 1.5 grams of agarose, poured into an Erlenmayer flask (200ml), added 150ml of 1xTAE buffer and heated the mixture several minutes in the microwave at 500 W until agarose completely melted. Melted agarose was cooling down to 50° C. Then I added 4.5µl of ethidium bromide (10µg/ml), mix it and pour it into a tray to harden. Electrophoresis was run in the tank for electrophoresis, which contained 1xTAE buffer.

On parafilm was loaded 5µl of loading buffer (described previously) and added 5µl of sample. The loading buffer was mixed with the sample and distributed it in the gel pockets; on each side of the gel one lane was loaded with suitable DNA weight standard. I covered the electrophoresis tank with a lid and turned on the electrical current. Voltage was 4 to 5

cm/cm and the electrophoresis was carried out in about 30 minutes. The gel was examined on the transilluminator, under UV light and took photographic images. Isolated DNA from barrel jellyfish was compared with the λ DNA / Hind III Marker, 2 (Fermentas). This marker has DNA fragments from 23130 bp to 125 bp. PCR products were compared with GeneRulerTM (Fermentas), which have DNA fragments from 1000 bp to 100 bp.

3.4 PCR AMPLIFICATION OF ITS1 AND ITS2 REGIONS

The whole process of preparing the mixtures for the PCR was carried out in a fume hood to prevent contamination with aerosols contained fragments of DNA. During the preparation all the reagents were kept on ice to prevent non-specific amplification. Firstly, a PCR »master mix« was prepared in 1.5ml tubes, without template DNA. All the reagents were briefly mixed and distributed to the PCR tubes, and then a proper quantity of template DNA was added into each tube. All the protocols for the PCR master mix preparation can be seen in the tables (Table 2 to Table 5). The basic recipe has remained the same, but it varies only on the amount and type of Taq polymerase and amount of template DNA. All mixtures were mixed, tightly sealed and placed in a thermal PCR cycler with a selected temperature profile.

Primer pairs for amplification of ITS 1 region (primer ITS1 and primer ITS2) and ITS 2 region (primer ITS3 and primer ITS4) described by White and colleagues [39]:

Forward primer - **ITS1** 5' GTTTCCGTAGGTGAACCTGC 3'

Reverse primer - **ITS2** 5' GCTGCGTTCTTCATCGATGC 3'

Forward primer - **ITS3** 5' GCATCGATGAAGAACGCAGC 3'

Reverse primer - **ITS4** 5' TCCTCCGCTTATTGATATGC 3'

Figure 9: PCR reagents on ice in the fume hood.

Figure 10: PCR cycler thermostat.

Table 6: »Touch up PCR« thermal profile for used for ITS1 and ITS2 regions.

Cycle	Temperature	Duration
1	95°C	3 min
2	95°C	1 min
3	45°C	30 sec
4	72°C	1 min
5	95°C	1 min
6	46°C	30 sec
7	72°C	1 min
8	95°C	1 min
9	47°C	30 sec
10	72°C	1 min
11	95°C	1 min
12	48°C	30 sec
13	72°C	1 min
14	95°C	1 min
15	49°C	30 sec
16	72°C	1 min
17	95°C	1 min
18	50°C	30 sec
19	72°C	1 min
20	Go to 17	30 cycles
21	72°C	2 min
22	4°C	∞

Touch up method was used in the process that increased the temperature in every cycle for 1°C in the phase of the annealing.

3.5 PCR AMPLIFICATION OF THE COI GENE

The universal primer pairs for invertebrates were used for PCR amplification of COI [12]. Primer LCO1490 F annealed on the light chain and primer HCO2198 R annealed on the heavy chain. PCR reactions were of the same composition as described in section 3.1.6. In some samples minor adjustments in the amount of Taq polymerase and amount of template DNA were made. For amplification of COI, were used the Top Taq polymeraze (Qiagen), Taq polymerase (Fermetntas) and Taq polymerase (Roche). The thermal profile from Table 7 was used for PCR amplification of COI.

Table 7: Thermal profile for COI amplification.

Cycle	Temperature	Duration
Initial denaturation	95°C	3 min
Denaturation	95°C	1 min
Annealing	40°C	1 min
Amplification	72°C	90 sec
	go to 3	35 cycles
Additional amplification	72°C	7 min
Cooling	7°C	∞

Universal invertebrate primer pair used for amplification of COI:

Forward primer - **LCO1490 F** 5'-GGTCAACAAATCATAAAGATATTGG-3'

Reverse primer - **HCO2198 R 5'**-TAAACTTCAGGGTGACCAAAAAATCA-3'

3.6 PROCESSING OF THE NUCLEOTIDE SEQUENCES

Sequencing was done with the same primer pairs as PCR amplification. 500 to 700 ng of PCR product in 15 µl was need for sequencing. Due to insufficient amount of PCR product in amplification of ITS region and COI it was necessary to re-amplify those samples. Amplification was done by the same PCR protocols, mixtures and temperature profiles, as in the previous procedure, as template was used PCR product. Samples were prepared according to the instructions given on the Macrogen website in the section for sequencing service (http://www.macrogen.com/eng/sequencing/automatic.jsp, 2005). Purification and sequencing was performed by a commercial service (Macrogen, Seoul, Korea). Both strands (sense and antisense) were sequenced to ensure the accuracy and precise detection of nucleotide polymorphism. The nucleotide sequences were received in the form of electropherograms and in suitable formats for further processing.

Nucleotide sequences were examined by eye with the program ChromasPro v1.34 [45] and sequences merged into contigs, which means that two sequences merged into a single consensus one. Ambiguous nucleotides in the contig were checked with the original sequence on electropherogram. Further processing of nucleotide sequences was performed with the software BioEdit [14]. Nucleotide sequences for ITS and COI regions were entered separately in the program.

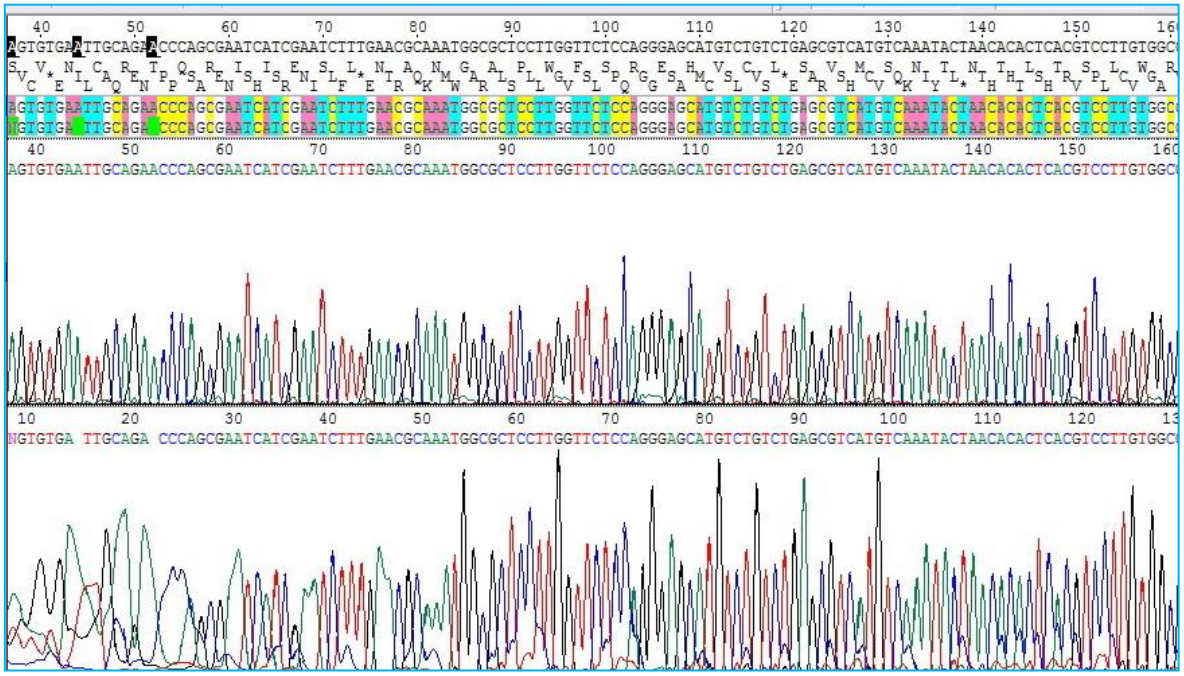

Figure 11: Example of a nucleotide sequence for the sample 11/06/RH2 ITS3-4 as seen with ChromasPro.

3.7 COMPARISON OF NUCLEOTIDE SEQUENCES WITH HOMOLOGOUS SEQUENCES IN THE GENBANK DATABASE

The nucleotide sequences were saved in FASTA format and then used to search for similar sequences in the GenBank database. I used the search algorithm BLAST, which is available on the website. At the time of search, no available sequences from *Rhizostoma pulmo* were available or from any closer species, the most similar nucleotide sequences, that I found, belonged to genera *Nemopilema, Rhopilema* and *Phyllorhiza* and the most appropriate nucleotide sequences for the phylogeographic analyses were included in alignment.

3.8 NUCLEOTIDE SEQUENCE ALIGNMENT

The alignments were made with the sequences of barrel jellyfish and selected similar sequences from GeneBank using Clustal [16]. In the application, settings were left by default. Before alignments, identical haplotypes were recognized by software Dambe [40].

The aligned sequences were prepared for further processing and calculations. The representative list of sequences used for alignment is in the Appendix 2 to Appendix 4.

3.9 PHYLOGEOGRAFIC ANALYSES

Phylogeographic analysis of barrel jellyfish (*Rhizostoma pulmo*) samples from lagoon Mar Menor (Spain), Lake Bizerte and Gulf of Tunis (Tunisia), Venetian Lagoon (Italy), Bay of Koper and Strunjan (Slovenia) was performed with software MEGA [38]. Phylogenetic relationships within the samples were calculated by using the Neighbor-Joining method. Sequences of species related species were used as outgroup. Outgroup nucleotide sequences for ITS1 were from *Rhopilema esculentum* (AB377586, AB377585), *Aurelia sp*. (AF461406) and *Aurelia aurita* (AY935206).

Nucleotide sequences from *Nemopilema nomurai* (EU373728), *Rhopilema esculentum* (EU373722), *Catostylus mosaicus* (AY319476), *Cassiopea xamachana* (AY319468), *Cassiopea andromeda* (AY319459) and *Phyllorhiza punctata* (EU363342) were used as outgroup for COI phylogeographic analyses.

4 RESULTS

DNA was isolated from 63 samples of barrel jellyfish were collected in several lagoons: Mar Menor (Spain), Lake Bizerte and Gulf of Tunis (Tunisia), Venetian, Varano (Italy) and in Bay of Koper and Strunjan (Slovenia). Samples were collected from 2005 to 2008 during winter blooms. Finally, only 36 samples were suitable for phylogeographic analyses (mainly due to insufficient quantity of isolated DNA amplification was not successful). In some samples, a considerably small amount of DNA was isolated in such a small amount that couldn't be seen on the agarose gel. Samples that weren't successfully amplified by PCR were removed from further analysis. The causes for low yield of DNA isolation could have been either due to sampling of dead jellyfish and/or poorly managed samples, such as low amount of tissue storage solution. This can lead to DNA degradation during the time of storage in the solution. Very short fragments (less than 500 bp) isolated from poor preserved tissue were visible on the gel. In Table 14 (Appendix 1) is the list of samples and their DNA concentrations (µg/ml) measured with fluorescence dye (Invitrogen, Quant-iT dsDNA BR Assay Kit). The Table 8 represent the list of the samples that were used for PCR amplification of ITS1 and ITS2 regions and COI.

Table 8: List of *Rhizostoma pulmo* samples used for amplification of ITS regions and COI.

Sampling site	Spain	Tunis		Italy	Slovenia	
	Mar Menor	Lake Bizerte	Gulf of Tunis	Venetian Lagoon	Bay of Koper	Bay of Strunjan
Sample name	20/08/RH/05	16/07/RH/01	15/07/RH/01	18/07/RH/01	13/07/RH/01	5/05/RH/01
	20/08/RH/07	16/07/RH/02	15/07/RH/02	18/07/RH/02	13/07/RH/02	5/05/RH/02
	20/08/RH/08	16/07/RH/03	15/07/RH/03	18/07/RH/03	12/06/RH/01	5/05/RH/03
	20/08/RH/13	16/07/RH/04	15/07/RH/05	18/07/RH/04	12/06/RH/02	5/05/RH/04
	20/08/RH/14	16/07/RH/05	15/07/RH/06		12/06/RH/03	
					11/06/RH/02	
					11/06/RH/03	
					11/06/RH/04	
					11/06/RH/05	
					9/06/RH/01	
					9/06/RH/02	

4.1 RESULTS OF DNA ISOLATION FROM BARREL JELLYFISH

Tissue samples from the umbrella margins and gonads were used to isolate DNA. Isolation was done by DNeasy Blood & Tissue Kit from Qiagen. The quality and quantity of isolated DNA were examined on agarose gel. The size of isolated DNA was determined by comparison with the DNA weight standard λ Hind III marker (Fermentas). An example of isolation is shown in Figure 12.

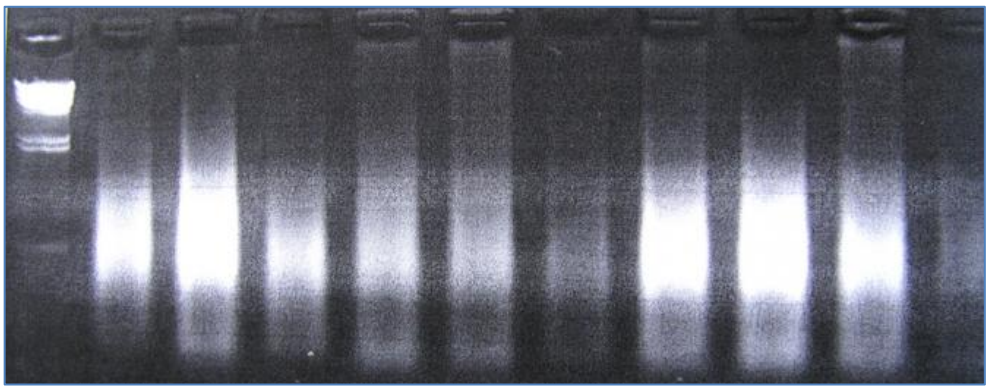

Figure 12: DNA isolation for sample 17/07/RH from Varano Lagoon (Italy).

DNeasy Blood & Tissue kit has proved to be suitable for isolating DNA from barrel jellyfish, but the yield was very low. The obtained amount of DNA ranged from 2.5 ng/ml to 216 ng/ml. Minimum of 10ng of DNA was needed for one PCR reaction (in volume of 50ul) Samples had relatively low DNA concentrations (measured with Qubit). Small quantity of DNA was mostly isolated from the umbrella margins, because the umbrella is composed mainly from mesoglea, which has a low amount of cells and consequently a lower DNA quantity. Mesoglea is made mostly from protein fibres and polysaccharides formed gelatinous substance, muscle fibres are also present at the umbrella margins. Quality of DNA is proportional to a long storage time of tissue, and it was one of the main factors for the increased degradation of DNA in the tissues. Storing period in the freezer was from one to four years, before the isolation was conducted.

4.2 RESULTS OF PCR AMPLIFICATION OF ITS1

ITS 1 region was amplified by primer pair F-ITS1 and R-ITS2. Reaction mixtures and temperature profile that were used are described in sections 3.1.6 and 3.4. The composition of the reaction mixture varied depending on the amount of template DNA and Taq polymerase used. In the experiment three different polymerases were used: Top Taq polymerase (Qiagen) and Taq polymerase of Roche (Cat. No. 11146173001) and Fermentas (Cat. No. EP0402). The amount of used Taq polymerase was 0,25 µl (1,25U per 50 µl reaction). Thermal profile for PCR of ITS regions was "touch up" with the initial annealing temperature from 45 and then raised to50^{o}C. ITS1 was successfully amplified in total of 21 samples collected at lagoon Mar Menor (Spain), Gulf of Tunis and Lake Bizerte Lagoon (Tunisia), Venetian Lagoon (Italy) and Bay of Koper and Strunjan (Slovenia), representing 33 percent from the initial 63 samples. The most effective was amplification with Top Taq DNA from Qiagen. Five samples from lagoon Mar Menor (Spain) were amplified with the Taq DNA polymerase from Fermentas. The list of successfully amplified samples is shown in the Table 9. The amount of PCR product visible on agarose gel was expressed with sign (+), determined by inspection and evaluation of product colour intensity on the agarose gel. Weak intensity was marked with +, medium with ++ and high intensity by +++. Amplified DNA was coloured with ethidium bromide, which intercalated between both chains and emitted fluoresce light when illuminated by UV. Differences in the amount of PCR product was caused by the quantity and quality of isolated DNA. Most PCR products had satisfactory intensity (Figure 13). The sizes of PCR products were determined by comparing with the DNA weight standard GeneRuler 100bp DNA ladder (Fermentas) on agarose gel. ITS1 product had length from 368 to 432 bp when primer sequences were removed. Efficiency of the PCR amplification process was controlled by a positive control. Isolated DNA from *Pelagia noctiluca* were used for positive control and purified distilled water instead of template DNA was used for negative control in PCR.

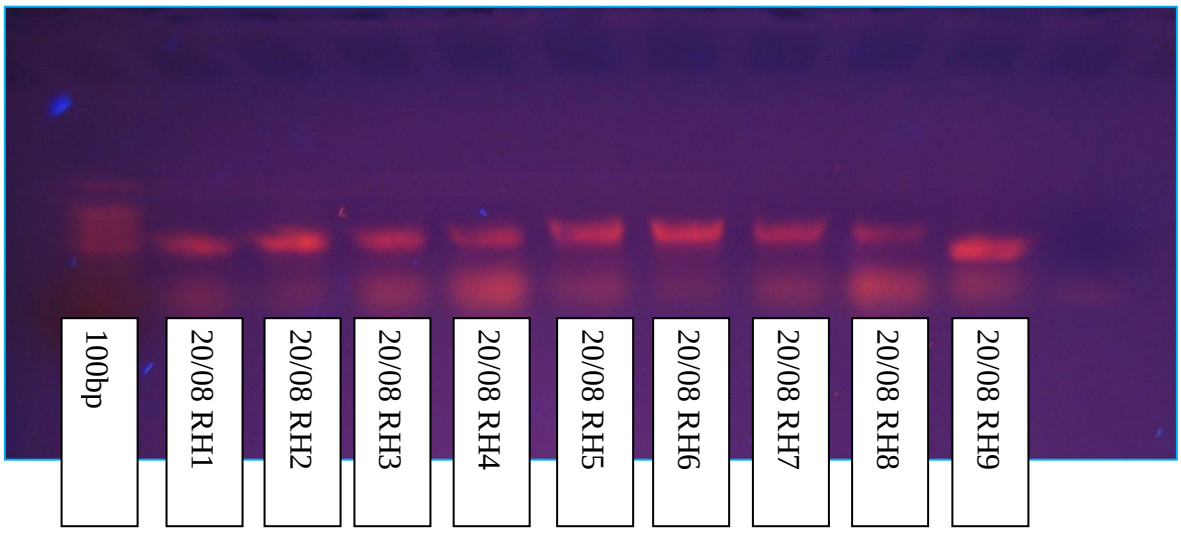

Figure 13: PCR amplification of ITS1 regions from samples of barrel jellyfish (*Rhizostoma pulmo*). First lane on left: marker 100bp, 20/08/RH/01, 20/08/RH/02, 20/08/RH/03, 20/08/RH/04, 20/08/RH/05, 20/08/RH/06, 20/08/RH/07, 20/08/RH/08 and 20/08/RH/09.

Some samples were not amplified in the amount sufficient for further manipulations (sequencing), those samples were re- amplified in PCR again under the same conditions as the first PCR. On the Figure 13 are re-amplified samples from the lagoon Mar Menor (Spain), samples were re-amplified from un-purified PCR product from the previous PCR reaction. The eleven samples were re-amplified in PCR to acquire sufficient amount of specific product (approximately 50 ng/µl of product) for sequencing. The following samples had already available sequences of ITS 1 region from previous analysis. (Spain: 20/08/RH5, RH7, RH8, RH13, RH14, Tunisia: 15/07/RH1, RH2, RH3, Italy: 18/07/RH1, RH2, RH3 RH4). In Figure 16 is visible smear, representing non-specific product or small fragments of isolated DNA, which did not affect the result of sequencing. In the Table 9 are the sorted the amplified DNA samples with their intensity.

Table 9: List of successfully amplified ITS1 region in samples.

Sampling site	Sample name	ITS1- Amplified product quantity
Mar Menor Lagoon (Spain)	20/08/RH/05	++
	20/08/RH/07	+
	20/08/RH/08	+
	20/08/RH/13	+++
	20/08/RH/14	+++
Lake of Bizerte (Tunisia)	16/07/RH/01	++
	16/07/RH/02	++
	16/07/RH/04	+++
	16/07/RH/05	+++
Gulf of Tunis (Tunisia)	15/07/RH/01	+
	15/07/RH/02	+
	15/07/RH/03	+
	15/07/RH/05	+
	15/07/RH/06	+
Venetian Lagoon (Italy)	18/07/RH/01	++
	18/07/RH/02	++
	18/07/RH/03	++
	18/07/RH/04	++
Bay of Koper (Slovenia)	9/06/RH/02	+
Bay of Strunjan (Slovenia)	5/05/RH/01	+
	5/05/RH/02	++

4.3 PCR AMPLIFICATION OF ITS2

ITS2 region was amplified by a pair of primers F-ITS3 and R-ITS4. Reaction mixtures and temperature profile that were used are described in sections 3.1.6 and 3.4. Three different Taq polymerases were used for amplification of ITS 2 region: Top Taq polymerase (Qiagen), Taq polymerase from Roche (Cat. No. 11146173001) and Fermentas (Cat. No. EP0402). The amount of used Taq polymerase was 0,25 µl (1,25U per 50 µl PCR reaction). PCR thermal profile was "touch up", with starting annealing of primers at 45^{o}C to50^{o}C.

ITS 2 region was successfully amplified in 34 samples, representing more than half of the initial 63 samples. The most efficient amplification was obtained by Top Taq polymerase from Qiagen and Taq polymerase of Fermentas (Cat. No. EP0402). Both polymerases have equal amplification efficiency and the quantity of product was similar. The size of amplified products was determined by comparing to a DNA weight standard GeneRuler 100bp DNA ladder (100-1000bp, Fermentas) on agarose gel. DNA isolated from *Pelagia noctiluca* was used as a positive control during PCR and purified water instead of template DNA was a negative control.

Table 10: List of successfully amplified ITS2 region in samples.

Sampling site		Sample name	ITS2- Amplified product quantity
Spain	Mar Menor	20/08/RH/05	+
		20/08/RH/07	+
		20/08/RH/08	+
		20/08/RH/13	+++
		20/08/RH/14	+++
Tunis	Lake Bizerte	16/07/RH/01	+++
		16/07/RH/02	+++
		16/07/RH/03	+++
		16/07/RH/04	+++
		16/07/RH/05	+++
	Gulf of Tunis	15/07/RH/01	+
		15/07/RH/02	+
		15/07/RH/03	+
		15/07/RH/05	+++
		15/07/RH/06	+++
Italy	Venetian Lagoon	18/07/RH/01	++
		18/07/RH/02	++
		18/07/RH/03	++
		18/07/RH/04	+
Slovenia	Bay of Koper	13/07/RH/01	+++
		13/07/RH/02	+
		12/06/RH/01	+++
		12/06/RH/02	++
		12/06/RH/03	+
		11/06/RH/02	+++
		11/06/RH/03	+++
		11/06/RH/04	++
		11/06/RH/05	++
		9/06/RH/01	++
		9/06/RH/02	+++
	Bay of Koper	5/05/RH/01	+++
		5/05/RH/02	+++
		5/05/RH/03	++
		5/05/RH/04	+++

Figure 14 represent re-amplified samples from Bay of Tunisia (Tunisia), using un-purified PCR product amplified from the previous PCR reaction. Twenty-two samples were re-amplified in order to get sufficient amount for sequencing. For the remaining 12 samples, I

got the ITS2 region sequences from a previous analysis. In the Figure 14 is clearly visible that strong intensity of the specific product is proportional with quantity of PCR product and no amplified non-specific products.

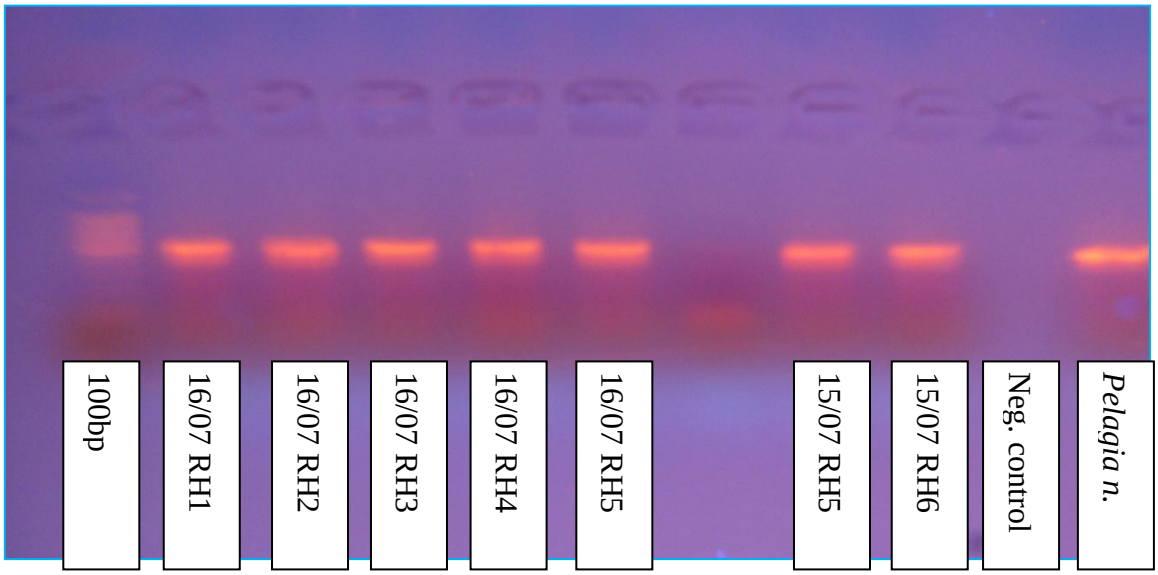

Figure 14: PCR amplification of ITS2 regions from samples of barrel jellyfish (*Rhizostoma pulmo*). First lane on left: marker 100bp, 16/07/RH/01, 16/07/RH/02, 16/07/RH/03, 16/07/RH/04, 16/07/RH/05, 15/07/RH/05, 15/07/RH/06, negative and positive control.

4.4 PCR AMPLIFICATION OF COI

COI was amplified by a pair of primers LCO1490 F and HCO2198 R [12]. Amplification of COI was done in the same way as described earlier for amplification of ITS regions. Similarly, three different polymerases were used for amplification and the most efficient Top Taq polymerase (Qiagen) in comparison with Taq polymerase from Roche (Cat. No. 11146173001) Fermentas (Cat. No. E00491). PCR reaction mixture and thermal profile that were used are described in sections 3.1.6 and 3.5. In the PCR reaction 1,25 U of Taq polymerase were used per 50 µl reaction and an amount of template DNA of 2-4 µl, depending on the DNA concentration.

Amplified products of COI were 700bp long, which correspond to predicted length. COI was successfully amplified in 14 samples that are roughly 22 percent from the initial 63 samples. The list of samples that gave a specific product is shown in the Table 11. The intensity of PCR product was expressed by + signs as described above for ITS regions. The strongest intensity on agarose gel had samples 16/07/RH/02, 16/07/RH/05, 18/07/RH/02, 18/07/RH/03 and 18/07/RH/04 that also had the highest quantity of specific product. The measured concentration of DNA for these samples was between 5 and 36 µg/ml. The most efficient in amplification was Top Taq polymerase from Qiagen. Q-solution (Qiagen) is likely to be the cause for its higher performance and better amplification results, thanks to betaine that alters the conformation and melting characteristics of DNA, allowing amplification of difficult templates. Amplification with Taq polymerase Fermentas and Roche was unsuccessful, without specific PCR product. The size of amplified products was determined by comparing with a DNA weight standard GeneRuler 100bp DNA ladder (Fermentas) on agarose gel. As a positive control in the DNA amplification, DNA from *Pelagia noctiluca* was used and for a negative control purified distilled water instead of template DNA.

Table 11: List of successfully amplified COI from samples.

Sampling site	Sample name	COI- Amplified product quantity
Lake of Bizerte (Tunisia)	16/07/RH/01	++
	16/07/RH/02	++
	16/07/RH/03	+
	16/07/RH/04	+
	16/07/RH/05	+++
Gulf of Tunis (Tunisia)	15/07/RH/01	+
	15/07/RH/02	++
	15/07/RH/03	+
	15/07/RH/05	++
	15/07/RH/06	++
Venetian lagoon (Italy)	18/07/RH/01	+
	18/07/RH/02	+++
	18/07/RH/03	+++
	18/07/RH/04	+++

Amplified COI products in the Figure 15 were re-amplified by using the non-purified PCR product from the previous PCR reaction. Eight samples were re-amplified to get sufficient amount (approximately 50 ng/ul of product) of PCR product for sequencing. Few COI sequences from a previous analysis were also included. PCR products are on Figure 15, samples differ from each other by the quantity of the specific product, related to the amount of template DNA. The amount of template DNA for the PCR was between 15 to 180ng, added in volume of 5µl into 50 µl reaction. On the bottom of the lane in some samples: 15/07/RH5, 15/07/RH6 and 16/07/RH1 are smear of non-specific product, apart from specific product, those were much shorter fragments.

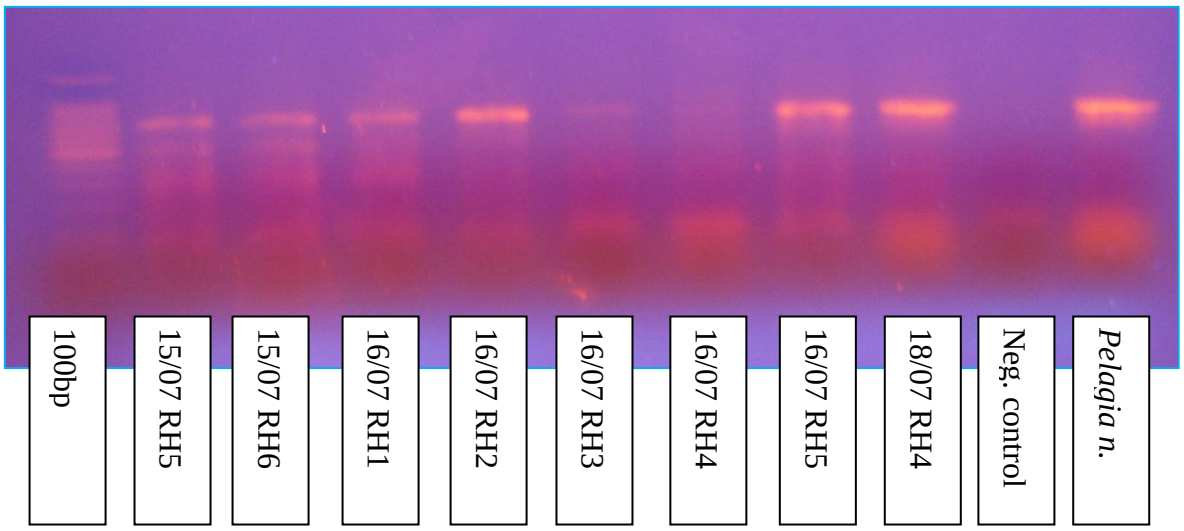

Figure 15: PCR amplification of COI from samples of barrel jellyfish (*Rhizostoma pulmo*). From left lane: 15/07/RH/05, 15/07/RH/06, 16/07/RH/01, 16/07/RH/02, 16/07/RH/03, 16/07/RH/04, 16/07/RH/05, 18/07/RH/04, negative control and positive control (DNA from *Pelagia noctiluca*).

4.5 SAMPLE SEQUENCING

The next step was to obtain nucleotide sequences for phylogeographic analysis. I sent the samples for sequencing to commercial service (Macrogen, Seoul, Korea). All nucleotide sequences were manually inspected, especially in places with ambiguous nucleotides. Both complementary strands were put together in consensus sequence by software ChromasPro v1.34 [45]. Primers were removed manually from consensus sequence. All ambiguous

bases in consensus sequences were checked again on original electropherograms. Data in Table 12 shows that COI sequences length was between 655 bp and 688 bp as predicted.

Table 12: Length polymorphism in COI region of *Rhizostoma pulmo.*

Sampling site	Code name	COI lenght (bp)
Venetian Lagoon-Italy	18/07/RH/01	671
	18/07/RH/02	667
	18/07/RH/04	664
Gulf of Tunis and Lake of Bizerte-Tunisia	15/07/RH/01	655
	15/07/RH/02	655
	15/07/RH/03	655
	16/07/RH/02	688
	16/07/RH/05	688
Bay of Koper-Slovenia	14/00/RH/03	655
	14/00/RH/04	655
	14/00/RH/07	655
	14/00/RH/08	655

More length variability was found in ITS1and ITS 2 sequences that are shown in the Table 13 and Table 14 below. The length of ITS 1 was from 368 to 432 bp, while the ITS 2 length was between 426 to 440 bp.

Table 13: Length polymorphism in ITS 1 region of *Rhizostoma pulmo.*

Sampling site	Code name	ITS1 lenght (bp)
Mar Menor Lagoon-Spain	20/08/RH/05	407
	20/08/RH/07	407
	20/08/RH/13	407
	20/08/RH/14	407
Lake of Bizerte-Tunisia	16/07/RH/01	419
	16/07/RH/02	432
	16/07/RH/04	421
	16/07/RH/05	425
Gulf of Tunis-Tunisia	15/07/RH/01	368
	15/07/RH/02	370
	15/07/RH/03	369
	15/07/RH/05	374
	15/07/RH/06	371
Venetian Lagoon-Italy	18/07/RH/01	411
	18/07/RH/02	409
	18/07/RH/03	408
	18/07/RH/04	408
Bay of Koper-Slovenia	9/06/RH/02	414
	14/00/RH/08	369
Bay of Strunjan-Slovenia	5/05/RH/01	409
	5/05/RH/02	410

Table 14: Length polymorphism in ITS 2 region of *Rhizostoma pulmo.*

Sampling site	Code name	ITS2 lenght (bp)
Mar Menor Lagoon-Spain	20/08/RH/05	426
	20/08/RH/07	426
	20/08/RH/08	426
	20/08/RH/13	426
	20/08/RH/14	426
Lake of Bizerte-Tunisia	16/07/RH/01	426
	16/07/RH/02	426
	16/07/RH/03	429
	16/07/RH/04	429
	16/07/RH/05	427
Gulf of Tunis-Tunisia	15/07/RH/01	426
	15/07/RH/02	426
	15/07/RH/03	426
	15/07/RH/05	426
	15/07/RH/06	426
Venetian Lagoon-Italy	18/07/RH/01	429
	18/07/RH/02	430
	18/07/RH/03	428
	18/07/RH/04	427
Bay of Koper-Slovenia	13/07/RH/01	427
	12/06/RH/01	426
	12/06/RH/02	431
	12/06/RH/03	436
	11/06/RH/02	426
	11/06/RH/03	427
	11/06/RH/04	429
	11/06/RH/05	428
	9/06/RH/01	428
	9/06/RH/02	433
Bay of Strunjan-Slovenia	5/05/RH/01	431
	5/05/RH/02	440
	5/05/RH/03	435

4.5.1 Sequence analyses

Contigs of each marker (ITS1, ITS2 and COI) were analysed separately. Identical haplotypes were searched by software Dambe [40] and later on all the identical sequences eliminated. Information on unique haplotypes and their frequency according to geographic origin is presented in Table 15 for COI. In total of 12 COI sequences, six different haplotypes with a length of 655 base pairs were found. The most frequent haplotype was RH1501z a representative from the Gulf of Tunis.

Table 15: Frequencies of COI haplotypes from *Rhizostoma pulmo* at different sampling sites.

Genetic marker COI					
Biogeographic region	Sampling site	Sample code	Haplotype name	Haplotype frequency	COI lenght (bp)
Northern Adriatic	Venetian Lagoon	18/07/RH/01	RH1801c	1	655
		18/07/RH/02	RH1802z	2	
		18/07/RH/04			
	Bay of Koper	14/00/RH/03	RH1403z	2	655
		14/00/RH/07			
		14/00/RH/04	RH1404z	2	655
		14/00/RH/08			
Central Mediterranean Sea	Gulf of Tunis	15/07/RH/01	RH1501z	3	655
		15/07/RH/02			
		15/07/RH/03			
	Lake Bizerte	16/07/RH/02	RH1602z	2	655
		16/07/RH/05			

Table 16: Frequencies of ITS 1 haplotypes from *Rhizostoma pulmo* at different sampling sites.

Genetic marker ITS1					
Biogeographic region	Sampling site	Sample code	Haplotype name	Haplotype frequency	ITS 1 lenght (bp)
Northern Adriatic	Venetian Lagoon	18/07/RH/01	RH502IT1z	4	410
		18/07/RH/02			
		18/07/RH/03			
		18/07/RH/04			
	Bay of Strunjan	5/05/RH/01	RH501IT1	1	409
		5/05/RH/02	RH502IT1z	1	410
	Bay of Koper	14/00/RH/08	RH1408IT1z	1	369
Western Mediterranean Sea	Mar Menor	20/08/RH/13	RH502IT1z	2	410
		20/08/RH/07			
		20/08/RH/05	RH2005IT1z	2	407
		20/08/RH/14			
Central Mediterranean Sea	Lake Bizerte	16/07/RH/01	RH1601	2	419
		16/07/RH/04			
		16/07/RH/02	RH1602	2	423
		16/07/RH/05			
	Gulf of Tunis	15/07/RH/01	RH1408IT1z	5	369
		15/07/RH/02			
		15/07/RH/03			
		15/07/RH/05			
		15/07/RH/06			

No variability was found among ITS 2 sequences from all 33 samples and one haplotype (RH1801IT4z) was present in all samples from different sampling locations (see Table 17). This particular haplotype had length of 426 bp. Since they were all identical, I didn't use them in phylogenetic analysis.

Table 17: Frequencies of ITS2 haplotypes from *Rhizostoma pulmo* at different sampling sites.

Genetic marker ITS2					
Biogeographic region	Sampling site	Sample code	Haplotype name	Haplotype frequency	ITS2 lenght (bp)
Northern Adriatic	Venetian Lagoon	18/07/RH/01	RH1801IT4z	33	426
		18/07/RH/02			
		18/07/RH/03			
		18/07/RH/04			
	Bay of Koper	13/07/RH/01			
		13/07/RH/02			
	Bay of Koper	12/06/RH/01			
		12/06/RH/02			
		12/06/RH/03			
	Bay of Koper	11/06/RH/02			
		11/06/RH/03			
		11/06/RH/04			
		11/06/RH/05			
	Bay of Koper	9/06/RH/01			
		9/06/RH/02			
	Bay of Strunjan	5/05/RH/01			
		5/05/RH/02			
		5/05/RH/03			
Central Mediterranean Sea	Lake Bizerte	16/07/RH/01			
		16/07/RH/05			
		16/07/RH/03			
		16/07/RH/04			
		16/07/RH/02			
	Gulf of Tunis	15/07/RH/01			
		15/07/RH/02			
		15/07/RH/03			
		15/07/RH/05			
		15/07/RH/06			
Western Mediterranean Sea	Mar Menor	20/08/RH/05			
		20/08/RH/13			
		20/08/RH/07			
		20/08/RH/08			
		20/08/RH/14			

Alignment of ITS 1 revealed deletions (37 or 10 bp long) in some of those sequences. More details were on Table 18. Most common is shorter deletion (10 bp) found in 15 haplotypes from all sampled locations. Final alignments were made by using BioEdit [14].

Table 18: List of haplotypes with deletion found in ITS 1 region of *Rhizostoma pulmo.*

	Type of indel in ITS 1 region	
	Deletion 10 bp (from site 289 to site 298)	Deletion 37 bp (from site 152 to site 189)
Northern Adriatic	RH501IT1 RH502IT1 RH1801IT1 RH1802IT1 RH1804IT1	RH1408IT1 RH1501IT1 RH1502IT1 RH1503IT1 RH1505IT1 RH1506IT1
Mar Menor	RH2005IT1 RH207IT1 RH2013IT1 RH2014IT1	
Tunis	RH1408IT1 RH1501IT1 RH1502IT1 RH1503IT1 RH1505IT1 RH1506IT1	

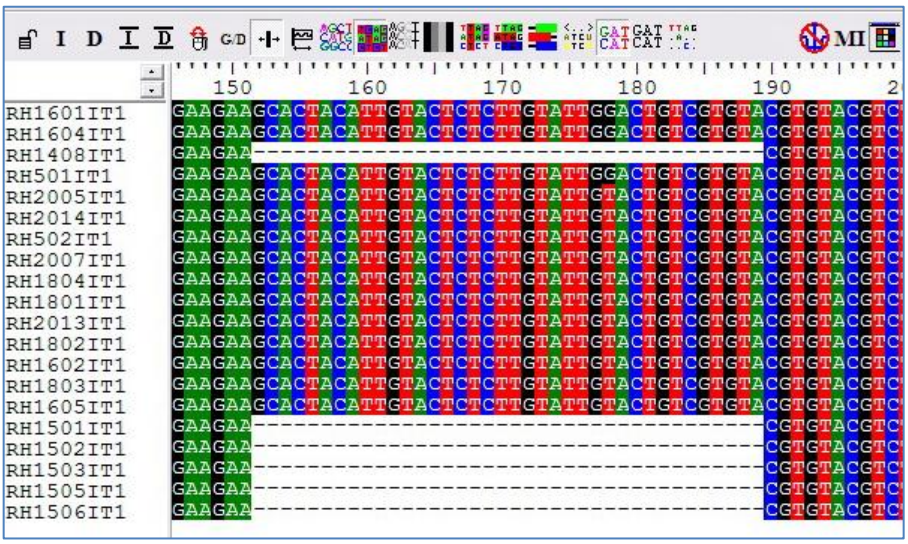

Figure 16: Example of deletion sites in nucleotide sequences for ITS.

4.6 PHYLOGEOGRAPHIC ANALYSES

Phylogeographic analyses were made with the software MEGA [38], and also used to draw phylogenetic trees for ITS1 and COI sequences. Only unique haplotypes were used. The method of Neighbor-Joining was used for phylogenetic calculations. The statistical reliability of tree branching was tested with bootstrap method with 1000 replications [11].

The phylogenetic tree was rooted with outgroup sequences of the following organisms:

ITS 1: *Rhopilema esculentum* (AB377586, AB377585), *Aurelia sp.* (AF461406) and *Aurelia aurita* (AY935206).

COI: *Nemopilema nomurai* (EU373728), *Rhopilema esculentum* (EU373722), *Catostylus mosaicus* (AY319476), *Cassiopea xamachana* (AY319468), *Cassiopea andromeda* (AY319459) and *Phyllorhiza punctata* (EU363342).

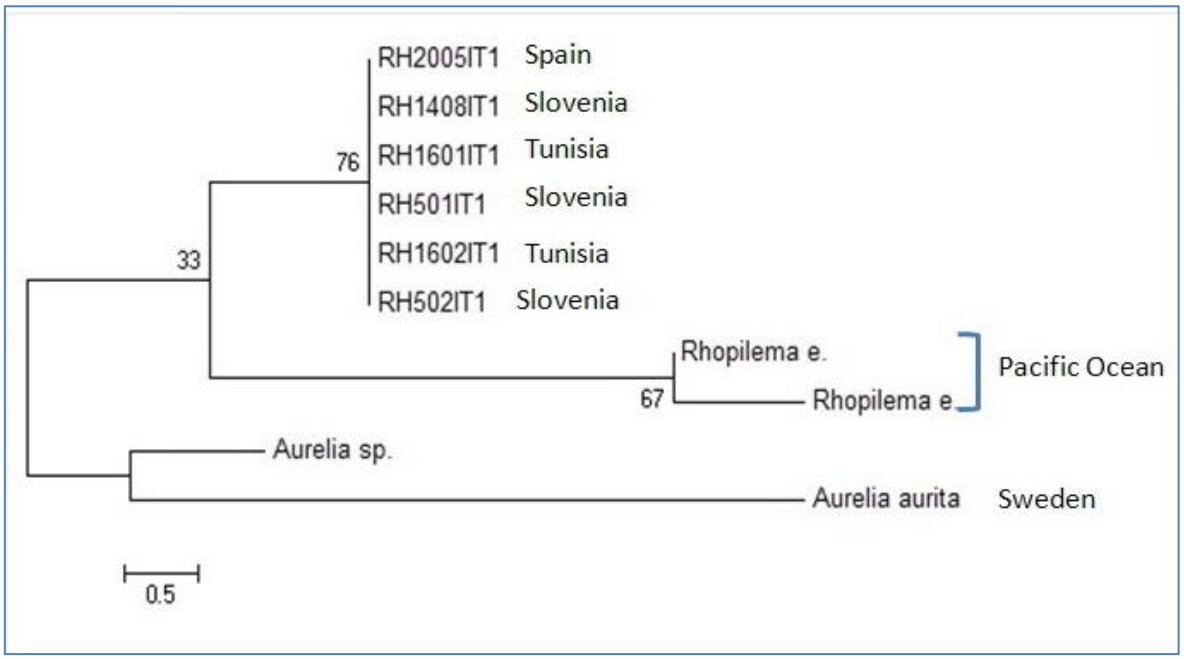

Figure 17: Phylogenetic tree for ITS1 done with Neighbour-Joining method.

Phylogenetic analysis of ITS1 made it clear that the nucleotide sequences of the barrel jellyfish from Tunisia, Spain and Slovenia, form the same phylogenetic group. The phylogenetic group was supported by low bootstrap value (76 probabilities). In ITS1 were 159 conserved, 246 variable and 237 phylogenetically informative sites.

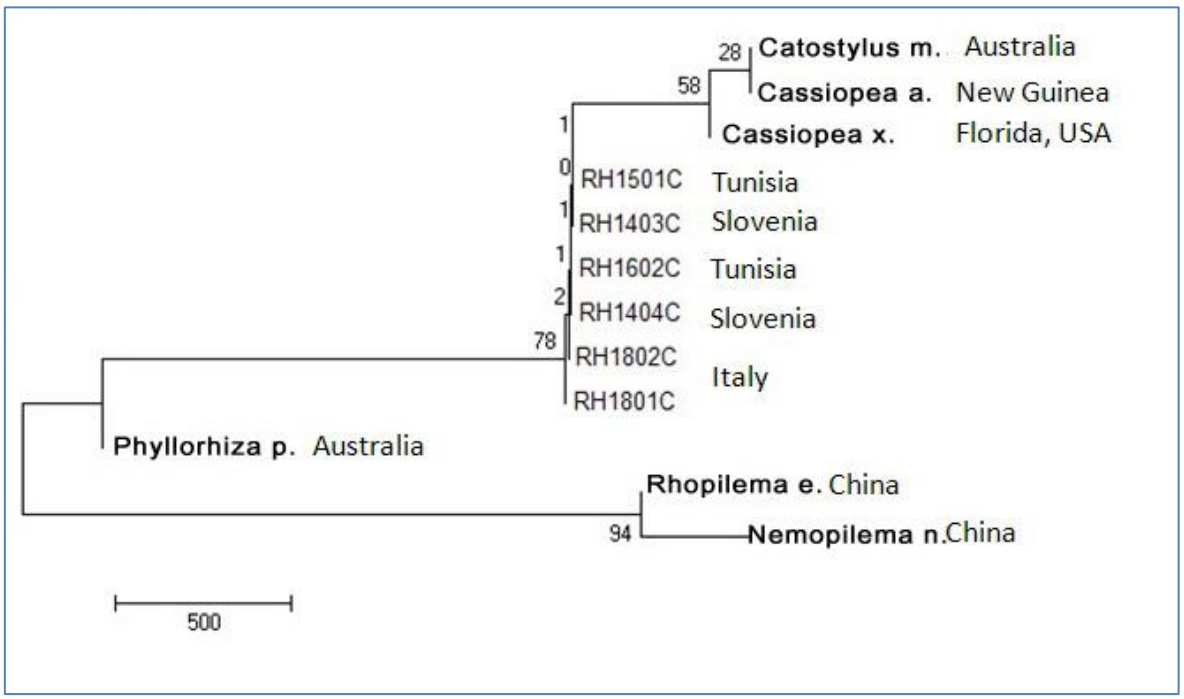

Figure 18: Phylogenetic tree for COI made with Neighbour-Joining method.

Phylogenetic analysis of COI had showed that haplotypes of barrel jellyfish from Tunisia, Italy and Slovenia, form the same phylogenetic group. This phylogenetic group was supported by bootstrap values (78 probabilities). COI sequences had 354 conserved sites, 256 variable and 211 phylogenetically informative sites, which are the basis for the phylogenetic analysis. Otherwise, samples from Tunisia and Slovenia showed a weak support of 1 to 2 percent makes it statistically insignificant.

5 SUMMARY

In recent decades, more and more attention focused on the mass occurrence or blooms of jellyfish. Although, dense jellyfish aggregations occur in natural and healthy pelagic ecosystems, reports on mass phenomenon are increasing. In the Mediterranean Sea and Atlantic Ocean is widespread the barrel jellyfish (*Rhizostoma pulmo*) species belong to class of Scyphozoa. In about 135 years the species, was occasionally observed in mass aggregations in the northern Adriatic, while the last few years are appearing more frequently in large numbers [22]. Many scientific papers indicate that the possible causes for the blooms are anthropogenic origins (eutrophication, hypoxia, climate change, destruction of natural ecosystems, and disruption in the food chain).

Recently, has been the life cycle of this organism re-described again with more details on polyp phase than in the previous descriptions [13]. In this master thesis I focused on the barrel jellyfish phylogeography across the Mediterranean Sea. At the beginning of the experiment there were no available sequences in GeneBank, to begin the analysis. In order to investigate the phylogeographic structure, I used two different genetic markers, one on mitochondrial DNA (COI) and another on nuclear DNA (ITS 1 and ITS 2 regions). Analysed samples were from the following biogeographical regions (Northern Adriatic, Central and Western Mediterranean). The genetic markers were amplified with PCR, and their nucleotide sequences were obtained. The universal primers [LCO1490 and HCO2198, 12] were used for COI amplifications. Regions ITS 1 and ITS 2 were amplified with the pairs of primers ITS1/ITS2 and ITS3/ITS4 [39]. COI regions were successfully amplified in 14 specimens, ITS1 from 23 specimens and ITS2 from 32 specimens.

Phylogenetic analyses were made with the program MEGA [38]. Calculations were performed with the method of Neighbour-Joining model. Results from the phylogenetic analysis showed, that the samples belong to a single homogenous group, supported by ITS 1 and COI markers. Haplotypes representing ITS2 regions were identical; therefore they weren't used in phylogenetic analyses. Based on phylogenetic analyses of COI and ITS1, no phylogeographic pattern among them was found across the Mediterranean Sea.

Recent phylogeographic analysis on *Rhizostoma pulmo*, *Pelagia noctiluca* and *Aurelia aurita* in the European seas showed an interesting fact. *Rhizostoma pulmo* and *Pelagia noctiluca* have a similar phylogenetic structure even that they both have different reproductive cycles. The phylogeographic analyses revealed that both species have no structuring connected with geographic area, but analysed haplotypes were mixed and there are no significant differences among them. Moreover, *Aurelia aurita* and *Rhizostoma pulmo* have the same biphasic reproductive cycle and no similar phylogenetic patterns.

The results clearly indicate the presence of only one big population of *Rhizostoma pulmo* in the Mediterranean Sea. It can be partially attributed to the species propagation cycle, with the presence of asexual generation in the form attached polyps and sexual generation in the form of free floating medusa. The results also confirmed the absence of significant barriers that would cause a restricted gene flow between the sampling sites around Mediterranean Sea.

6 LITERATURE

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7 APPENDIX

Appendix 1: Results of fluorimetric measurements of and calculated DNA concentrations.

Sampling Site	Sample name	Qubit measurements	DNA Concentration
			DNA(ug/mL)
Spain	20/08/RH/01	0.0339	6.78
	20/08/RH/02	0.0122	2.44
	20/08/RH/03	0.0359	7.18
	20/08/RH/04	0.237	47.40
	20/08/RH/05	0.0233	4.66
	20/08/RH/06	0.282	56.40
	20/08/RH/07	0.0801	16.02
	20/08/RH/08	0.196	39.20
	20/08/RH/09	0.111	22.20
	20/08/RH/10	0.127	25.40
	20/08/RH/11	0.139	27.80
	20/08/RH/12	0.0424	8.48
	20/08/RH/13	0.226	45.20
	20/08/RH/14	0.023	4.60
Tunisia	16/07/RH/01	0.0744	14.88
	16/07/RH/02	0.0271	5.42
	16/07/RH/03	No signal	
	16/07/RH/04	0.0772	15.44
	16/07/RH/05	0.104	20.80
	16/07/RH/06	No signal	
	15/07/RH/01	0.0532	10.64
	15/07/RH/02	0.0762	15.24
	15/07/RH/03	0.0606	12.12
	15/07/RH/04	No signal	
	15/07/RH/05	0.0164	3.28
	15/07/RH/06	0.035	7.00
Italy	17/08/RH/01	0.0833	16.66
	17/08/RH/02	0.0286	5.72
	17/08/RH/03	0.127	25.40
	17/08/RH/04	0.0843	16.86
	17/08/RH/05	0.0656	13.12
	17/08/RH/06	0.086	17.20
	17/08/RH/07	0.173	34.60
	17/08/RH/08	0.223	44.60
	17/08/RH/09	0.133	26.60
	17/08/RH/10	0.0648	19.26
	18/07/RH/01	0.205	41.00
	18/07/RH/02	0.123	24.60
	18/07/RH/03	0.112	22.40
	18/07/RH/04	0.181	36.20
Slovenia	13/07/RH/01	0.22	44.00
	13/07/RH/02	0.207	41.40
	12/06/RH/01	0.0826	16.52
	12/06/RH/02	0.0828	16.56
	12/06/RH/03	0.079	15.80
	11/06/RH/01	No signal	
	11/06/RH/02	0.0417	8.34
	11/06/RH/03	0.323	64.60
	11/06/RH/04	0.0891	17.82
	11/06/RH/05	0.163	32.60
	11/06/RH/06	No signal	
	9/06/RH/01	0.249	49.80
	9/06/RH/02	0.327	65.40
	9/06/RH/03	No signal	
	5/05/RH/01	0.226	45.20
	5/05/RH/02	0.776	155.20
	5/05/RH/03	0.278	55.60
	5/05/RH/04	1.08	216.00
	5/05/RH/06	0.286	57.20
	5/05/RH/07	0.316	63.20

Appendix 2: Representative ITS1 sequences of barrel jellyfish (*Rhizostoma pulmo*) in FASTA format (name of haplotype; GenBank accession number)

BAY OF STRUNJAN (SLOVENIA)

>RH501IT1; GQ999576

GGAAGGATCATTACTGATATATTGAGGATGACCGTTCGATCACGAACAGCCGTCTGTCGATTGC
TTGTCACACTGATTGAAAAACCAGTTTTGACAAACACCACTGTGAACCTGTACCCATCCGTGTG
AGGTGGACAGAGTGCACGAAGAAGCACTACATTGTACTCTCTTGGATTGGTACTGTCGTGTACG
TGTACGTCTGTGCTTAAATGAAAGGCGATTCCCTTTCGGCCTCACATTGGAGTTTTCTTATATCG
TATTCGGTCGGTTGTTTTCGAATTCGTTCGAAAACAGTCAGCCACACTTTGCACACATAAATATT
TACTGAATATTTTGGACGTGCTTGCTTTGCTGGCACGGCGATGGAAAAATGAAATACAACTTCT
AACGATGGATACTCTTGGCTCGT

>RH502IT1; GQ999574

GGAAGGATCGATTAGCTGCATATATTGAGGATGACCGTTCGATCACGAACAGCCGTCTGTCGAT
TGCTTGTCACACTGATTGAAAAACCAGTTTTGACAAACACCACTGTGAACCTGTACCCATCCGT
GTGAGGTGGACAGAGTGCACGAAGAAGCACTACATTGTACTCTCTTGTATTGTACTGTCGTGTA
CGTGTACGTCTGTGCTTAAATGAAAGGCGATTCCCTTTCGGCCTCACATTGGAGTTTTCTTATAT
CGTATTCGGTCGGTTGTTTTCGAATTCGTTCGAAAACAGTCAGCCACACTTTGCACACATAAATA
TTTACTGAATATTTTGGACGTGCTTGCTTTGCTGGCACGGCGATGGAAAAATGAAATACAACTT
CTAACGGTGGATCTCTTGGCTCGT

BAY OF KOPER (SLOVENIA)

>RH1408IT1; GQ999575

GGAAGGATCATTACTGATATATTGAGGATGACCGTTCGATCACGAACAGCCGTCTGTCGATTGC
TTGTCACACTGATTGAAAAACCAGTTTTGACAAACACCACTGTGAACCTGTACCCATCCGTGTG
AGGTGGACAGAGTGCACGAAGAACGTGTACGTCTGTGCTTAAATGAAAGGCGATTCCCTTTCGG
CCTCACATTGGAGTTTTCTTATATCGTATTCGGTCGGTTGTTTTCGAATTCGTTCGAAAACAGTC
AGCCACACTTTGCACACATAAATATTTACTGAATATTTTGGACGTGCTTGCTTTGCTGGCACGGC
GATGGAAAAATGAAATACAACTTCTAACGGTGGATCTCTTGGCTCGT

VENETIAN LAGOON (ITALY)

>RH1801IT1; GQ999574

GGAAGGATCATGTAGCTGATATATTGAGGATGACCGTTCGATCACGAACAGCCGTCTGTCGATT
GCTTGTCACACTGATTGAAAAACCAGTTTTGACAAACACCACTGTGAACCTGTACCCATCCGTG
TGAGGTGGACAGAGTGCACGAAGAAGCACTACATTGTACTCTCTTGTATTGTACTGTCGTGTAC
GTGTACGTCTGTGCTTAAATGAAAGGCGATTCCCTTTCGGCCTCACATTGGAGTTTTCTTATATC
GTATTCGGTCGGTTGTTTTCGAATTCGTTCGAAAACAGTCAGCCACACTTTGCACACATAAATAT
TTACTGAATATTTTGGACGTGCTTGCTTTGCTGGCACGGCGATGGAAAAATGAAATACAACTTC
TAACGGTGGATACTCTTGGCTCGT

GULF OF TUNIS (TUNISIA)

>RH1501IT1; GQ999575

GGAAGGATCATTACTGATATATTGAGGATGACCGTTCGATCACGAACAGCCGTCTGTCGATTGC
TTGTCACACTGATTGAAAAACCAGTTTTGACAAACACCACTGTGAACCTGTACCCATCCGTGTG
AGGTGGACAGAGTGCACGAAGAACGTGTACGTCTGTGCTTAAATGAAAGGCGATTCCCTTTCGG
CCTCACATTGGAGTTTTCTTATATCGTATTCGGTCGGTTGCTTTCGAATTCGTTCGAAAACAGTC
AGCCACACTTTGCACACATAAATATTTACTGAATATTTTGGACGTGCTTGCTTTGCTGGCACGGC
GATGGAAAAATGAAATACAACTTCAAACGGTGGATCTCTTGGCTCGT

LAKE BIZERTE (TUNISIA)

>RH1601IT1; GQ999577

GGAAGGATCATTACTGATATATTGAGGATGACCGTTCGATCACGAACAGCCGTCTGTCGATTGC
TTGTCACACTGATTGAAAAACCAGTTTTGACAAACACCACTGTGAACCTGTACCCATCCGTGTG
AGGTGGACAGAGTGCACGAAGAAGCACTACATTGTACTCTCTTGTATTGGACTGTCGTGTACGT
GTACGTCTGTGCTTAAATGAAAGGCGATTCCCTTTCGGCCTCACATTGGAGTTTTCTTATATCGT
ATTCGGTCGGTTGTTTTCGAATTCGTTCGAATTCGTTCGAAAACAGTCAGCCACACTTTGCACAC
ATAAATATTTACTGAATATTTTGGACGTGCTTGCTTTGCTGGCACGGCGATGGAAAAATGAAAT
ACAACTTCTAACGGTGGCAATCTCTTGGCTCGT

>RH1602IT1; GQ999578

GGAAGGATCATGTACTAGCAGTATATTGAGGATGACCGTTCGATCACGAACAGCCGTCTGTCGA
TTGCTTGTCACACTGATTGAAAAACCAGTTTTGACAAACACCACTGTGAACCTGTACCCATCCG
TGTGAGGTGGACAGAGTGCACGAAGAAGCACTACATTGTACTCTCTTGTATTGTACTGTCGTGT
ACGTGTACGTCTGTGCTTAAATGAAAGGCGATTCCCTTTCGGCCTCACATTGGAGTTTTCTTATA
TCGTATTCGGTCGGTTGTTTTCGAATTCGTTCGAATTCGTTCGAAAACAGTCAGCCACACTTTGC
ACACATAAATATTTACTGAATATTTTGGACGTGCTTGCTTTGCTGGCACGGCGATGGAAAAATG
AAATACAACTTCTAACGGTAGCGATCTCTTGGCTCGT

MAR MENOR (SPAIN)

>RH2005IT1; GQ999576

GGAAGGATCATTACTGATATATTGAGGATGACCGTTCGATCACGAACAGCCGTCTGTCGATTGC
TTGTCACACTGATTGAAAAACCAGTTTTGACAAACACCACTGTGAACCTGTACCCATCCGTGTG
AGGTGGACAGAGTGCACGAAGAAGCACTACATTGTACTCTCTTGTATTGTACTGTCGTGTACGT
GTACGTCTGTGCTTAAATGAAAGGCGATTCCCTTTCGGCCTCACATTGGAGTTTTCTTATATCGT
ATTCGGTCGGTTGTTTTCGAATTCGTTCGAAAACAGTCAGCCACACTTTGCACACATAAATATTT
ACTGAATATTTTGGACGTGCTTGCTTTGCTGGCACGGCGATGGAAAAATGAAATACAACTTCTA
ACGATGGATCTCTTGGCTCGT

>RH2007IT1; GQ999574

GGAAGGATCATTACTGATATATTGAGGATGACCGTTCGATCACGAACAGCCGTCTGTCGATTGC
TTGTCACACTGATTGAAAAACCAGTTTTGACAAACACCACTGTGAACCTGTACCCATCCGTGTG
AGGTGGACAGAGTGCACGAAGAAGCACTACATTGTACTCTCTTGTATTGTACTGTCGTGTACGT
GTACGTCTGTGCTTAAATGAAAGGCGATTCCCTTTCGGCCTCACATTGGAGTTTTCTTATATCGT
ATTCGGTCGGTTGTTTTCGAATTCGTTCGAAAACAGTCAGCCACACTTTGCACACATAAATATTT
ACTGAATATTTTGGACGTGCTTGCTTTGCTGGCACGGCGATGGAAAAATGAAATACAACTTCTA
ACGGTGGATCTCTTGGCTCGT

Appendix 3: Representative ITS2 sequences of barrel jellyfish (*Rhizostoma pulmo)* in FASTA format (name of haplotype; GenBank accession number)

>RH1801IT2; GQ999572

CCAGCTGCGATAGAGTAGTGTGAATTGCAGAACCCAGCGAATCATCGAATCTTTGAACGCAAAT
GGCGCTCCTTGGTTCTCCAGGGAGCATGTCTGTCTGAGCGTCATGTCAAATACTAACACACTCA
CGTCCTTGTGGCGTACGTGTGGCGTTGAGACTTCACGGCAGAAGCATGGACGATCATCGTTCGC
GCGGTGCCGCGTGTCTTCAAATGGAGTCAGTACAGCTCGTGTGTCTCTCACCTTGTGTGTGTGTA
CGATCGAGAGCAGCGACAAGCTATCAGCATTGAAGTCTGCCTCTCGGCTCGTACTACCCATACT
TTAACGGTGTCGAGCTTGCGATGCTGTCACAGTGACGCGTGCGTGCTTTATTGCCGCCGCACTGT
TGTTTTGACCTCAGATCAGGCAGGACTACCACGCTGAATTTAA

Appendix 4: Representative COI sequences of barrel jellyfish (*Rhizostoma pulmo*) in FASTA format (name of haplotype; GenBank accession number).

BAY OF KOPER (SLOVENIA)

>RH1403C; GQ999571

ACTATATTTAATATTTGGAGCCTTTTCCGCTATGATAGGAACTGCTTTCAGTATGATTATAAGAT
TAGAATTATCTGGTCCAGGATCCATGTTGGGTGATGATCAACTATATAATGTTGTAGTGACAGC
CCACGCATTGATTATGATATTTTTCTTTGTTATGCCAGTGTTGATAGGAGGTTTTGGAAACTGGT
TAGTTCCTTTATATATAGGGGCACCAGACATGGCCTTCCCAAGGTTAAATAATATTAGTTTTTGA
TTACTTCCTCCTGCACTACTACTATTGCTAGGTTCTTCTCTTGTAGAACAAGGTGCGGGAACAGG
TTGGACAATTTATCCTCCTCTAAGTTCGATCCAGGCCCATTCAGGAGGTTCTGTGGATATGGCCA
TCTTCAGTTTACACTTAGCAGGAGCTTCCTCTATAATGGGAGCTATTAATTTCATTACTACTATC
TTGAATATGAGGGCTCCCGGTATGACTATGGATAAAATTCCTTTGTTTGTATGGTCTGTTCTAGT
CACAGCAATATTATTGTTATTGTCCTTACCTGTTTTAGCTGGGGCTATTACAATGCTACTTACAG
ATAGAAATTTTAATACTTCTTTCTTCGACCCAGCGGGAGGAGGAGATCCTATTTTATTTCAGCAT
TTGTTT

VENETIAN LAGOON (ITALY)

>RH1801C; GQ999568

CACGTATAGTTTAATATTTGGAGCACTTTGTCCGCTATGACCAGGAACTGCTTTCAGTATGATTA
TAAGATTAGAATTATCTGGTCCAGGATCCATGTTGGGTGATGATCAACTATATAATGTTGTAGT
GACAGCCCACGCATTGATTATGATATTTTTCTTTGTTATGCCAGTGTTGATAGGAGGTTTTGGAA

ACTGGTTAGTTCCTTTATATATAGGGGCACCAGACATGGCCTTCCCAAGGTTGAATAATATTAG
TTTTTGATTACTTCCTCCTGCACTACTACTATTGCTAGGTTCTTCTCTTGTAGAACAAGGTGCGG
GAACAGGTTGGACAATTTATCCTCCTCTAAGTTCGATCCAGGCCCATTCAGGAGGTTCTGTGGA
TATGGCCATCTTCAGTTTACACTTAGCAGGAGCTTCCTCTATAATGGGAGCTATTAATTTCATTA
CTACTATCTTGAATATGAGGGCTCCCGGTATGACTATGGATAAAATTCCTTTGTTTGTATGGTCT
GTTCTAGTCACAGCAATATTATTGTTATTGTCCTTACCTGTTTTAGCGGGGGCTATTACAATGCT
ACTTACAGATAGAAATTTTAATACTTCTTTCTTCGACCCAACTGGTGAGGTAGGAGATCCTTATT
ATGTAGTCTTCAGACGAGTTTGTTT

>RH1802C; GQ999569

TAGCTGATATTTAGATTATTTGGAGCCTTTTCCGCTATGACTAGGAACTGCTTTCAGATATGATT
ATAAGATTAGAATTATCTGGTCCAGGATCCATGTTGGGTGATGATCAACTATATAATGTTGTAG
TGACAGCCCACGCATTGATTATGATATTTTTCTTTGTTATGCCAGTGTTGATAGGAGGTTTTGGA
AACTGGTTAGTTCCTTTATATATAGGGGCACCAGACATGGCCTTCCCAAGGTTAAATAATATTA
GTTTTTGATTACTTCCTCCTGCACTACTACTATTGCTAGGTTCTTCTCTTGTAGAACAAGGTGCA
GGAACAGGTTGGACAATTTATCCTCCTCTAAGTTCGATCCAGGCCCATTCAGGAGGTTCTGTGG
ATATGGCCATCTTCAGTTTACACTTAGCAGGAGCTTCCTCTATAATGGGAGCTATTAATTTCATT
ACTACTATCTTGAATATGAGGGCTCCCGGTATGACTATGGATAAAATTCCTTTGTTTGTATGGTC
TGTTCTAGTCACAGCAATATTATTGTTATTGTCCTTACCTGTTTTAGCTGGGGCTATTACAATGCT
ACTTACAGATAGAAATTTTAATACTTCTTTCTTCGACTCCTAGCGGGATGGAGGTAGATCCTATT
TTATTCTCAGCATTTGTTT

GULF OF TUNIS (TUNISIA)

>RH1501C; GQ999570

GCTATATTTAATATTTGGAGCCTTCTCCGCTATGATAGGAACTGCTTTCAGTATGATTATAAGAT
TAGAATTATCTGGTCCAGGATCCATGTTGGGTGATGATCAACTATATAATGTTGTAGTGACAGC
CCACGCATTGATTATGATATTTTTCTTTGTTATGCCAGTGTTGATAGGAGGTTTTGGAAACTGGT
TAGTTCCTTTATATATAGGGGCACCAGACATGGCCTTCCCAAGGTTAAATAATATTAGTTTTTGA
TTACTTCCTCCTGCACTACTACTATTGCTAGGTTCTTCTCTTGTAGAACAAGGTGCGGGAACAGG
TTGGACAATTTATCCTCCTCTAAGTTCGATCCAGGCCCATTCAGGGGGTTCTGTGGATATGGCCA
TCTTCAGCTTACACTTAGCAGGAGCTTCCTCTATAATGGGAGCTATTAATTTCATTACTACTATC
TTGAATATGAGGGCTCCCGGTATGACTATGGATAAAATTCCTTTGTTTGTATGGTCTGTTCTAGT
CACAGCAATATTATTGTTATTGTCCTTACCTGTTTTAGCTGGGGCTATTACAATGCTACTTACAG
ATAGAAATTTTAATACTTCTTTCTTCGACCCAGCGGGAGGAGGAGATCCTATTTTATTTCAGCAT
TTGTTT

LAKE BIZERTE (TUNISIA)

>RH1602C; GQ999571

TCATAAAGATATTAGAACACTTATGATTTAATATTTGGAGCCTTTTCCGCTATGCCATAGGAACT
GCTTTCAGTATGATTATAAGATTAGAATTATCTGGTCCAGGATCCATGTTGGGTGATGATCAACT
ATATAATGTTGTAGTGACAGCCCACGCATTGATTATGATATTTTTCTTTGTTATGCCAGTGTTGA
TAGGAGGTTTTGGAAACTGGTTAGTTCCTTTATATATAGGGGCACCAGACATGGCCTTCCCAAG
GTTAAATAATATTAGTTTTTGATTACTTCCTCCTGCACTACTACTATTGCTAGGTTCTTCTCTTGT
AGAACAAGGTGCGGGAACAGGTTGGACAATTTATCCTCCTCTAAGTTCGATCCAGGCCCATTCA
GGAGGCTCTGTGGATATGGCCATCTTCAGCTTACACTTAGCAGGAGCTTCCTCTATAATGGGAG
CTATTAATTTCATTACTACTATCTTGAATATGAGGGCTCCCGGTATGACTATGGATAAAATTCCT
TTGTTTGTATGGTCTGTTCTAGTCACAGCAATATTATTGTTATTGTCCTTACCTGTTTTAGCTGGG
GCTATTACAATGCTACTTACAGATAGAAATTTTAATACTGGTCWTTCTTCGATCCTAGCGGGTA
GGTAGGTAGATCCTATTTTAGTATTACAGACTATTTGTTT