GENETIC DIVERSITY OF PORPITIDAE (CNIDARIA, HYDROZOA) IN THE AZORES

Dissertação de Mestrado

Francisca Oliveira Leonardes

Mestrado em

Estudos Integrados dos Oceanos

Horta
2021

GENETIC DIVERSITY OF PORPITIDAE (CNIDARIA, HYDROZOA) IN THE AZORES

Dissertação de Mestrado

Francisca Oliveira Leonardes

Orientadores

Professor Doutor João Manuel dos Anjos Gonçalves

Doutor Carlos Filipe Justo Moura

Dissertação de Mestrado submetida como requisito parcial para obtenção do grau de Mestre em Estudos Integrados dos Oceanos

Abstract

Porpitidae is a family of neustonic hydrozoans scarcely investigated genetically, with most scientific publications mentioning solely their occurrence. In order to study the diversity of this family in the Azores, a total of 277 individuals were analysed. These animals were collected on beach areas of two islands (Faial and São Miguel) and morphologically identified as *Velella velella* or *Porpita porpita.* To confirm species identification and to investigate their genetic diversity, phylogeographic associations and population structure, the samples were sequenced using three different molecular markers: COI, 16S and ITS. The MinION (Oxford Nanopore Technologies) sequencing device was utilized, providing long and fast reading sequencing in real-time.

Haplotype networks and phylogenetic trees were built. The analysis of the sequences revealed genetic diversity in the Porpitidae in the Azores. However, the intra-genus nucleotidic variability was practically null in the nuclear gene (ITS) when compared to mitochondrial genes (COI and 16S). When confronting with other sequences available in the databases, it was possible to verify a greater similarity with individuals sampled in locations closer to the Azores (Mediterranean and Sargasso Sea). In the genes with high genetic diversity, it was also possible to distinguish two sampled *Porpita* individuals which exhibited a high genetic distance compared to the others. Although the species delimitation analysis exhibited different results in both methods, the outcome suggested that there may exist two to sixteen species, but there is a higher possibility that there are only two species.

This study provided important information at the taxonomic level of the Porpitidae family. Through this work a good representation of the genetic diversity of Porpitidae was known. Although the number of individuals sampled was quite large, the low geographical representation of the samples in relation to the distribution of the genera may condition the results. It would be important to investigate individuals from other locations as well as to use other molecular markers in order to provide more complete information.

Key-words: genetic, Azores, Porpitidae, *Velella velella*, *Porpita porpita*

Resumo

Porpitidae é uma família de hidrozoários neustónicos pouco investigados geneticamente, onde a maioria das publicações menciona somente a sua ocorrência. Para estudar a diversidade desta família nos Açores, foram recolhidos 277 indivíduos em praias de duas ilhas (Faial e São Miguel) e morfologicamente identificados como *Velella velella* ou *Porpita porpita*. Para confirmar a identificação das espécies e investigar a sua diversidade genética, associações filogeográficas e a estrutura da população, as amostras foram sequenciadas utilizando três marcadores moleculares diferentes: COI, 16S e ITS. O dispositivo MinION (Oxford Nanopore Technologies) foi utilizado, proporcionando uma sequenciação de leitura longa e rápida em tempo real.

Foram construídas redes de haplótipos e árvores filogenéticas. A análise das sequências revelou diversidade genética dos Porpitidae nos Açores. No entanto, a variabilidade intra-género foi praticamente nula no gene nuclear (ITS) quando comparada com os genes mitocondriais (COI e 16S). Comparando com sequências disponíveis nas bases de dados, foi possível verificar uma maior semelhança com os indivíduos amostrados em locais mais próximos dos Açores (Mediterrâneo e Mar dos Sargassos). Nos genes com elevada diversidade genética, também foi possível distinguir dois indivíduos do género *Porpita* que exibiam uma distância genética significativa quando comparados com outros do mesmo local. Apesar da análise da delimitação das espécies ter apresentado resultados diferentes para ambos os métodos, o resultado sugeriu que podem existir duas a dezasseis espécies, mas há uma maior possibilidade de haver apenas duas espécies.

Este estudo forneceu informações importantes ao nível taxonómico da família Porpitidae. Através deste trabalho foi conhecida uma boa representação da diversidade genética dos Porpitidae. Embora o número de indivíduos amostrados seja bastante grande, a baixa representação geográfica das amostras relativamente à distribuição dos géneros pode condicionar os resultados. Seria importante investigar indivíduos de outros locais e utilizar outros marcadores para providenciar informações mais completas.

Key-words: genética, Açores, Porpitidae, *Velella velella*, *Porpita porpita*

Acknowledgements

To Prof. Dr. João Gonçalves, for introducing me to this project. For helping me and pushing me through the less good times of the work. For his great team spirit and trust presented to me. For accompanying me in these two years of master's degree as my teacher and my mentor.

To Dr. Carlos Moura, for teaching me new laboratory techniques and introducing me to molecular biology and genetics.

To Bruno Ivo Magalhães, for sharing with me his knowledge of jellyfish. For trusting me and sharing his work with me. For helping and giving me the best tips to improve my work.

To my master's colleagues: David, Sandra and Joana. A special thanks to Joana for being the best housemate and older sister. For always saying the right words and for her endless support.

To my friends in Faial and to the DOP staff. To Mariana, who brought joy to my days and who was always available for a good laugh and for giving me encouragement. To Sandra, who helped me immensely, sharing with me all her knowledge in genetics and always helping me throughout the project. To all the good people I met and worked with in DOP.

To my whole family, especially my parents who gave me the necessary tools for this work and who always supported me, never letting me give up. To my mother, who made tireless efforts to help me and who often stopped everything she was doing, to only stand by me. For always showing me that after the storm comes the rainbow; she didn’t allow me to doubt my value and capacity. My parents were undoubtedly the most important support in this work.

To Miguel, who though distance was always close. For pushing me all the time. For the little gestures and words that made all the difference. For being the safe harbour on stormy days. Thank you for never giving up or letting me give up.

To my friends in São Jorge, especially Ana. For giving me the best advice and never letting me quit. For being there in the best and the worst moments. For

helping me prove myself and for encouraging me to do even better. To Deolinda who shared with me the experience of writing a master’s thesis and by sharing with me awesome tips. For the common tastes that have brought us together.

To everyone I came across in these two years, to those who added something new in my life and even to those who didn't believe I could and made me doubt myself. They all gave me the strength to conclude this work with success.

To all, a huge thank you.

Contents

List of figures

List of tables

List of abbreviations

16S - 16S ribosomal ribonucleic acid

ASAP - Assemble Species by Automatic Partitioning

BIC – Bayesian information criteria

BOLD - Barcode of life data system (https://www.boldsystems.org/)

(mt)COI - (mitocondrial) cytochrome c oxidase I

DNA - Deoxyribonucleic acid

GENBANK - NIH genetic sequence data base (https://www.ncbi.nlm.nih.gov/genbank/)

ITS - Internal transcribed spacer

MCMC - Markov chain Monte Carlo

PCR – Polymerase chain reaction

PHYML - Phylogenetic Maximum Likelihood (http://www.atgc montpellier.fr/phyml/)

PTP - Poisson Tree Processes

RNA - Ribonucleic acid

SSU – Small subunit

1. Introduction

1.1 DNA Barcoding

1.1.1 Taxonomy

One of the most basic questions of life on Earth is how many species are there. This question remains without a precise answer. A species is the fundamental unit in biology as well as biodiversity (Mayr 1985). Thus, the correct identification of a species is extremely important. It is estimated that there may be 8.7 million species, most of them awaiting description. In the oceans alone, 91% of the species are still undiscovered (Mora *et al.* 2011). In other words, the knowledge that we currently have on these marine environments represents a low number of the existing reality. So far, only almost 240 thousand marine species have been registered (WoRMS 2021).

A precondition for most biological studies is to know exactly what are the species under study (Böttger-Schnack & Machida 2011). Consistently, species have been identified and described based on the comparison between individuals, by direct observation of their distinctive morphological characters (Wiens 2007). Consequently, most of what we know today about the phylogeny of life is due to morphological data. However, identification based solely on this methodology can be insufficient.

Morphology by itself may easily lead to the appearance of cryptic species. These are distinct but morphologically indistinguishable species which are classified (or hidden) within a single species name (Bickford *et al.* 2007). If this cryptic diversity is ignored, many species can be misclassified, leading to biodiversity conclusions beyond reality. This misestimation leads to unsuitable efforts of conservation and managing, that may hasten the extinction of previously unknown endangered species. (Heath *et al.* 2008; Theodoridis *et al.* 2019).

1.1.2 Molecular methods

Although DNA was first identified in the late 1860s by Swiss chemist Friedrich Miescher, its applications were only explored many years later (Pray 2008). The great technological advance that occurred at the beginning of the 21st

century led to the development of new techniques and applications in all areas, including taxonomy. The identification of species through molecular data, not based on morphological characters emerged, leading to what is known as "DNA taxonomy" (Tautz *et al.* 2002, 2003).

Soon after, a new molecular analysis technique was presented: "DNA barcoding". This approach differs from the previous one because it focuses on associating a known species to unidentified organisms. The principle of “DNA barcoding” is to associate species with a specific DNA sequence that can be interpreted as a genetic “barcode” (Hebert *et al.* 2003a; Hebert *et al.* 2003b). Despite the numerous benefits of this new method, doubts have arisen about its ability to replace the traditional taxonomy (Will *et al.* 2005). This discussion led to a consensual technique identified as integrative taxonomy. (Dayrat 2005; Padial & De La Riva 2010; Schwentner *et al.* 2011). This approach suggests that species identification may be simpler and more correct by integrating data from complementary perspectives such as: morphology, phylogenetics, phylogeography, evolutionary biology, ecology, etc…

1.1.3 Barcoding regions

The barcoding process begins with the capture and the tissue removal from a sample, moving on to the extraction of DNA. Then a specific region of the nuclear, chloroplast or mitochondrial DNA is amplified from the genome using Polymerase Chain Reaction (PCR). The ideal DNA barcoding region must have a variability that allows to differentiate between species but it also has to be preserved within the species itself. It should also be robust, with a highly conserved priming site, to provide high reliability. Lastly, it’s essential that the gene can be standardized, using the same DNA region for the largest number of taxonomic groups possible (Bandyopadhyaya *et al.* 2014). The first identification system known was the cytochrome oxidase I (COI), a gene used as a standard DNA barcode marker (Hebert *et al.* 2003b).

This universal DNA barcode region, mitochondrial cytochrome oxidase I (mtCOI), is a region of just over 600 pairs of length bases encoded in the mitochondrial genome (Hebert *et al.* 2003b). It is not only an easily amplified gene

but it also appears to have a better phylogenetic signal than the other mitochondrial genes. The great rate of evolution of this gene makes it possible to easily distinguish very close species as well as phylogeographic groups within the same species (Hebert *et al.* 2003a). However, there are some taxonomic groups where mitochondrial genome evolution occurs slowly, making this gene less appropriate for DNA barcoding. For example, animals of the phylum Cnidaria, mostly of the class Anthozoa, show very low levels of sequence divergence, so this gene is not very suitable for DNA barcoding on this taxon. (France & Hoover 2002; Huang *et al.* 2008).

Depending on the target species, there are other suitable genes that can be used for DNA barcoding. The internal transcribed spacer (ITS) rRNA is widely used for fungi (Schoch *et al.* 2012). In plants, however, two sections of coding regions within the chloroplast are used: part of the genes rbcL and matK (de Vere *et al.* 2015). The small subunit ribosomal RNA (SSU rRNA) genes are also universally used: 16S in bacteria and 18S in eukaryotes (Karst *et al.* 2018). However it has not been yet discovered an universal gene for DNA barcoding as there isn't a single gene that is conserved in all life domains (Purty & Chatterjee 2016).

1.1.4 Sequencing methods

The DNA is sequenced after verifying the success and quality of the PCR procedure. Although barcoding methods are widely used in various fields, it is still relatively difficult to obtain DNA sequences, due to the fact that expensive and highly specialised equipment is needed. The first method for sequencing DNA was "chain-termination method" (Sanger & Coulson 1975) but at the same time a new method was also being developed: "chemical sequencing method" (Maxam & Gilbert 1977). These methodologies were designated first-generation sequencing (FGS). The FGS were associated with a high cost and low yield and they have been used for more than 20 years.

At the beginning of the 20th century, with the arose of new technological development, new methods were quickly developed. The so-called second-generation sequencing (SGS) was able to generate amounts of sequence data

very quickly and at relatively limited costs. With these methods the sequence of a human genome was completed in a few weeks (Venter 2001). In order to make the sequencing method faster, third generation methods (TGS) were developed. Based on a constant analysis of a single DNA molecule, the main goal was to minimize errors and, consequently, produce high quality readings (Benítez-Páez *et al.* 2016).

The search for technologies that operate at a higher speed and produce longer readings led to the discovery of new sequencing approaches. In 2014, the MinION (Oxford Nanopore Technologies) sequencer was launched, which is a device that allows long and fast real-time reading sequencing of nucleic acids (Tyler *et al.* 2018). MinION identifies the bases of DNA by measuring changes in electrical conductivity generated when DNA chains pass through a biological pore. Weighing only 90g and measuring 10 cm, MinION is the smallest sequencing device available in the market (Jain *et al.* 2016). The main advantages of the device are being portable, accessible and with quick data production. It has also got several applications in barcoding, genome assembly and metagenomic identification (Bates *et al.* 2016; Benítez-Páez *et al.* 2016; Wang *et al.* 2017; Ho *et al.* 2020; Tan *et al.* 2020; Tsugama & Fujino 2020; Azinheiro *et al.* 2021; Brancaccio *et al.* 2021; Groen *et al.* 2021; Mann *et al.* 2021; Ngo *et al.* 2021).

1.2 Gelatinous zooplankton

One of the fields where integrative taxonomy can display an important role is the identification of marine zooplankton species, especially the gelatinous ones. Although gelatinous animals are distributed in all the planet's oceans in large numbers and all through the water column, they are still the least known of all planktonic animals (Condon *et al.* 2012). In general, they are poorly investigated mainly due to their fragile and delicate structure, as well as lack of taxonomic expertise (Hosia *et al.* 2017). To overcome this problem, new *in situ* technologies have been developed, allowing the study without interference, as

well as the collection of living organisms. These methods include the capture of images by scuba diving or using submersibles/remote vehicles (Raskoff *et al.* 2003; Corgnati *et al.* 2016). However, the search for new sampling methodologies continues, seeking for more effective and low-cost protocols (Aubert *et al.* 2018).

Gelatinous zooplankton include a wide range of animal groups with a planktonic life style, such as ctenophores, cnidarians and pelagic tunicates (Madin & Harbison 2001). Gelatinous organisms are composed mostly of water (about 95%), having a soft and transparent body, without hard structures. These unique features provide these animals with numerous benefits such as protection from the colossal pressure in the deep and the ability to float and swim on the water column. As the material is non-living, it can also survive under situations of food scarce, and can reproduce and grow at outstanding rates when food is abundant (Madin & Harbison 2001). The blooms of these individuals may cause considerable impacts on ecosystems as well as in fisheries, aquaculture and tourism (Graham *et al.* 2001; Brodeur *et al.* 2016; Bosch-Belmar *et al.* 2017; Bosch-Belmar *et al.* 2021). Nevertheless, gelatinous zooplankton have an important role in the ecosystem with implications for carbon cycle (Condon *et al.* 2011) and the food web. They are an important component for the diet of several species and also compete with other species for their own food (Hay 2006). All these important animals are the target of some lucrative fisheries, because they become a significant food source, mainly in the Asian countries (Omori & Nakano 2001). In addition, the gelatinous zooplankton also have a lot of useful applications on biomedicine (Addad *et al.* 2011; Prieto *et al.* 2018).

1.2.1 Phylum Cnidaria

With a diversified group of relatively simple animals, Cnidaria is the most representative phylum of the gelatinous zooplankton. The phylum comprises a great diversity of species, mainly marine, including corals, jellyfish, anemones and hydrozoans (Ruppert *et al.* 2004). Cnidarian animals share an exclusive feature: the presence of highly specialized cells called nematocysts. These cells are mainly located in the tentacles, allowing these animals to efficiently capture

their preys, although they lack a complex nervous system. Nematocysts are also used for locomotion and defence. (Beckmann & Özbek 2012). Cnidarian bodies can display radial or biradial symmetry. Almost all their tissues have a double layer basic structure, the epidermis (outside) and the gastrodermis (inside). Between the two layers, a gelatinous substance (mesoglea) maintains the integrity of the tissues and the body (Shikina & Chang 2018). Reproduction of cnidarians can be either asexual by budding or sexual using gametes. Some cnidarians can cycle between a medusa stage and a polyp stage during their life cycle, exhibiting two body forms. Anemones are examples of polypoid forms, while jellyfish are examples of medusoid forms. (Figure 1).

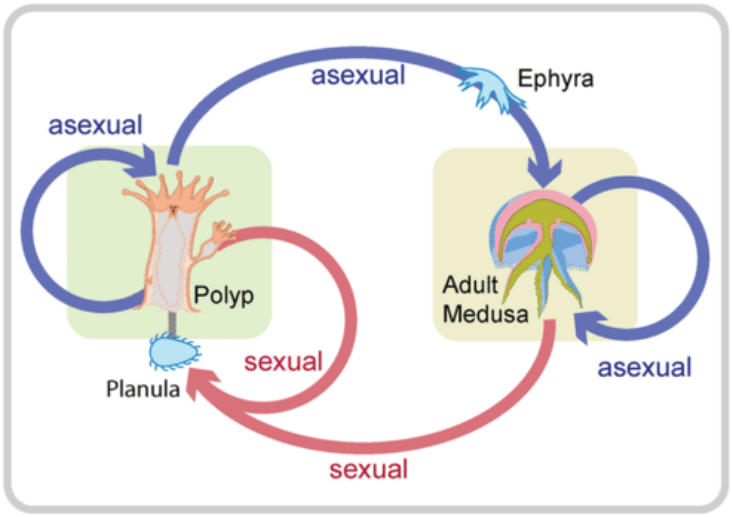


Figure 1 - A generalized life cycle of a cnidarian: medusae primarily reproduce by sexual reproduction with the formation of a larval stage called the planula. The planula then develops into a polyp that can reproduce either sexually or asexually. Adapted from CK-12 Foundation (2016)

Despite of the recent increase in the investigation of marine gelatinous, the phylogenetic classification of cnidarians represents one of the major problems of invertebrate zoology (Bridge *et al.* 1992). The large population sizes, high larval dispersion and the lack of physical barriers can contribute for weak genetic diversity in Cnidaria (Miglietta *et al.* 2011). The development of the phylum Cnidaria probably occurred in the Ediacaran period. Since then, it has evolved in a variety of forms and a diversity of adaptation strategies (Peterson & Butterfield 2005; Cartwright & Collins 2007). Based on the alternation of its life cycle,

structure and DNA sequences, the phylum Cnidaria can be divided in two large clades: Anthozoa and Medusozoa (Collins 2002; Daly *et al.* 2006; Technau & Steele 2011). Anthozoa is a class comprising coral and anemone species, while Medusozoa is a sub-phylum named with a reference to the typical adult pelagic medusa state of the group (Collins *et al.* 2006). Medusozoa incorporate four distinct classes: Cubozoa (about 60 species of box jellyfish), Hydrozoa (about 3800 species of hydrozoans), Scyphozoa (about 300 species of true jellyfish) and Staurozoa (about 70 species of benthic jellyfish) (WoRMS 2021). (Figure 2)

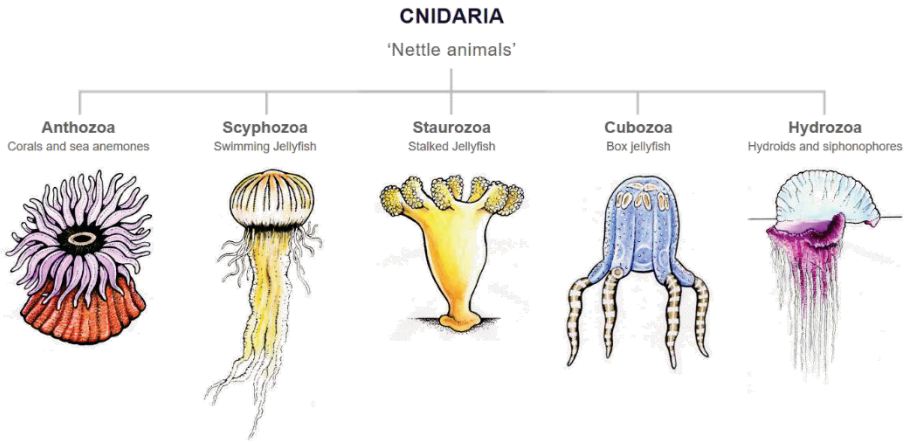


Figure 2 - Five classes of the phylum Cnidaria: Anthozoa, Schphozoa, Staurozoa, Cubozoa, Hydrozoa. Adapted from Dennis Gordon, 'Corals, anemones and jellyfish - Cnidaria – the nettle animals', Te Ara - the Encyclopedia of New Zealand. (2016)

1.2.2 Class Hydrozoa

The largest and most diversified class within Medusozoa is Hydrozoa, with more than 3700 species currently described (WoRMS 2021). The polyp stage usually predominates in this class, with the medusa small or absent (Bouillon *et al.* 2006). Another special and common feature found in Hydrozoa is colonial organization (Nawrocki & Cartwright 2012). In colonial hydroids, the individual polyps exhibit different functions: the gastrozooids are responsible for the feeding task, the dactylozoids capture prey, and gonozooids are responsible to produce the medusoids with the gametes. Most of the colonies behave like a single animal and consequently are often mistaken for jellyfish (UCMP 2021).

A consequence of these peculiarities is that the Hydrozoan taxonomy is very controversial and problematic: often the species are described using only

one of the life cycle parts. There are cases, for instance, where the hydroid stage is placed in one taxon and the medusa stage in another. Coupled with very poor investigation and sampling (mainly in the deep sea), this leads to a serious misperception of the taxonomy within this class (Boero 1980). The most recent studies in the taxonomy of hydrozoans used molecular sequencing methods, such as ribosomal nuclear (Cartwright *et al.* 2008; Collins *et al.* 2008) and mitochondrial (Kayal *et al.* 2013) sequences, suggesting that Hydrozoa consists of two main sub classes: Trachylina and Hydroidolina.

Despite having low specific diversity, the subclass Trachylina includes the most enigmatic cnidarians, which differ greatly from typical hydrozoans in their morphology and life cycle (Osadchenko & Kraus 2018). The orders currently accepted for this suborder are Limnomedusae, Narcomedusae, Trachymedusae and Actinulida (Collins *et al.* 2008). The suborder Hydroidolina contains the remaining hydrozoans. This group display an immense variety and diversity, presenting a total of approximately 3,350 species, distributed in three orders: Antoathecata, Leptothecata and Siphonophora (Cartwright *et al.* 2008). Of these three orders, only Antoathecata has no support to monophyly, provided by phylogenetic analyses (Dunn *et al.* 2006; Leclère *et al.* 2009). The order Antoathecata is so called due to the absence of theca cells. Although there is no support for monophyly, three suborders can currently be distinguished: Aplanulata (lack a planula stage), Filifera (filiform distribution of nematocysts) and Capitata (presence of capitate tentacles) (Cartwright & Nawrocki 2010).

Currently the suborder Capitata aggregates 19 families (Schuchert 2021). Several taxonomic studies, based on morphology, have already been done (Bouillon & Boero 2000; Petersen 2008), but more recently they have been based on molecular data (Collins 2002; Collins *et al.* 2005). Molecular studies have gained importance because the animals of this suborder are morphologically diverse. This group includes floating pelagic colonies, species with free swimming medusae or fixed polyps. A family that is an example of the ambiguity of the morphological characters within Capitata is Porpitidae, commonly mistaken over the years with siphonophores and chondrophores (Calder 1988).

1.3 Family Porpitidae

Porpitidae is a family of colonial pelagic hydroids, highly polymorphic and specialized, being able to live on the surface of the oceans (neuston) , where there is exposition to extreme environmental conditions (Munro *et al.* 2019). This family was first described by Goldfuss (1818) as Porpitae but ten years later Guilding corrected the name to Porpitidae. In 1829 another family was described by Eschscholtz who named it Velellidae. Later, in 1888, three new families became known within the order Siphonophora: Discallidae, Porpitellidae and Porpallidae (Haeckel 1888). In 1954, several differences were found between these families and the order Siphonophora, and a new order was created to accommodate them: Chondrophora (Totton 1954). The idea of this new order was abandoned when several authors suggested that this family species were athecate hydroids. Thus, Velellidae was then considered a family and it included *Velella* and *Porpita* (Brinckmann-Voss 1970). Currently it is accepted as Porpitidae and it includes two genus: *Velella* and *Porpita* (Calder 1988; Schuchert 2021).

Velella and *Porpita* were described in 1801 (Lamarck 1801a). However, over the years, new genus were described. Nowadays, *Velella* includes two synonymized names: *Armenista* Haeckel, 1888 and *Rataria* Eschscholtz, 1829 whereas *Porpita* has ten synonyms: *Acies* Lesson, 1830; *Chrysomitra* Gegenbaur, 1857; *Discalia* Haeckel, 1888; *Discomitra* Haeckel, 1888; *Dystonia* Haeckel, 1888; *Polybrachionia* Guilding, 1828; *Porpalia* Haeckel, 1888*; Porpema* Haeckel, 1888; *Porpitella* Haeckel, 1888; *Ratis* Lesson, 1830 (WoRMS 2021). One of the main systematic problems within this family is the species attributed to *Porpita*. Firstly, all species of the genus *Porpita* were united in just one species: *Porpita porpita* and it was also accepted the genus *Porpema* Heackel 1888. More recently *Porpita* has two accepted species: *Porpita porpita* and *Porpita prunella*. However, there is very little information about *Porpita prunella* because it was only sighted once by Heackel, who described it in 1888 (Figure 3).

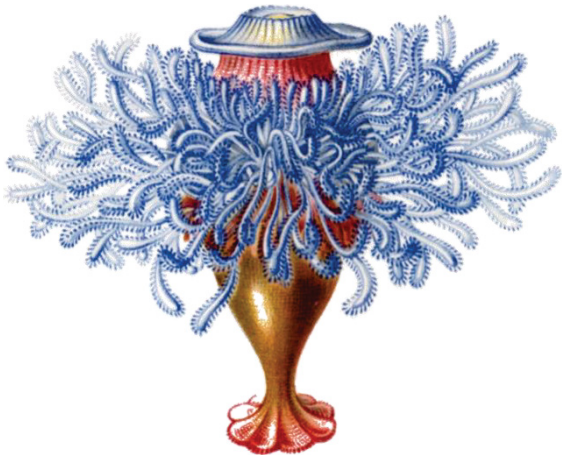


Figure 3 - Illustration of *Porpita prunella* (first described as Porpema prunella). Ernst Haeckel, Kunstformen der Natur (1900).

1.3.1 Porpita porpita

First described as *Medusa porpita* in the Indian Ocean by (Linné & Salvius 1758), *Porpita porpita*, commonly known as the blue button, is a pleustonic colonial hydroid. The first records found in the Azores for *Porpita porpita* were in 1895.07.26 in Porto Pim Bay, Horta, Faial Island. The species identified was *Porpita umbella* by O. F. Müller (Bedot 1904). Until 1888 more than 32 species were described as belonging to *Porpita* (Appendix A). However, Moser carefully observed a large number of *Porpita* from different locations and concluded that all these species of Porpita were only variations of one species: *Porpita porpita* (Deutsche & Drygalski 1912). Other studies have emerged in the identification of *Porpita porpita* species, mainly with the aid of molecular methods. Although there are some DNA sequences available (about 25 total – Bold Systems and GenBank), most of them are for morphological identification studies (Cartwright & Nawrocki 2010; Ortman *et al.* 2010; Furfaro *et al.* 2017; Khalturin *et al.* 2019). As for its use for taxonomic studies, there is no study dedicated only to *Porpita porpita*. However, there are several publications that investigated phylogenetic relationships within Hydrozoa, including Porpitidae, in which the family was found to be monophyletic (Collins 2002; Collins *et al.* 2005; Collins *et al.* 2006; Dunn *et al.* 2006).

This species is distributed in tropical and temperate seas and it is reported to be found in several localizations in the Atlantic Ocean, Mediterranean Sea and Indo-Pacific Ocean (Kirkendale & Calder 2003; Gul & Gravili 2014; Msn *et al.* 2016; Lillo *et al.* 2019; Madkour *et al.* 2019; Sivalingam 2019). The hydroid phase is a dark blue floating colony with diameter up to 30 cm; it has a disk-shaped mantle and internal float consisting of several concentric chambers. The cnidocytes are located between the float and the central gastrozooid. The dactylozoids have four main capitate tentacles and a varying number of small tentacles in vertical rows. The medusa has eight radial canals with a conical manubrium and octagonal base. It has also got two opposite marginal capitate tentacles and six non tentaculate (Bouillon *et al.* 2006) (Figures 4 and 5).

The development of the *Porpita porpita* hydroids evolves through three phases: the conaria, ratarula and rataria. Although *Porpita porpita* has nematocysts it appears to have an imperceptible sting in humans (Gershwin *et al.* 2010).

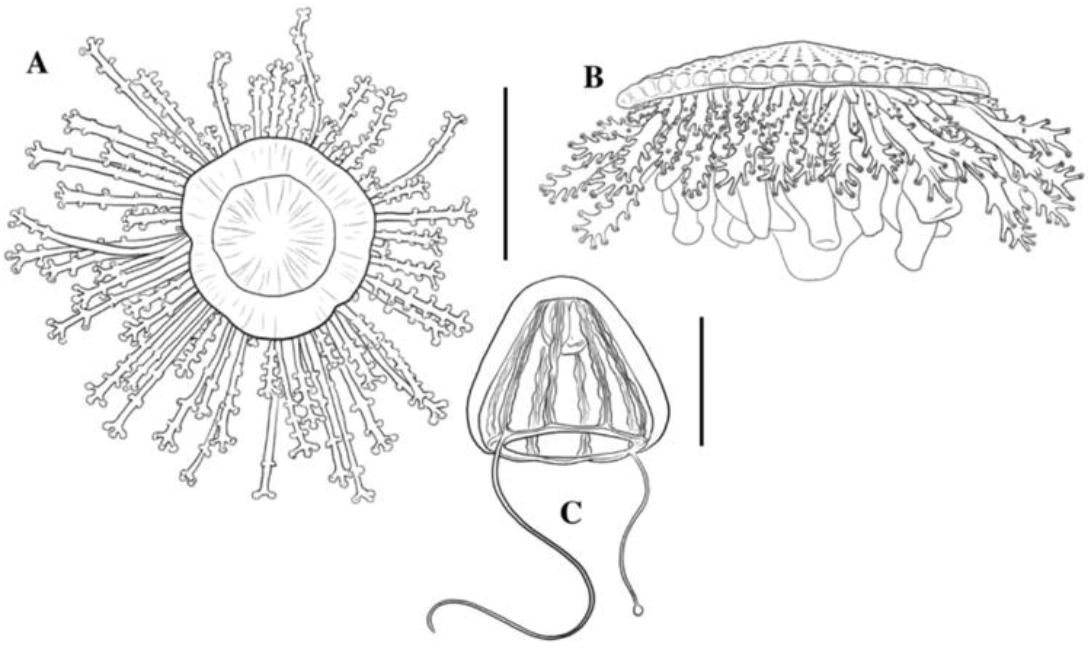


Figure 4 - *Porpita porpita*: A, colony (seen from above); B, mature colony (lateral view); C, medusa. Adapted from Schuchert (1996) and Pagès et al. (1992)

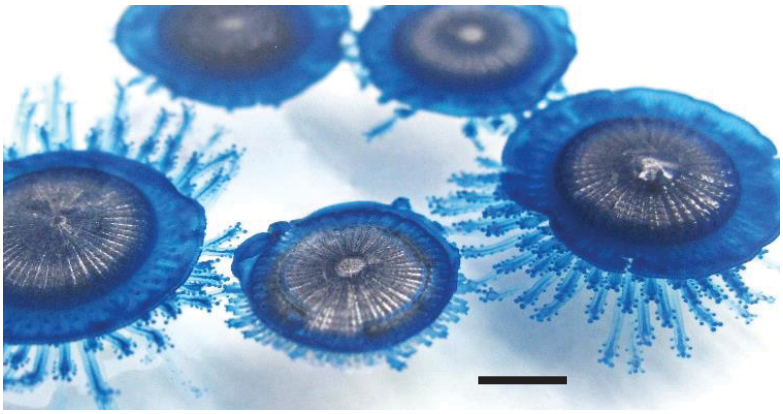


Figure 5 – Several Porpita porpita in water, from Porto Pim beach, Faial Island – Azores. The scale represents 1 cm. (author: Bruno Ivo Magalhães)

1.3.2 Velella velella

The other genus of the Porpitidae family is *Velella* Lamarck 1801. In the Azores, the species *Velella spirans* Forskal is the first reported record on 1895.06.22 in the eastern edge of the EEZ Azores (36º54' N 20º46'15" W) (Bedot 1904). Over 24 species have been described (Appendix B) in this genus but currently is accepted that they all belong to a single one: *Velella velella* (Brinckmann-Voss 1970; Kirkpatrick & Pugh 1984; Calder 1988). Similar to its sister species, *Porpita porpita*, the study of *Velella velella* is focused on identification work, and studies with DNA sequencing are the same for both species. *Velella velella* hydroid is an oval floating colony with a triangular sail. It can reach up to 70 mm long, being higher in the centre. When it is alive this animal shows a deep blue colour. There are two mirror images of the animal (the left and the right sail). The sail is kept rigid by a chitin support, covered by a mantle tissue. In the centre of the underside is a single gastrozooid, surrounded by gastro-gonozooids that are responsible for the medusa production. There is also a peripheral band of dactylozoids. (Bouillon *et al.* 2006) (Figure 6)

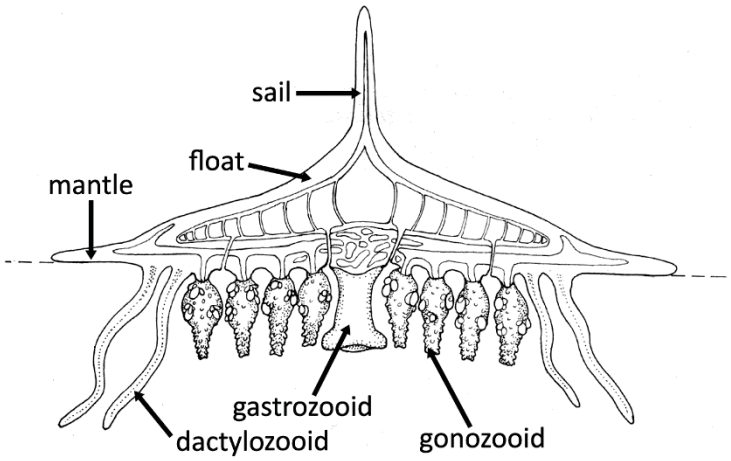


Figure 6 - Lateral view of a *Velella velella*. Adapted from Siphonophores and Velellids by Kirkpatrick and Pugh (1984).

Velella velella life cycle follows the developmental stages of a hydroid: it includes the colonial asexual stage and a medusa stage. The floating polyp colony frees small medusa. These medusa buds are yellow-olive coloured due to the symbiotic algae and are about 1mm long. They possess a conical manubrium with quadrate base, including four radial canals. They sink to 600-1000 meters depth and perform the sexual reproduction. A small larva, named Conaria, after being formed, metamorphoses, floating to the surface and then grows up, reaching the adult size (Calder 1988; Schuchert 2010). (Figure 7 and 8). These medusae are rarely seen but there are some records for Mediterranean Sea and North Atlantic (Brinckmann-Voss 1970; Larson 1980).

These animals do not have their own locomotion, moving only by wind and ocean currents. This process has an important potential for dispersal of the species beyond biogeographic boundaries (Mackie 1962). Although they were first described in the Mediterranean Sea (Linnaeus, 1758) they are distributed in temperate and warm waters across the world (Purcell *et al.* 2012; Araya & Aliaga 2018; Carrera *et al.* 2019). *Velella velella* occurs all year round, with periods of greater abundance, where post-larval individuals show, mostly in the winter months of December/January (Bieri 1959). Mass strandings may occur, easily

seen in sandy beaches, spreading over to millions of individuals (Sibley 2007; Flux 2008; Betti *et al.* 2019).

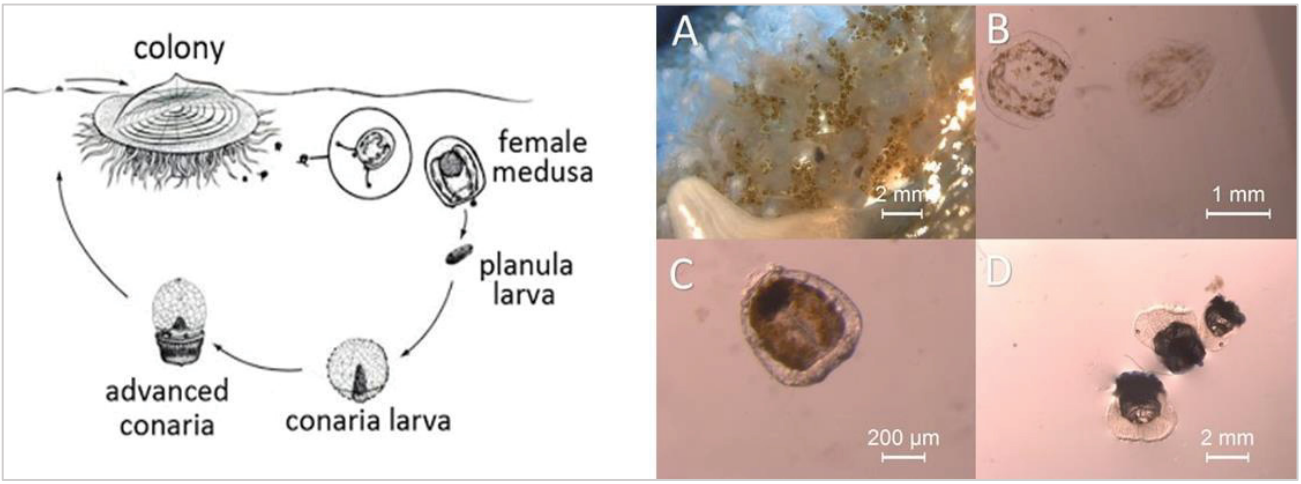


Figure 7 - Left: schematic representation of *Velella velella* life cycle. Adapted from Langstroth and Langstroth (2000)

Right: - A – medusa being released from the hydroid colony; B – medusa after release; C – medusa two days after release (exposed to sun light); D- advanced conaria. Microscopic photography obtained with Leica (CTR 600 Microscope) by Carlos J. Moura

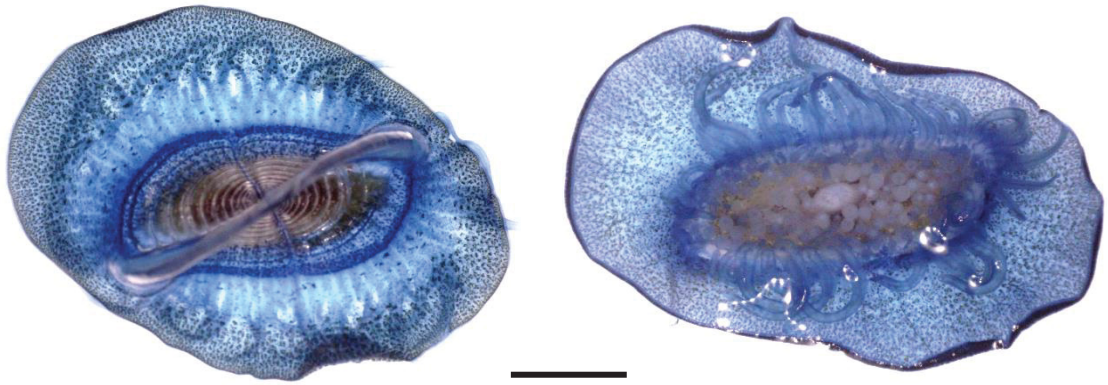


Figure 8 – Dorsal and ventral view of adult *Velella velella* colony, from Santa Barbara beach in São Miguel Island - Azores. The scale represents 1 cm. (author: Bruno Ivo Magalhães)

Therefore, DNA barcoding techniques have been widely tested on solving some of the hydrozoan taxonomy problems, such as the presence of cryptic species (Moura *et al.* 2008; Postaire *et al.* 2016b; Maggioni *et al.* 2020). The gene most applied as molecular marker in these studies is 16S. In addition to being easily amplified, this gene also provides a lot of information about phylogenetic relationships at various taxonomic levels (Collins *et al.* 2005;

Schuchert 2005; Leclère *et al.* 2007; Moura *et al.* 2008; Miglietta *et al.* 2009; Nawrocki *et al.* 2010; Peña Cantero *et al.* 2010; Moura *et al.* 2011; Moura *et al.* 2012a; Zheng *et al.* 2014; Postaire *et al.* 2016b; Ronowicz *et al.* 2017; Schuchert *et al.* 2017). However, other markers are also used, including multi-marker analyses, particularly the universal COI and the nuclear ITS (Govindarajan *et al.* 2005; Schuchert 2014; Cunha *et al.* 2015; Schwentner & Bosch 2015; Postaire *et al.* 2016a; Schuchert 2018; Maggioni *et al.* 2020).

1.4 Aim of the study

The main goal of this research was to study the genetic diversity of the Porpitidae species sampled in the Azores: *Velella velella* and *Porpita porpita*. Applying a new and innovative sequencing technique, three genes were analysed: COI, 16S and ITS. In a first approach the genetic diversity of the sampled individuals was calculated in order to understand if there was some diversity among the three molecular markers. Secondly phylogenetic trees and haplotype networks were built to compare the Azorean sequences with other sequences available on databases. Finally, the sequences were evaluated by species delimitation methods in order to check the number of species among the studied sequences.

2. Materials and Methods

2.1 Study area and sampling methods

The sampling took place between February 2019 and February 2021 in six different collecting points, within Faial and São Miguel Island, in the Azores archipelago, NE Atlantic (Figure 9). This work had permissions to field study approvals of Direção Regional dos Assuntos do Mar (SAI-DRAM/2018/1247 SGC0010/2018/919 Proc. 120.12.09/61) and Direção Regional da Ciência e Tecnologia (ADENDA AMP/2018/021), of the Azores Government.

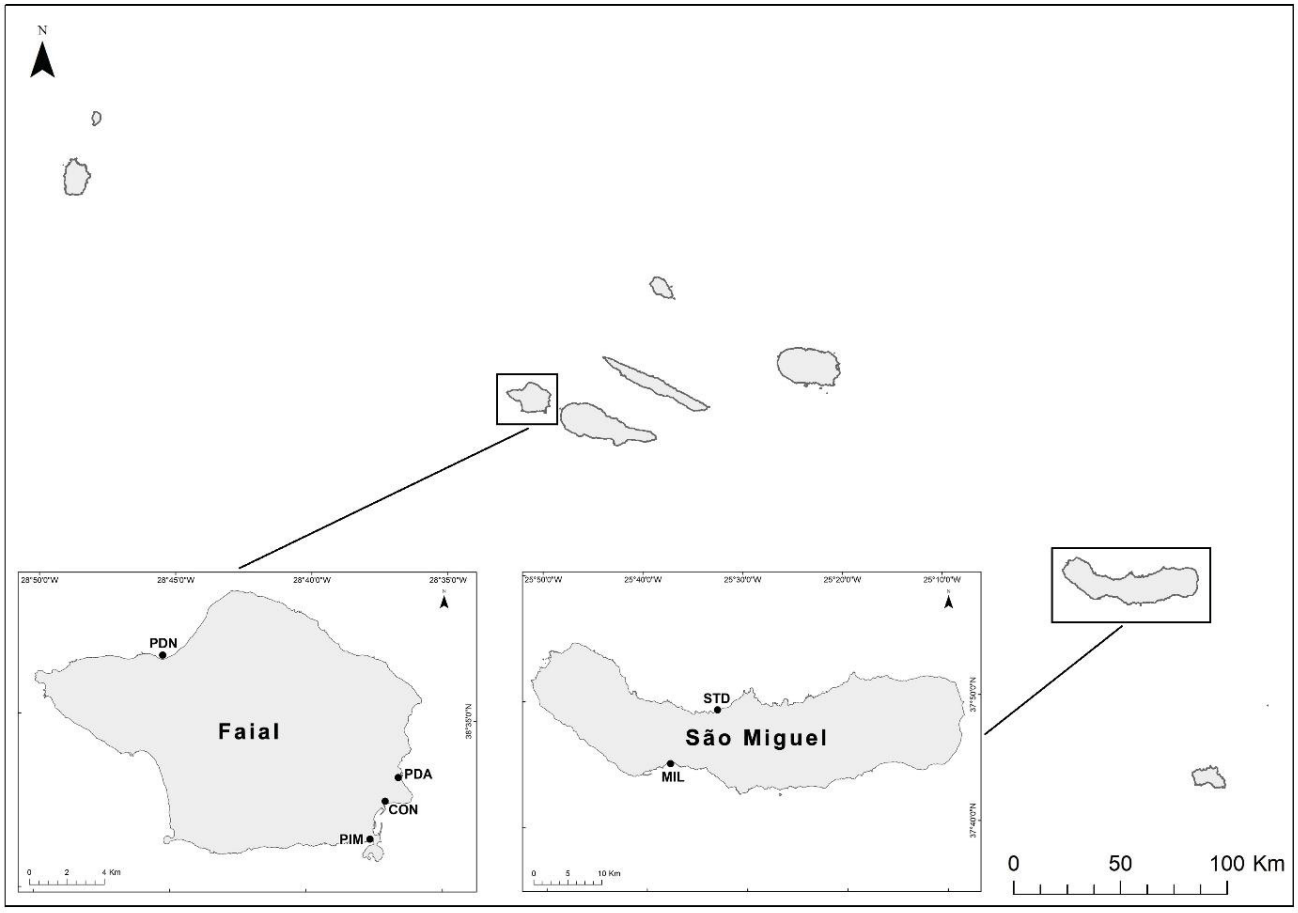


Figure 9 - Map illustrating the six collecting points (Faial: PIM - Praia de Porto Pim; PDN - Praia do Norte; CON - Praia da Conceição; PDA - Praia do Almoxarife; São Miguel: MIL - Praia das Milicias; STB - Praia de Santa Bárbara). R.Medeiros © ImagDOP

The methodology was based on terrestrial visual transects. It consisted of walking along the beach during low tide, more specifically along the water's edge to the end of the transect and returning along the upper limit of the tide, collecting and identifying the animals (Doyle *et al.* 2007; Houghton *et al.* 2007; Fleming *et al.* 2013; Ionescu *et al.* 2016). The specimens were morphologically identified based on the descriptions from (Brinckmann-Voss 1970; Kirkpatrick & Pugh 1984; Calder 1988; Bouillon 1999; Bouillon 2006; Boyra A 2008; Schuchert 2010; Ferreira 2011; Conway 2012; Piraino 2014; Licandro *et al.* 2017). A total of 277 animals were collected for this study: 67 *Porpita porpita* and 210 *Velella velella*. The samples were preserved in ethanol 96% and stored for further processing and DNA extraction.

2.2 Molecular methods

2.2.1 DNA extraction

The DNA extraction was performed using small fragments of the specimen tissue. The fragments were first washed with Milli-Q® water and set in a new vial. Approximately 10 µl of QuickExtract™ (Lucigen) was added. Following a stir for 15 s in the vortex and a short spin, to make sure all the material was submersed, the samples were incubated at 65°C for 15 min and 98°C for 2 min. A new homogenization was performed and the vials were preserved at -18°C.

2.2.2 DNA amplification – Primers and PCR

After extraction, three molecular markers were amplified by polymerase chain reactions: COI, 16S and ITS. The primers LCO1490 and HCO2198 were used to amplify cytochrome oxidase subunit I (COI) (Folmer *et al.* 1994). The primers used for 16S mRNA were SHA and SHB (Cunningham & Buss 1993). ITS1 was amplified using the primers CAS18sF1 and CAS5 p8sB1d (Pontin & Cruickshank 2012) (Table 1). All primers had a 13-bp tag for demultiplexing. Each PCR product was amplified using 0,5 µl of DNA, 0,4 µl of each primer and 6,5µl of MasterMix (CWBio). The final volume was adjusted to 15µl by addition of 5µl

nuclease free water. The PCR technique was used with the same thermal profile for the three markers: 95°C/5min followed by 35 cycles (94°C/30s, 46,5°C/45s, 72°C/45s) with final extension of 72°C/5min. Finally, each PCR product was run on an agarose gel to ensure that PCRs were successful. The quality of each PCR product in the gel was evaluated on a colour scale in which green was good, yellow for intermediate, orange for weak and red when there was no signal.

Table 1 - Primer for amplification: sequence in 5' → 3' order and references

Gene	Primer	Sequence 5' → 3'	Source
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	(Folmer *et al.* 1994)
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	
16S	SHA	ACGGAATGAACTCAAATCATGT	(Cunningham & Buss 1993)
	SHB	TCGACTGTTTACCAAAAACATA	
ITS	CAS5p8sB1d	ATGTGCGTTCRAAATGTCGATGTTCA	(Pontin & Cruickshank 2012)
	CAS18SF1	TACACACCGCCCGTCGCTACTA	

2.2.3 DNA Purification and quantification

To purify PCR products and prepare the samples for the MinION sequencing, a defined quantity of each product was extracted for vials, not exceeding the amount of 1 ml total in each. The quantity taken from each PCR product took into account the quality result in the agarose gel. Thus, a higher volume was removed from the products with lower quality. The vials with the mixture of samples were purified with the AMPURE kit® (Agencourt®). After the purification protocol was successfully completed, the DNA of each tube was quantified using a microvolume spectrophotometer (Simpli-Nano™ - GE). Purified products were used for MinION sequencing library preparation.

2.2.4 Library preparation and sequencing

For library preparation and sequencing, a SQK-LSK109 ligation sequencing kit (Oxford Nanopore Technologies®) was chosen. For the first experiment the total of DNA used for preparation was 724 ng. Library preparation followed all steps of the manufacturer's protocol. The sequencing was performed with the MinION sequencer, using the MinKNOW software (Figure 10). The obtained sequences were finally demultiplexed.

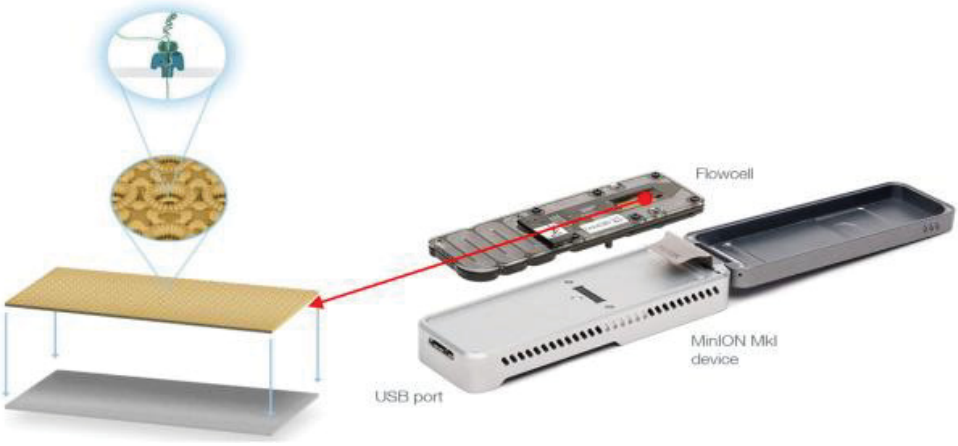


Figure 10 - The MinION sequencing device. (author: Hengyun Lu 2016)

2.3 Data analysis

2.3.1 Editing, alignment and quality control of the sequences

The obtained sequences were aligned and edited with Geneious Prime 2021.2.2 (https://www.geneious.com). MAFFT (Katoh *et al.* 2002) (algorithm: Auto; scoring matrix: 200PAM/K=2; gap open penalty=1) was used as alignment algorithm. In first place, each sequence was run with the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology

Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi), to check for eventual contaminations. All the obtained reads for each sequence were aligned with the correspondent consensus sequence, in order to eliminate contaminated reads and check each position on the consensus. The sequences were grouped in six different data sets: each specie (*Velella velella* and *Porpita porpita*) had one data set per molecular marker. Once the sequences on a data set were all corrected, an align was performed and the amino acid composition was analysed for further quality checking.

2.3.2 Phylogeographic analysis

The number of polymorphic sites, haplotypes and nucleotide and haplotypic diversity in the alignment was determined with DnaSP v6 (Rozas *et al.* 2017). To infer the haplotypic genetic diversity, six haplotype networks were constructed using the software PopArt 1.7 (Population Analysis with Reticulate Trees -http://popart.otago.ac.nz/index.shtml) (Leigh & Bryant 2015) based on Median Joining test (Bandelt *et al.* 1999). The networks were coloured differently according to the sampling site, displaying the obtained sequences from the study as well as the existing sequences in the databases for the species under study, in order to check for a correlation between the haplotypes and local. The MEGAX software (Kumar *et al.* 2018) was used to establish the p-distance (Felsenstein 1984). For the three alignments the determination of the optimal nucleotide substitution model was performed with the programme jModelTest version 2.1.10 (Darriba *et al.* 2012). With the model choice, ML analyses were conducted in PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/)(Guindon *et al.* 2010), with BIC (Bayesian Information Criterion) for 1000 bootstrap replications. The obtained phylogenetic trees were manipulated with FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and ITOL (https://itol.embl.de/). The phylogenetic trees were built with bootstrap, providing statistical support to the branches.

2.3.2 Species delimitation

In order to investigate the genetic differentiation of species within the sampled sequences, two different analyses were applied in two different data sets: the COI and 16S alignment with all the studied sequences (including the ones retrieved from the databases). The first delimitation method was Assemble Species by Automatic Partitioning (ASAP)(Puillandre *et al.* 2021). ASAP is an analysis by hierarchical clustering algorithm that only uses pairwise genetic distances, without taking into account the phylogenetic reconstructions. The number of hypothesized species is calculated using genetic distances and ranked by an “ASAP-score”. The best score is the lowest value, that suggests the best partition. The second performed analysis was PTP (Poisson Tree Processes) (https://species.h-its.org/). This method combines phylogenetic relationships with genetic distances in order to delimit species, using Maximum-Likelihood methods. The input trees were the ones obtained with PhyML. The analyses were run with the following parameters: 5×10^5 MCMC (Markov chain Monte Carlo) generations, thinning value of 100 and burn-in of 25%.

3. Results

3.1 Genetic diversity indices

The aim of this work was to study the genetic diversity of the Porpitidae family in the Azores. A total of 280 individuals were sampled (67 *Porpita porpita* and 213 *Velella velella*). Once corrected and aligned the sequences were separated by species and molecular marker, for a first analysis regarding the polymorphism (Table 2).

Table 2 - Comparison of the information contained in the observed polymorphism of partial sequences for the three genes analysed (COI, 16S and ITS), by species. Nseq – Number of sequences; Bp – Total base pairs; S - Number of polymorphic (segregating) sites; h - Number of haplotypes; Hd - Haplotype (gene) diversity; π - Nucleotide diversity.

	Porpita porpita			***Velella velella***		
	COI	16S	ITS	COI	16S	ITS
N_{seq}	64	65	61	162	168	153
Bp	585	589	439	658	590	458
S	99	64	4	140	94	10
h	61	49	5	150	108	11
H_d	0.988	0.974	0.080	0.995	0.914	0.064
π	0.01718	0.00919	0.00019	0.01030	0.00391	0.00014

The large subunit ribosomal RNA gene (16S) was the molecular marker with the largest number sequences in both species. The region that showed a higher degree of polymorphism was COI, unlike ITS that exhibited a low genetic variation. These results translate into a variable number of haplotypes within the different molecular markers. Haplotype and nucleotide diversity also display highest values for the mitochondrial markers: COI and 16S rRNA.

3.2 Phylogeographical analyses

To better visualize the haplotidic diversity of each marker, haplotype median-joining networks were constructed. The sequences obtained in the study as well as some database withdrawals were used (see Appendix C). The division was made according to the sampling site of each individual.

The phylogenetic trees allow the visualization of an hypothesis of the evolutionary relationships among the studied specimens, with a great advantage of showing statistical support. The following phylogenetic trees were built using only sequences of the Porpitidae family. Thus, some sequences of the genus *Porpita* were used as an outgroup of the genus *Velella*, and vice versa.

Before performing an evolutionary analysis, the most suitable evolutionary model was selected for each molecular marker (Table 3).

Table 3 - Selected evolutionary model for COI, 16S and ITS, suggested by jModeltest.

COI	16S	ITS
GTR+G+I	GTR+G	HKY+G

3.2.1 *Porpita porpita*

3.2.1.1 COI

The network for COI *Porpita porpita* (Figure 11) demonstrates a clear differentiation of populations by main biogeographic area. Four main clusters can be distinguished, two of which are occurring in the Azores. It is also possible to observe two haplotypes (PPO 30 and PPO38) from the Azores that differ greatly in number of base pairs from the other Azorean *Porpita*. These two distinctive lineages (PPO 30 and PPO 38) seem closely related genetically, and seem actually to cluster in a clade conjuntly with two main sub-clades present in the Indo-Pacific. Curiously, one Porpita haplotype present in the Caribbean seems to

relate closely with one of the Indo-Pacific cluster. The Mediterranean and NO Atlantic sequenced are in the main clade, associated with the Azorean sequences.

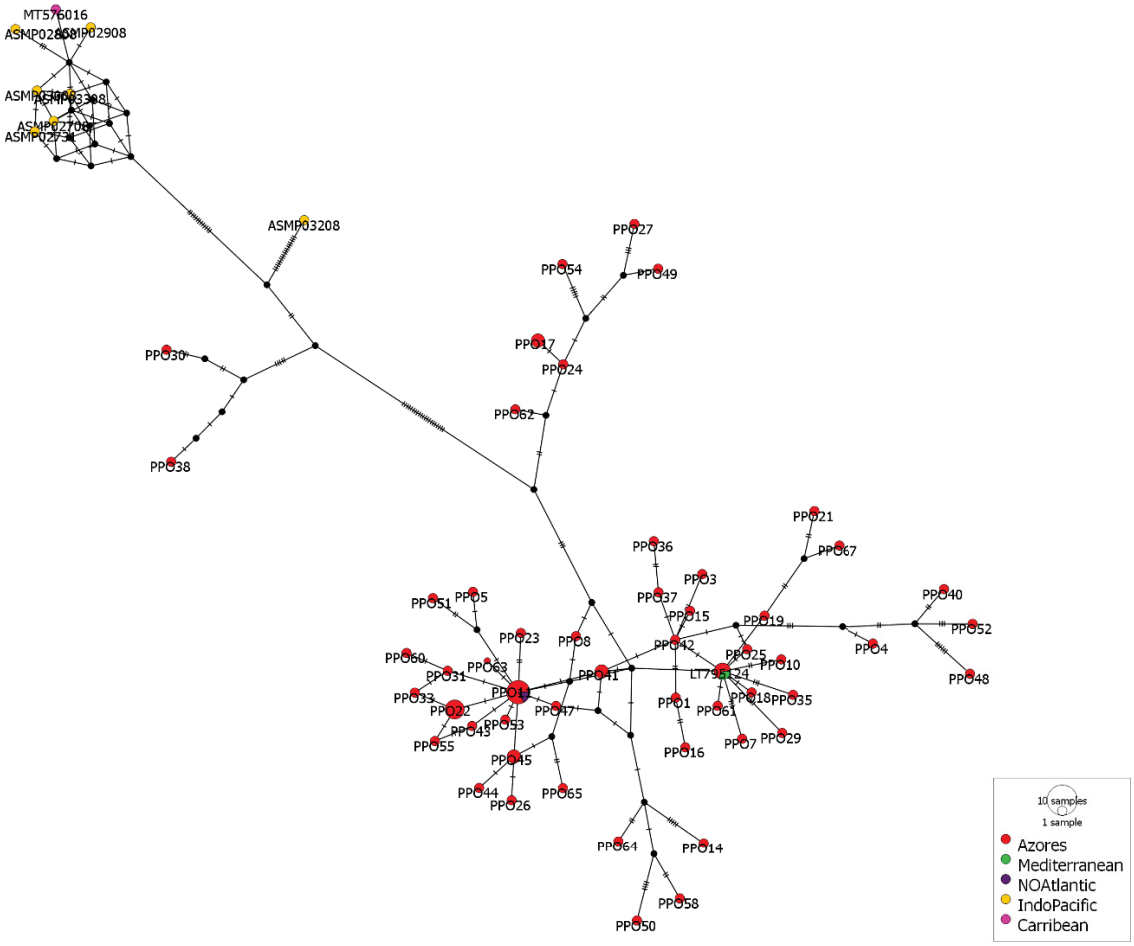


Figure 11 - Haplotype network (Median-Joining) with COI sequences for the different populations of *Porpita porpita*.

The obtained tree for *Porpita* sequences show similar results to the equivalent haplotype network, also exhibiting four main clades. In the COI tree (Figure 12) there is a more genetically distant cluster within the *Porpita* branch with medium bootstrap support, above 750. This group includes the sequences from Indo-Pacific, Caribbean and the Azorean sequences PPO 30 and PPO 38. The Mediterranean and NO Atlantic sequences are genetically closer to the other Azorean sequences. There is evidence of the sharing of haplotypes between localities, suggesting for these genes, some relatively recent genetic flow amongst these locations.

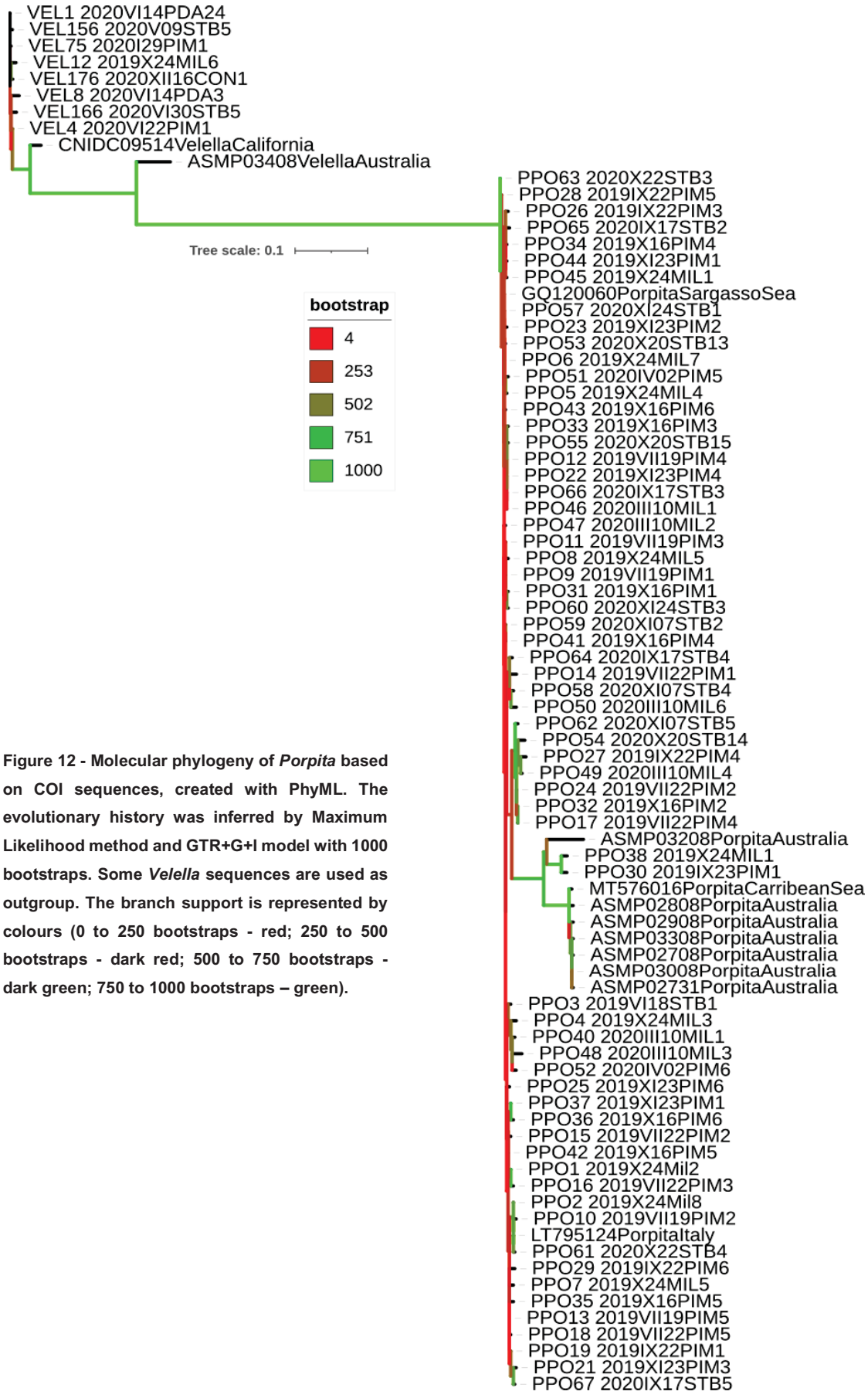


Figure 12 - Molecular phylogeny of *Porpita* based on COI sequences, created with PhyML. The evolutionary history was inferred by Maximum Likelihood method and GTR+G+I model with 1000 bootstraps. Some *Velella* sequences are used as outgroup. The branch support is represented by colours (0 to 250 bootstraps - red; 250 to 500 bootstraps - dark red; 500 to 750 bootstraps - dark green; 750 to 1000 bootstraps – green).

3.2.1.2 16S

In the 16S haplotype network of *Porpita porpita* is possible to distinguish four main clades, two of them including the sequences of Azores. The two large clades include the samples from the Azores (red). Closer to these clades, is the single sequence clade with the W Pacific sequence. Finally, with more nucleotidic differentiation, is represented a clade that includes the samples from the Pacific (green and purple) and two of the specimens sampled in the Azores (PPO30 and PPO 38).

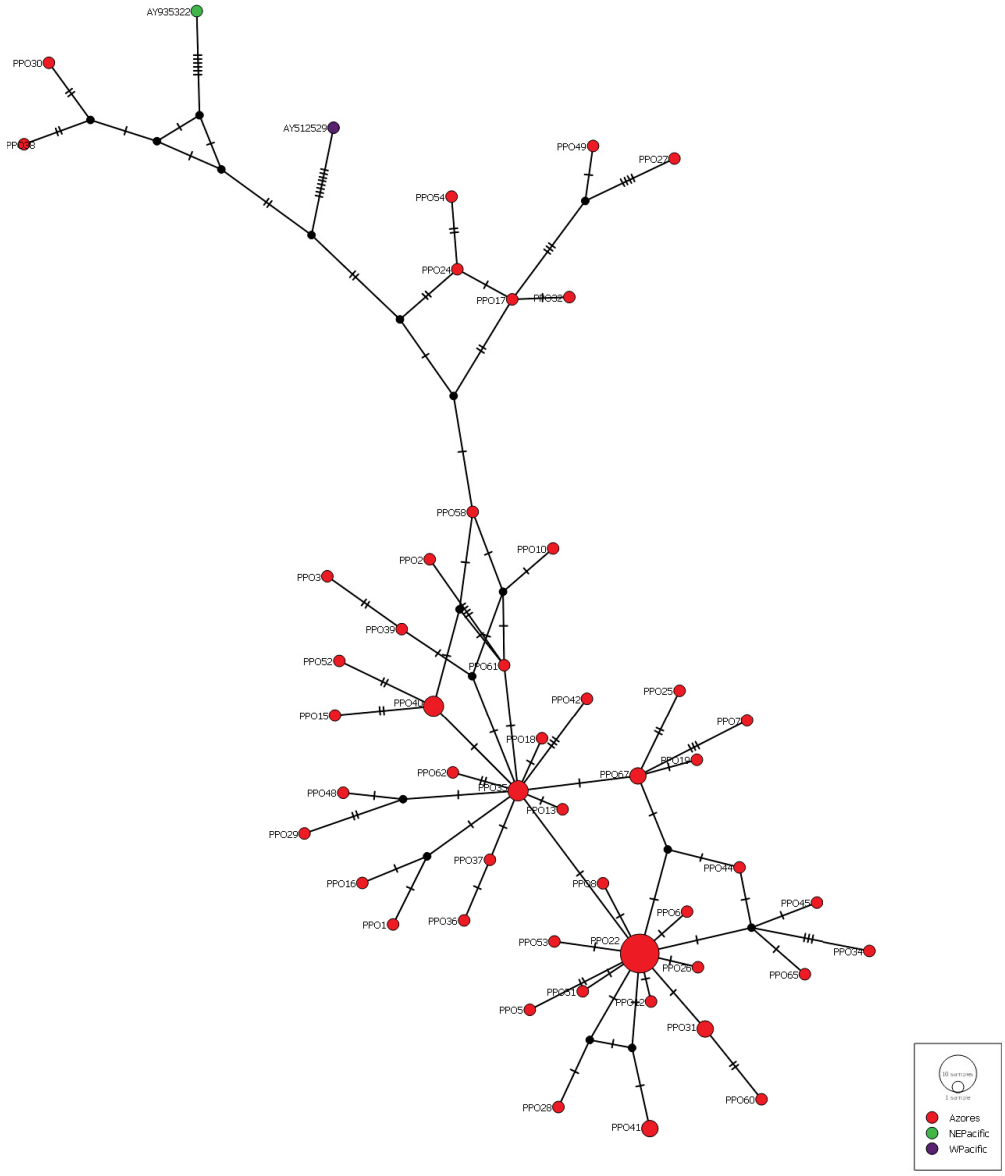


Figure 13 - Haplotype network (Median-Joining) with 16S sequences for the different populations of *Porpita porpita.*

The *Porpita* 16S tree also exhibit four main lineages. The sequences from NE and W Pacific are genetically closer to the Azorean sequences PPO 30 and PPO 38 (Figure 14), with a moderate bootstrap support (about 750).

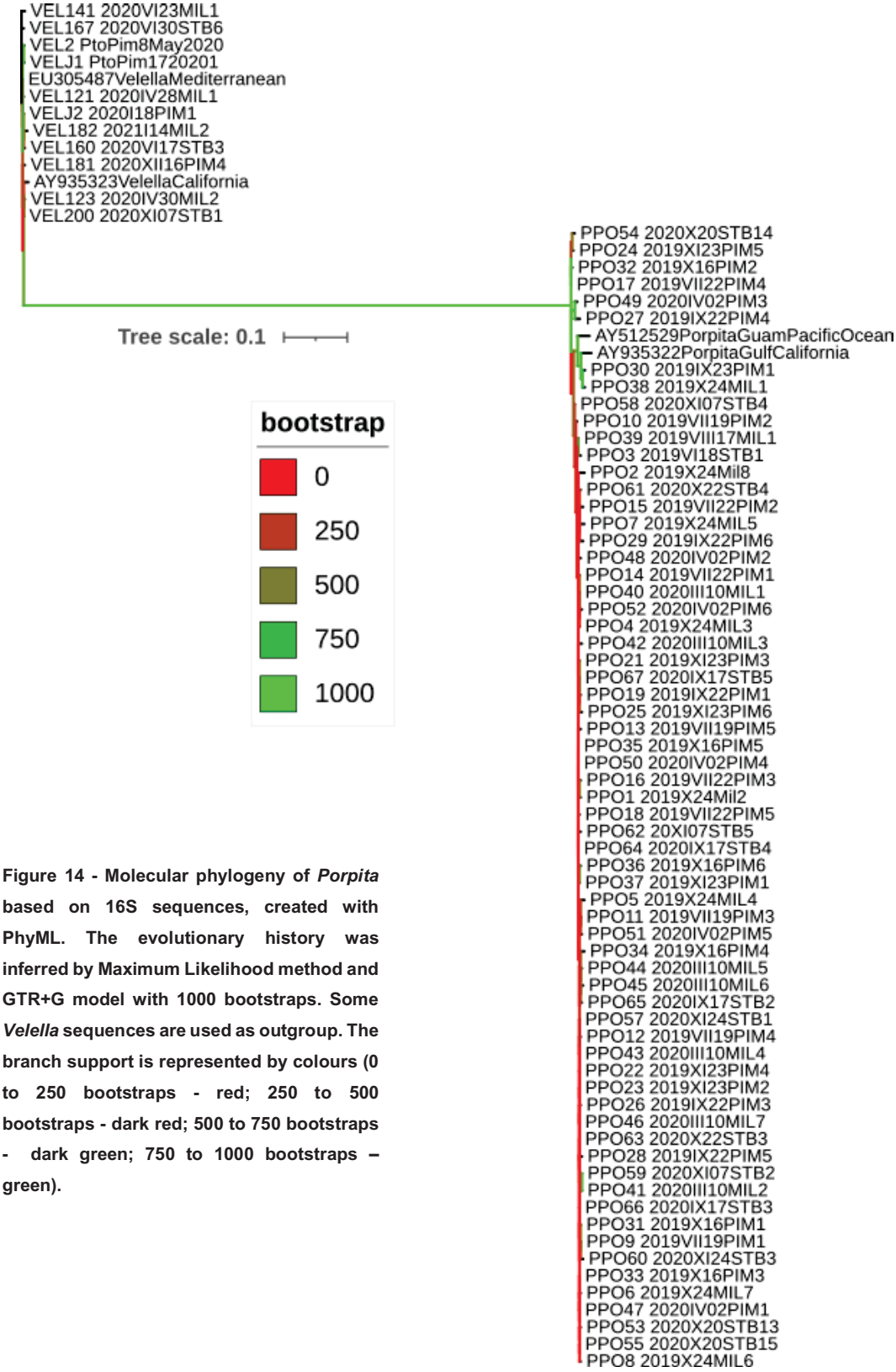


Figure 14 - Molecular phylogeny of *Porpita* based on 16S sequences, created with PhyML. The evolutionary history was inferred by Maximum Likelihood method and GTR+G model with 1000 bootstraps. Some *Velella* sequences are used as outgroup. The branch support is represented by colours (0 to 250 bootstraps - red; 250 to 500 bootstraps - dark red; 500 to 750 bootstraps - dark green; 750 to 1000 bootstraps – green).

3.2.1.3. ITS

Unlike both mitochondrial genes, ITS nuclear gene revealed a very low haplotypic diversity (Figure 15). The haplotype network suggests an haplotype comprehending all the sequences, including the Azorean sequences and one sequence from Caribbean.

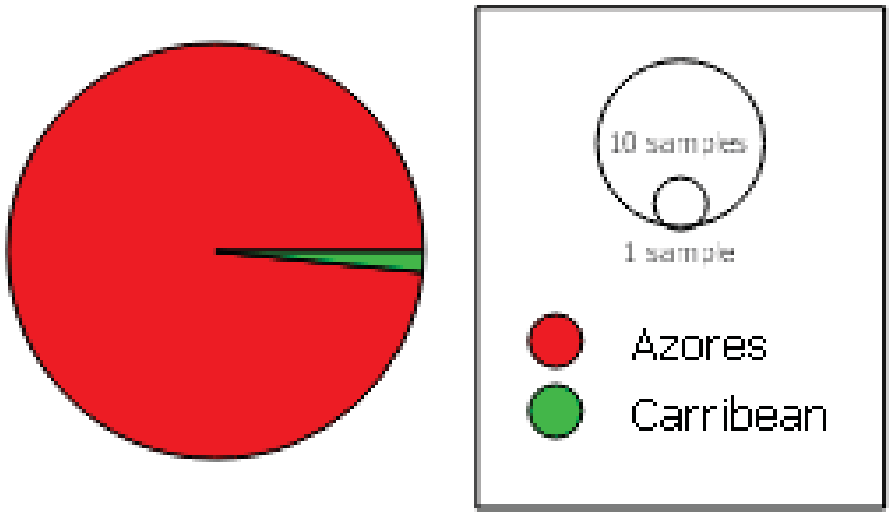


Figure 15 - Haplotype network (Median-Joining) with ITS sequences for the different populations of *Porpita porpita.*

The phylogenetic tree confirms little or no evidence of genetic variation in this locus (Figure 16). The displayed tree exhibits the same genetic distance for all the sequences, including the Caribbean and the Azorean sequences.

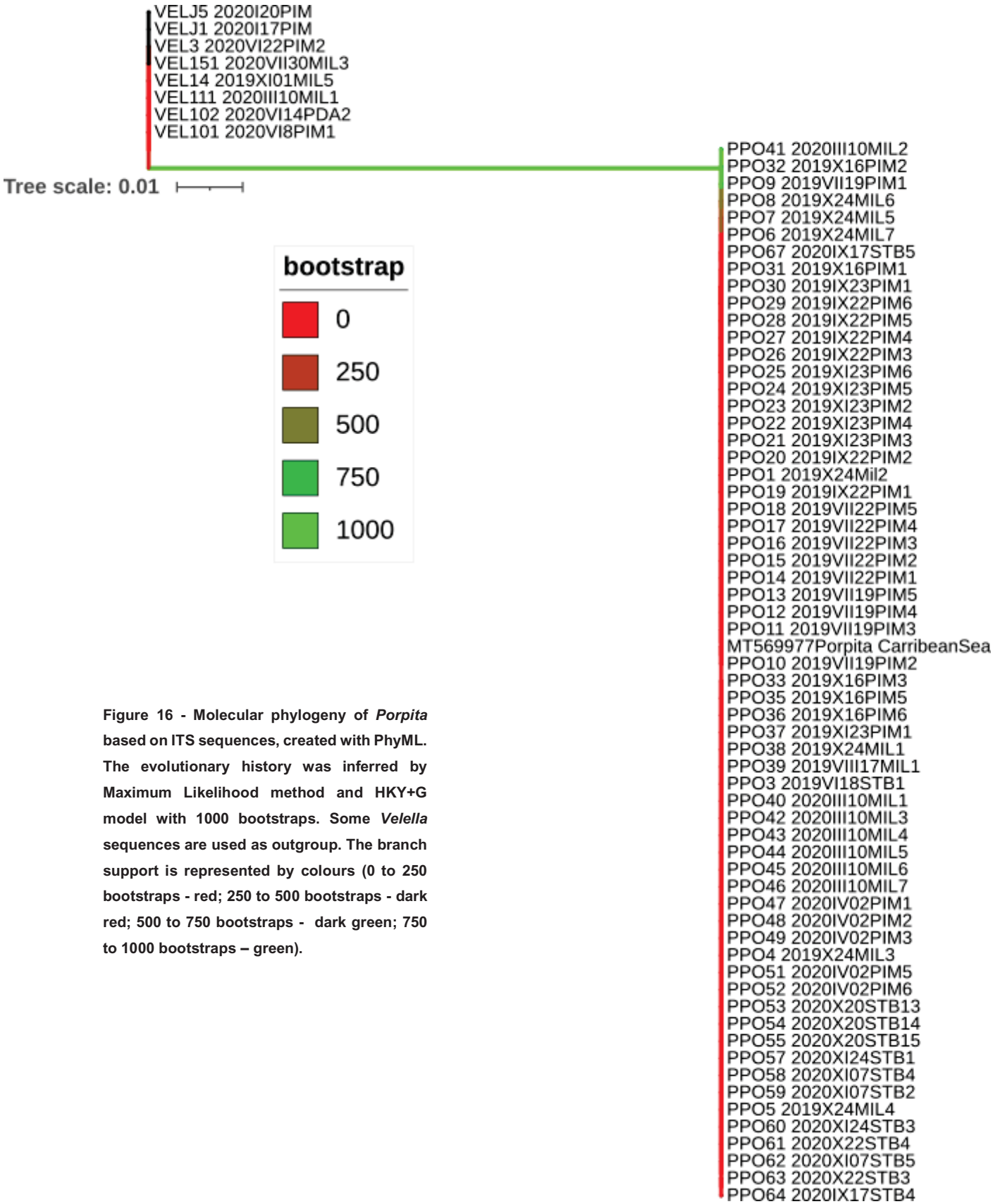


Figure 16 - Molecular phylogeny of *Porpita* based on ITS sequences, created with PhyML. The evolutionary history was inferred by Maximum Likelihood method and HKY+G model with 1000 bootstraps. Some *Velella* sequences are used as outgroup. The branch support is represented by colours (0 to 250 bootstraps - red; 250 to 500 bootstraps - dark red; 500 to 750 bootstraps - dark green; 750 to 1000 bootstraps – green).

3.2.2. *Velella velella*

3.2.2.1 COI

The *Velella velella* COI network (Figure 17) is more complex, due to the increase in the number of sequences and sampling sites. It is possible to distinguish two main clades: one with samples from Indo-Pacific (red), and other presenting a remarkable haplotypic diversity without much nucleotidic differentiation that includes the *Porpita* sampled in the Azores (purple). Remarkably, a specimen from the NE Pacific (green) presents an haplotype closely incorporated in the clade with the samples from the Azores.

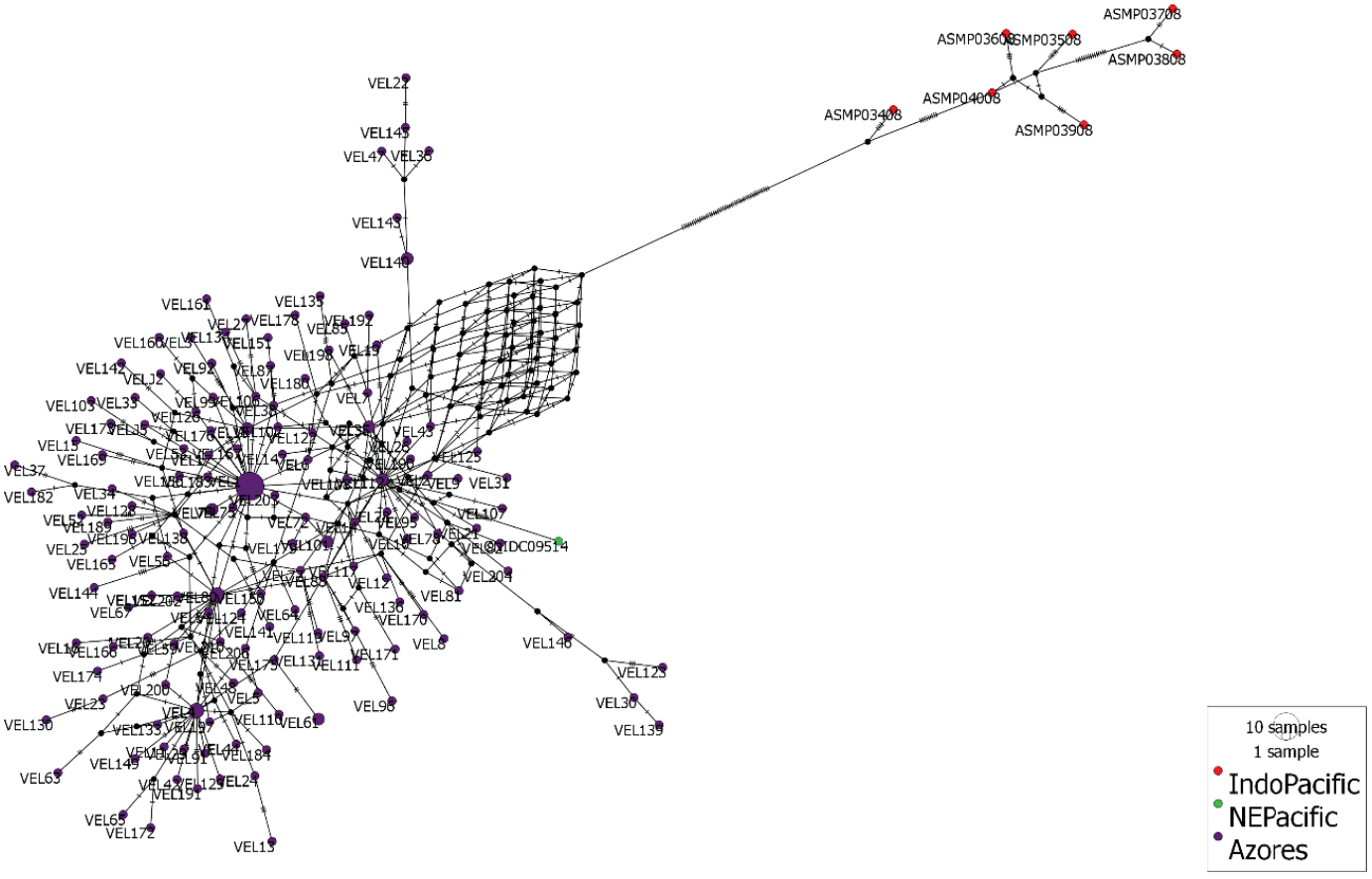


Figure 17 - Haplotype network (Median-Joining) with COI sequences for the different populations of *Velella velella.*

In the *Velella* COI tree (Figure 18) is possible to distinguish three main branches within the *Velella* sequences. The first branch includes the specimens from Indo-Pacific but with a low bootstrap support. The others branches display, with a high bootstrap support, a relation between the sample from NE Pacific and the Azorean sequences.

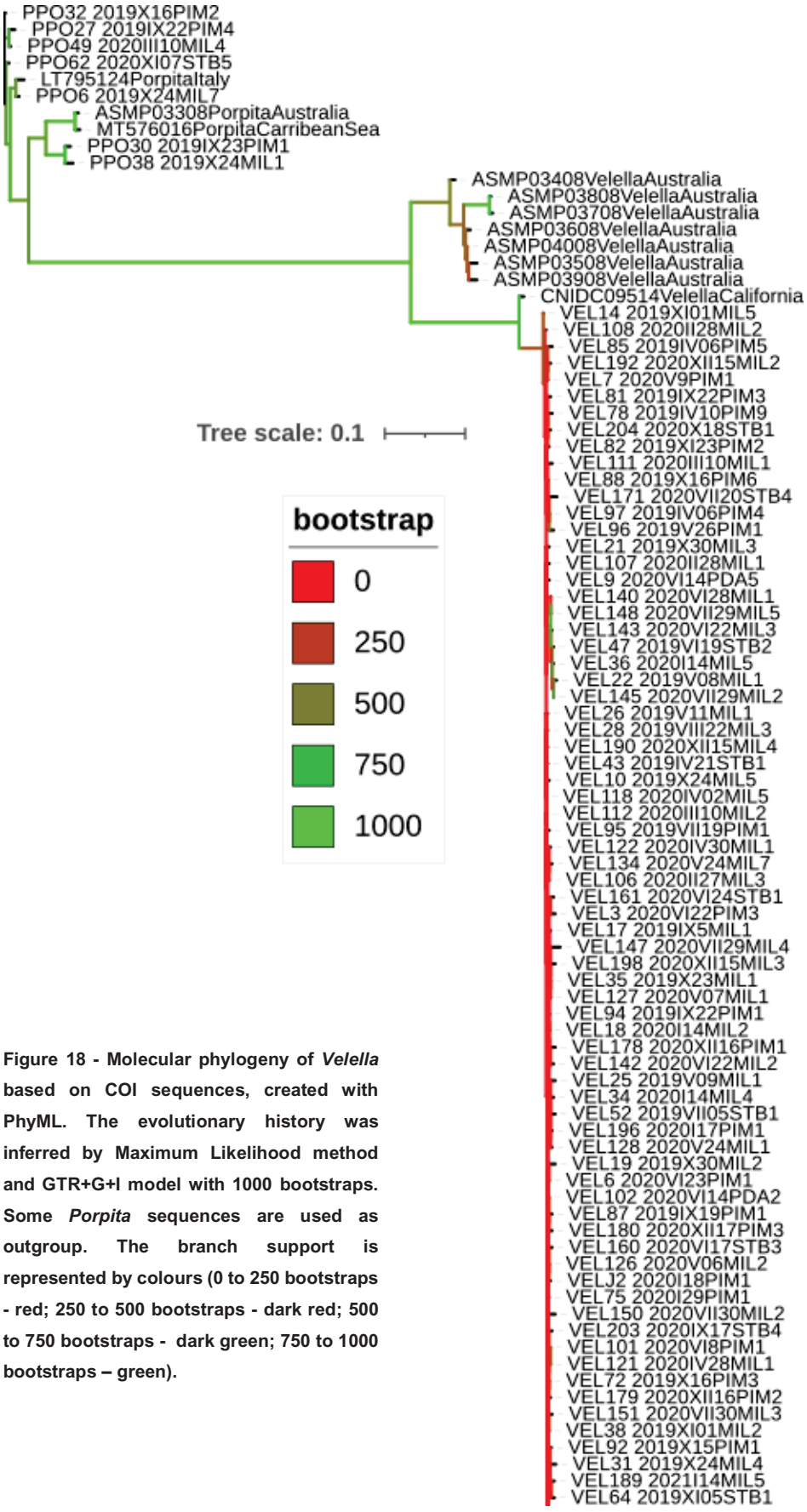
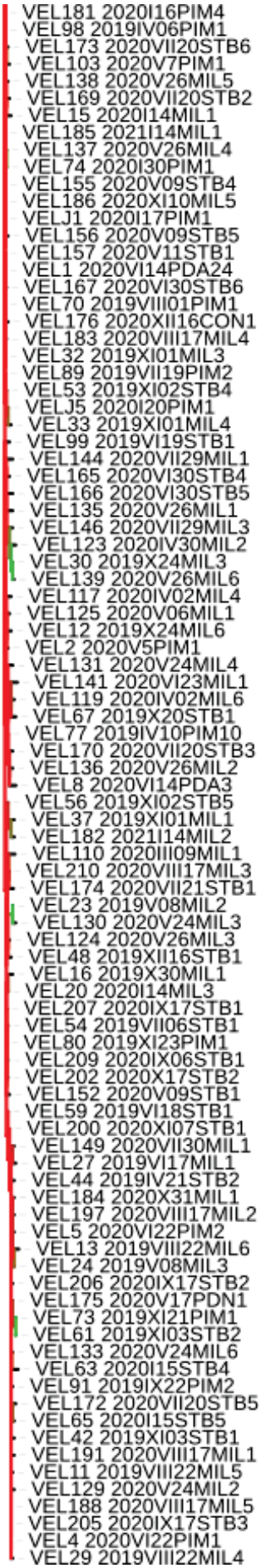
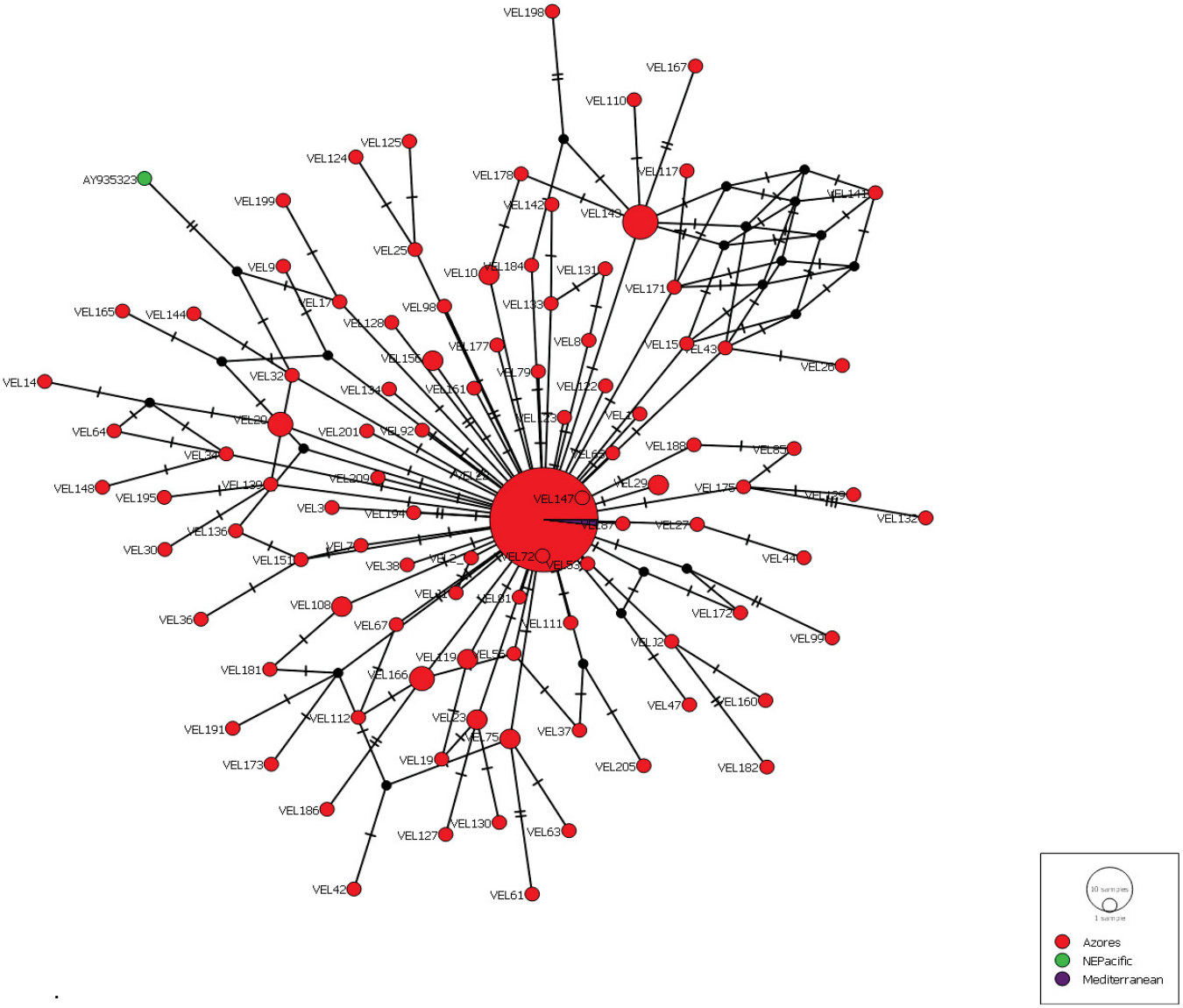


Figure 18 - Molecular phylogeny of *Velella* based on COI sequences, created with PhyML. The evolutionary history was inferred by Maximum Likelihood method and GTR+G+I model with 1000 bootstraps. Some *Porpita* sequences are used as outgroup. The branch support is represented by colours (0 to 250 bootstraps - red; 250 to 500 bootstraps - dark red; 500 to 750 bootstraps - dark green; 750 to 1000 bootstraps – green).


VEL181 2020I16PIM4
VEL98 2019IV06PIM1
VEL173 2020VII20STB6
VEL103 2020V7PIM1
VEL138 2020V26MIL5
VEL169 2020VII20STB2
VEL15 2020I14MIL1
VEL185 2021I14MIL1
VEL137 2020V26MIL4
VEL74 2020I30PIM1
VEL155 2020V09STB4
VEL186 2020XI10MIL5
VELJ1 2020I17PIM1
VEL156 2020V09STB5
VEL157 2020V11STB1
VEL1 2020VI14PDA24
VEL167 2020VI30STB6
VEL70 2019VIII01PIM1
VEL176 2020XII16CON1
VEL183 2020VIII17MIL4
VEL32 2019XI01MIL3
VEL89 2019VII19PIM2
VEL53 2019XI02STB4
VELJ5 2020I20PIM1
VEL33 2019XI01MIL4
VEL99 2019VI19STB1
VEL144 2020VII29MIL1
VEL165 2020VI30STB4
VEL166 2020VI30STB5
VEL135 2020V26MIL1
VEL146 2020VII29MIL3
VEL123 2020IV30MIL2
VEL30 2019X24MIL3
VEL139 2020V26MIL6
VEL117 2020IV02MIL4
VEL125 2020V06MIL1
VEL12 2019X24MIL6
VEL2 2020V5PIM1
VEL131 2020V24MIL4
VEL141 2020VI23MIL1
VEL119 2020IV02MIL6
VEL67 2019X20STB1
VEL77 2019IV10PIM10
VEL170 2020VII20STB3
VEL136 2020V26MIL2
VEL8 2020VI14PDA3
VEL56 2019XI02STB5
VEL37 2019XI01MIL1
VEL182 2021I14MIL2
VEL110 2020III09MIL1
VEL210 2020VIII17MIL3
VEL174 2020VII21STB1
VEL23 2019V08MIL2
VEL130 2020V24MIL3
VEL124 2020V26MIL3
VEL48 2019XII16STB1
VEL16 2019X30MIL1
VEL20 2020I14MIL3
VEL207 2020IX17STB1
VEL54 2019VII06STB1
VEL80 2019XI23PIM1
VEL209 2020IX06STB1
VEL202 2020X17STB2
VEL152 2020V09STB1
VEL59 2019VI18STB1
VEL200 2020XI07STB1
VEL149 2020VII30MIL1
VEL27 2019VI17MIL1
VEL44 2019IV21STB2
VEL184 2020X31MIL1
VEL197 2020VIII17MIL2
VEL5 2020VI22PIM2
VEL13 2019VIII22MIL6
VEL24 2019V08MIL3
VEL206 2020IX17STB2
VEL175 2020V17PDN1
VEL73 2019XI21PIM1
VEL61 2019XI03STB2
VEL133 2020V24MIL6
VEL63 2020I15STB4
VEL91 2019IX22PIM2
VEL172 2020VII20STB5
VEL65 2020I15STB5
VEL42 2019XI03STB1
VEL191 2020VIII17MIL1
VEL11 2019VIII22MIL5
VEL129 2020V24MIL2
VEL188 2020VIII17MIL5
VEL205 2020IX17STB3
VEL4 2020VI22PIM1
VEL29 2019VIII22MIL4

3.2.2.2. 16S

The 16S haplotype network reconstruction for *Velella velella* resulted in a single clade without definition of populations by the areas (Figure 19). The sequence from the Pacific (green) represents a distinct haplotype. Contrary, the Mediterranean (purple) is equal to a dominant haplotype in the Azores.



.

Figure 19 - Haplotype network (Median-Joining) with 16S sequences for the different populations of *Velella velella*

The 16S tree for *Velella* (Figure 20) has only one branch with all sequences, including all the Azoreans, Mediterranean and the NE Pacific. It is possible to verify that there is a low genetic diversity between the sequences, with NE Pacific being the most distant. The Mediterranean sequence is genetically closer to the Azorean sequences. The results are in accordance with the haplotype network, is similar to the dominant Azorean haplotype.

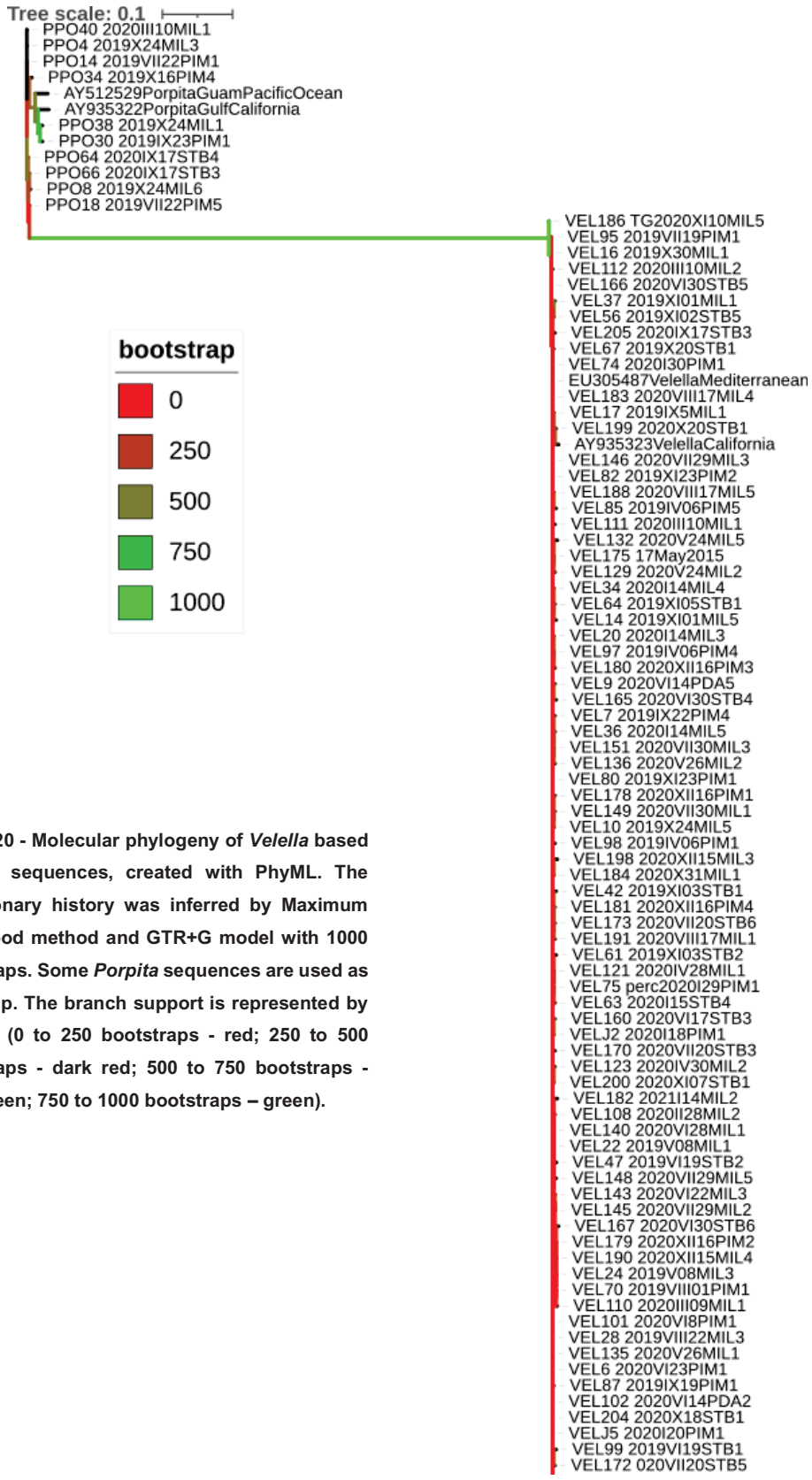
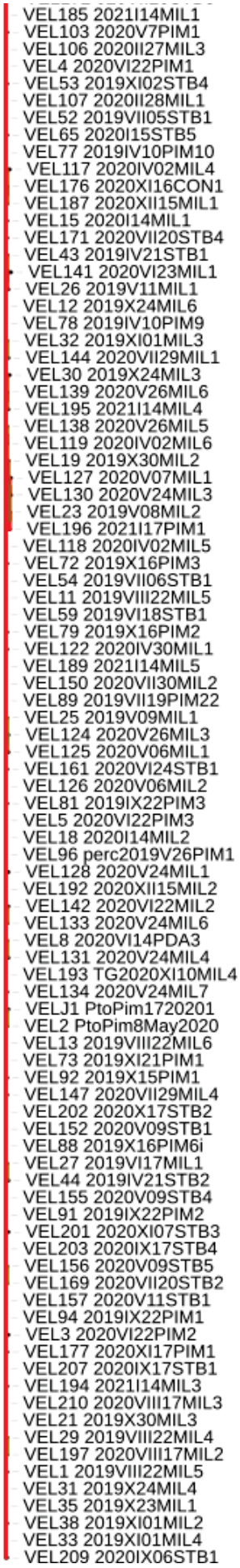


Figure 20 - Molecular phylogeny of *Velella* based on 16S sequences, created with PhyML. The evolutionary history was inferred by Maximum Likelihood method and GTR+G model with 1000 bootstraps. Some *Porpita* sequences are used as outgroup. The branch support is represented by colours (0 to 250 bootstraps - red; 250 to 500 bootstraps - dark red; 500 to 750 bootstraps - dark green; 750 to 1000 bootstraps – green).


VEL185 2021I14MIL1
VEL103 2020V7PIM1
VEL106 2020II27MIL3
VEL4 2020VI22PIM1
VEL53 2019XI02STB4
VEL107 2020II28MIL1
VEL52 2019VII05STB1
VEL65 2020I15STB5
VEL77 2019IV10PIM10
VEL117 2020IV02MIL4
VEL176 2020XI16CON1
VEL187 2020XII15MIL1
VEL15 2020I14MIL1
VEL171 2020VII20STB4
VEL43 2019IV21STB1
VEL141 2020VI23MIL1
VEL26 2019V11MIL1
VEL12 2019X24MIL6
VEL78 2019IV10PIM9
VEL32 2019XI01MIL3
VEL144 2020VII29MIL1
VEL30 2019X24MIL3
VEL139 2020V26MIL6
VEL195 2021I14MIL4
VEL138 2020V26MIL5
VEL119 2020IV02MIL6
VEL19 2019X30MIL2
VEL127 2020V07MIL1
VEL130 2020V24MIL3
VEL23 2019V08MIL2
VEL196 2021I17PIM1
VEL118 2020IV02MIL5
VEL72 2019X16PIM3
VEL54 2019VII06STB1
VEL11 2019VIII22MIL5
VEL59 2019VI18STB1
VEL79 2019X16PIM2
VEL122 2020IV30MIL1
VEL189 2021I14MIL5
VEL150 2020VII30MIL2
VEL89 2019VII19PIM22
VEL25 2019V09MIL1
VEL124 2020V26MIL3
VEL125 2020V06MIL1
VEL161 2020VI24STB1
VEL126 2020V06MIL2
VEL81 2019IX22PIM3
VEL5 2020VI22PIM3
VEL18 2020I14MIL2
VEL96 perc2019V26PIM1
VEL128 2020V24MIL1
VEL192 2020XII15MIL2
VEL142 2020VI22MIL2
VEL133 2020V24MIL6
VEL8 2020VI14PDA3
VEL131 2020V24MIL4
VEL193 TG2020XI10MIL4
VEL134 2020V24MIL7
VELJ1 PtoPim1720201
VEL2 PtoPim8May2020
VEL13 2019VIII22MIL6
VEL73 2019XI21PIM1
VEL92 2019X15PIM1
VEL147 2020VII29MIL4
VEL202 2020X17STB2
VEL152 2020V09STB1
VEL88 2019X16PIM6i
VEL27 2019VI17MIL1
VEL44 2019IV21STB2
VEL155 2020V09STB4
VEL91 2019IX22PIM2
VEL201 2020XI07STB3
VEL203 2020IX17STB4
VEL156 2020V09STB5
VEL169 2020VII20STB2
VEL157 2020V11STB1
VEL94 2019IX22PIM1
VEL3 2020VI22PIM2
VEL177 2020XI17PIM1
VEL207 2020IX17STB1
VEL194 2021I14MIL3
VEL210 2020VIII17MIL3
VEL21 2019X30MIL3
VEL29 2019VIII22MIL4
VEL197 2020VIII17MIL2
VEL1 2019VIII22MIL5
VEL31 2019X24MIL4
VEL35 2019X23MIL1
VEL38 2019XI01MIL2
VEL33 2019XI01MIL4
VEL209 2020IX06STB1

3.2.2.3 ITS

The haplotype network for *Velella velella* ITS includes only Azorean sequences, since there are no available sequences for *Velella velella* ITS in the databases. As in *Porpita porpita* ITS network there in no haplotypic diversity in the gene (Figure 21).

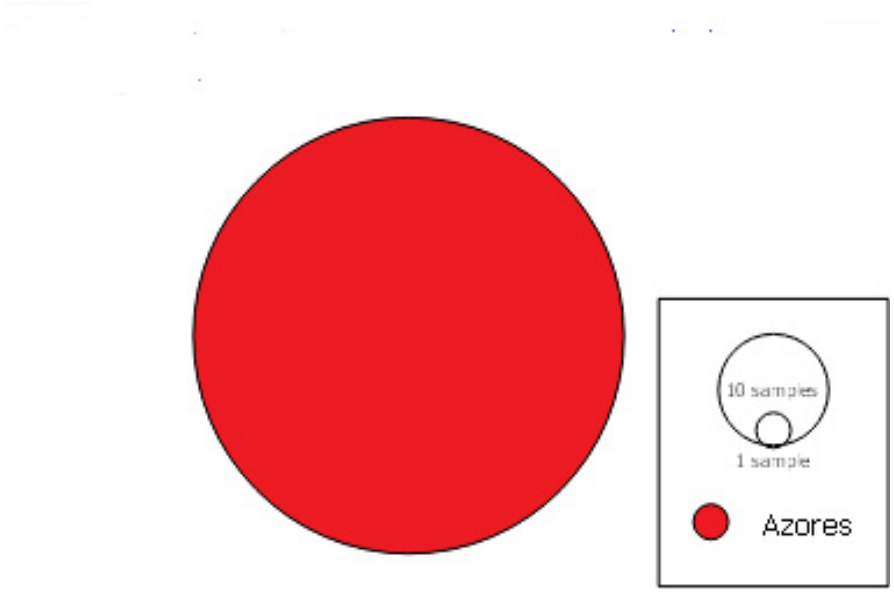


Figure 21 - Haplotype network (Median-Joining) with ITS sequences for the different populations of *Velella velella.*

Lastly, the ITS tree (Figure 22) exhibits no genetic distance between the sequences, all from the Azores. However, it is important to point out that since there are no sequences available from other sampling sites for this species, in this specific gene, only a regional analysis could be made.

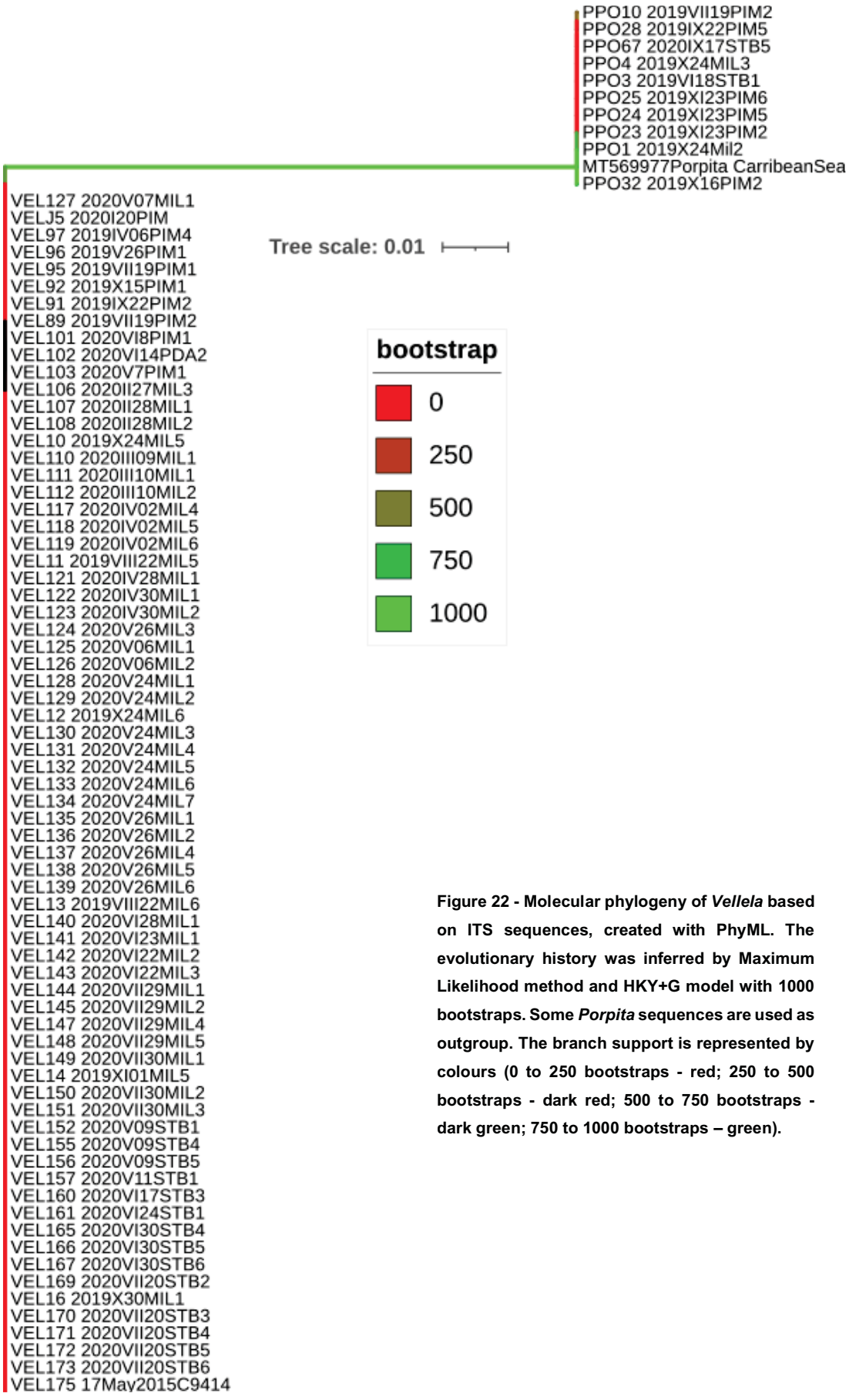


Figure 22 - Molecular phylogeny of *Vellela* based on ITS sequences, created with PhyML. The evolutionary history was inferred by Maximum Likelihood method and HKY+G model with 1000 bootstraps. Some *Porpita* sequences are used as outgroup. The branch support is represented by colours (0 to 250 bootstraps - red; 250 to 500 bootstraps - dark red; 500 to 750 bootstraps - dark green; 750 to 1000 bootstraps – green).

VEL176 2020XI16CON1
VEL178 2020XII16PIM1
VEL179 2020XII16PIM2
VEL17 2019IX5MIL1
VEL180 2020XII16PIM3
VEL181 2020XII16PIM4
VEL182 2021I14MIL2
VEL183 2020VIII17MIL4
VEL184 2020X31MIL1
VEL185 2021I14MIL1
VEL186 TG2020XI10MIL5
VEL187 2020XII15MIL1
VEL188 2020VIII17MIL5
VEL189 2021I14MIL5
VEL18 2020I14MIL2
VEL190 2020XII15MIL4
VEL191 2020VIII17MIL1
VEL192 2020XII15MIL2
VEL193 TG2020XI10MIL4
VEL194 2021I14MIL3
VEL195 2021I14MIL4
VEL196 2021I17PIM
VEL197 2020VIII17MIL2
VEL198 2020XII15MIL3
VEL19 2019X30MIL2
VEL1 2020VI14PDA1
VEL200 2020XI07STB1
VEL203 2020IX17STB4
VEL205 2020IX17STB3
VEL206 2020IX17STB2
VEL207 2020IX17STB1
VEL208 2020X17STB1
VEL20 2020I14MIL3
VEL210 2020VIII17MIL3
VEL21 2019X30MIL3
VEL23 2019V08MEL2
VEL24 2019V08MEL3
VEL25 2019V09MEL1
VEL26 2019V11MEL1
VEL27 2019VI17MIL1
VEL28 2019VIII22MIL3
VEL30 2019X24MIL3
VEL31 2019X24MIL4
VEL32 2019XI01MIL3
VEL33 2019XI01MIL4
VEL35 2019X23MIL1
VEL36 2020I14MIL5
VEL37 2019XI01MIL1
VEL38 2019XI01MIL2
VEL3 2020VI22PIM2
VEL42 2019XI03STB1
VEL43 2019IV21STB1
VEL44 2019IV21STB2
VEL48 2019XII16STB1
VEL4 2020VI22PIM1
VEL53 2019XI02STB4
VEL54 2019VII06STB1
VEL56 2019XI02STB5
VEL59 2019VI18STB1
VEL61 2019XI03STB2
VEL63 2020I15STB4
VEL64 2019XI05STB1
VEL65 2020I15STB5
VEL67 2019X20STB1
VEL6 2020VI23PIM1
VEL70 2019VIII01PIM1
VEL72 2019X16PIM3
VEL73 2019XI21PIM1
VEL74 2020I30PIM1
VEL75 2020I29PIM1
VEL76 2019VIII01PIM2
VEL77 2019IV10PIM10
VEL78 2019IV10PIM9
VEL79 2019X16PIM2
VEL7 2020V9PIM1
VEL80 2019XI23PIM1
VEL81 2019IX22PIM3
VEL82 2019XI23PIM2
VEL85 2019IV06PIM5
VEL87 2019IX19PIM1
VEL88 2019X16PIM6
VEL94 2019IX22PIM1
VELJ1 2020I17PIM

3.3 Species delimitation

The ASAP procedure with COI, identified as the first partition (best hypothesis) the presence of three hypothetical species. The suggested species were: (1) all the *Porpita* sequences; (2) the *Velella* sequences from the Indo-Pacific and (3) all the remaining *Velella* sequences. This partition was at the threshold distance of 7.33% (p-distance) which has the same ASAP-score (2.50) as the second partition, that exhibited a threshold distance of 3.92% (p-distance). Six species were suggested by the second partition including: three different species within the sequences of *Velella* Indo-Pacific, one specie for the PPO 30 and PPO 38 and the other two species with the remaining *Porpita* and *Velella* sequences.

For 16S dataset, the result with the lowest ASAP-score (1.50), proposed two species at a threshold distance of 8.62% (p-distance). For the second-best partition, with an ASAP-score of 2.00 and threshold value of 1.42% (p-distance) the number of considered species was three (Table 4). This analysis suggests three groups of hypothetical species: one with all the *Velella velella* sequences (Azorean, Mediterranean and NE Pacific), another with PPO 30 and PPO 38 and the last one with the remaining *Porpita porpita* sequences (Azorean, NE and W Pacific).

Table 4 - Results obtained in the delimitation of species for the sequences of *Velella velella* and *Porpita porpita*, applying the ASAP procedure.

Gene	Number of species	Threshold distance (p-distance)	ASAP-score
COI	**3**	7.33	2.5
	6	3.92	2.5
16S	**2**	8.62	1.5
	3	1.43	2.0

Finally, the PTP analysis by Maximum-Likelihood for COI suggested the presence of 16 species (Figure 23).

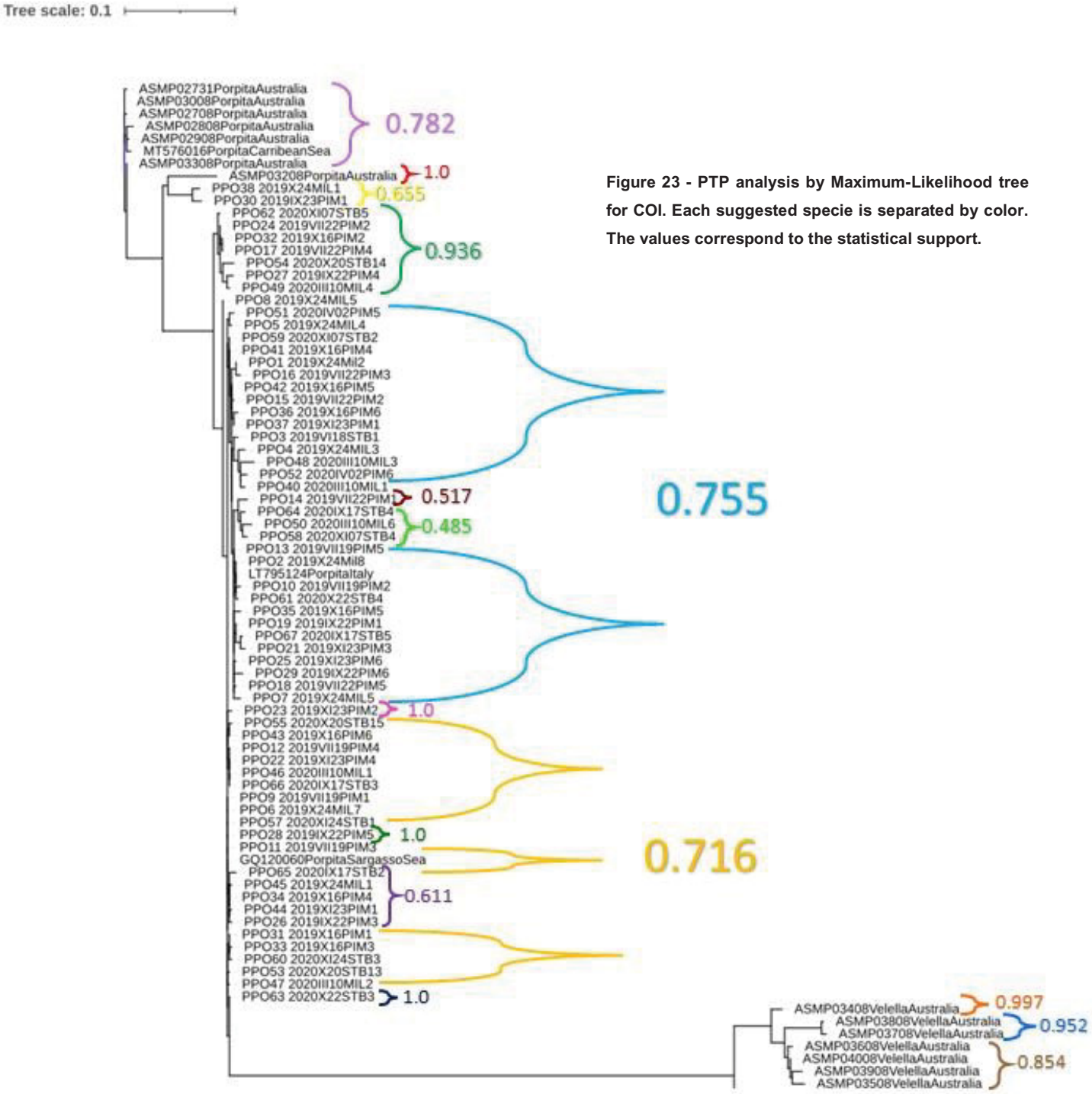
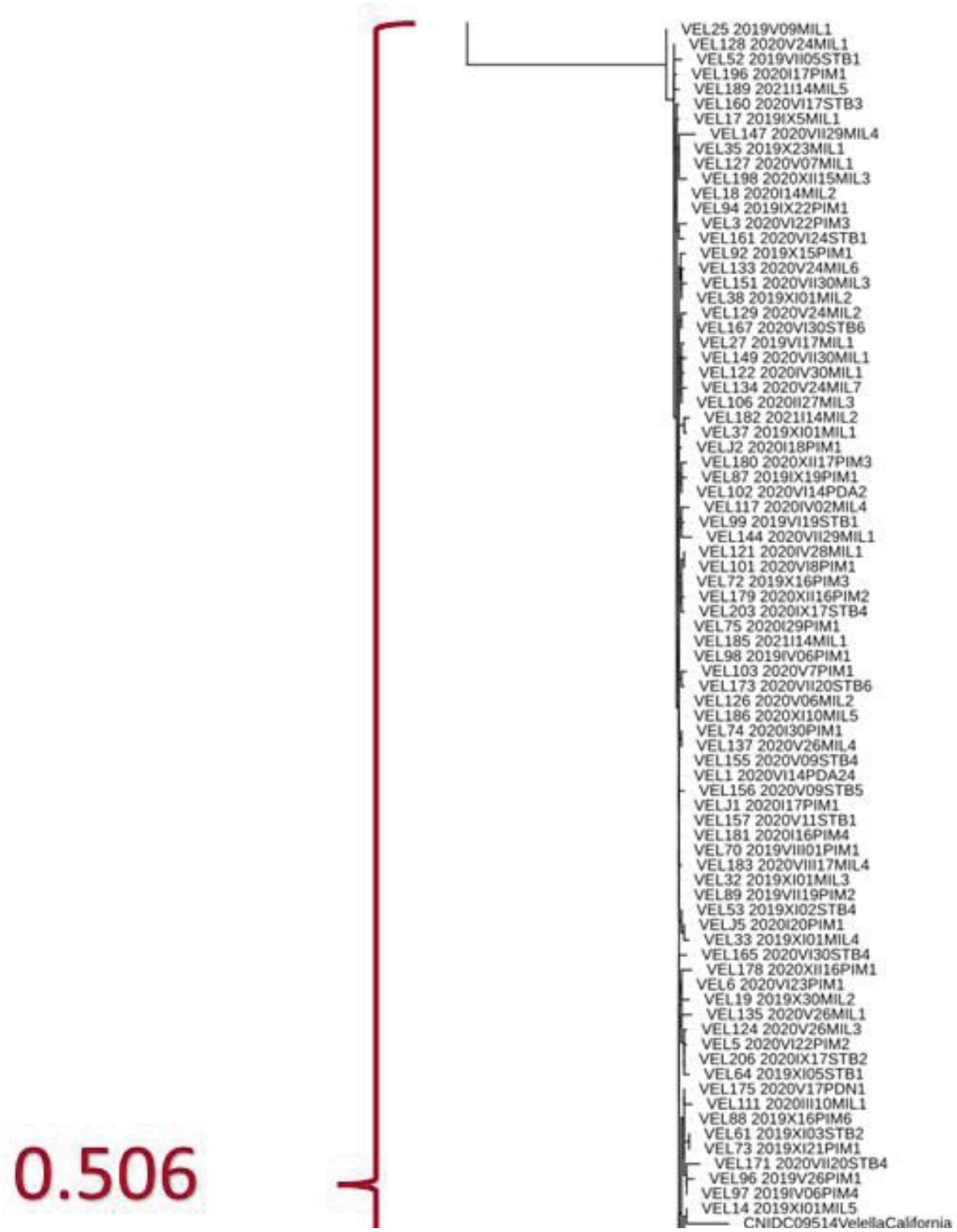


Figure 23 - PTP analysis by Maximum-Likelihood tree for COI. Each suggested specie is separated by color. The values correspond to the statistical support.


0.506
VEL25 2019V09MIL1
VEL128 2020V24MIL1
VEL52 2019VII05STB1
VEL196 2020I17PIM1
VEL189 2021I14MIL5
VEL160 2020VI17STB3
VEL17 2019IX5MIL1
VEL147 2020VII29MIL4
VEL35 2019X23MIL1
VEL127 2020V07MIL1
VEL198 2020XII15MIL3
VEL18 2020I14MIL2
VEL94 2019IX22PIM1
VEL3 2020VI22PIM3
VEL161 2020VI24STB1
VEL92 2019X15PIM1
VEL133 2020V24MIL6
VEL151 2020VII30MIL3
VEL38 2019XI01MIL2
VEL129 2020V24MIL2
VEL167 2020VI30STB6
VEL27 2019VI17MIL1
VEL149 2020VII30MIL1
VEL122 2020IV30MIL1
VEL134 2020V24MIL7
VEL106 2020II27MIL3
VEL182 2021I14MIL2
VEL37 2019XI01MIL1
VELJ2 2020I18PIM1
VEL180 2020XII17PIM3
VEL87 2019IX19PIM1
VEL102 2020VI14PDA2
VEL117 2020IV02MIL4
VEL99 2019VI19STB1
VEL144 2020VII29MIL1
VEL121 2020IV28MIL1
VEL101 2020VI8PIM1
VEL72 2019X16PIM3
VEL179 2020XII16PIM2
VEL203 2020IX17STB4
VEL75 2020I29PIM1
VEL185 2021I14MIL1
VEL98 2019IV06PIM1
VEL103 2020V7PIM1
VEL173 2020VII20STB6
VEL126 2020V06MIL2
VEL186 2020XI10MIL5
VEL74 2020I30PIM1
VEL137 2020V26MIL4
VEL155 2020V09STB4
VEL1 2020VI14PDA24
VEL156 2020V09STB5
VELJ1 2020I17PIM1
VEL157 2020V11STB1
VEL181 2020I16PIM4
VEL70 2019VIII01PIM1
VEL183 2020VIII17MIL4
VEL32 2019XI01MIL3
VEL89 2019VII19PIM2
VEL53 2019XI02STB4
VELJ5 2020I20PIM1
VEL33 2019XI01MIL4
VEL165 2020VI30STB4
VEL178 2020XII16PIM1
VEL6 2020VI23PIM1
VEL19 2019X30MIL2
VEL135 2020V26MIL1
VEL124 2020V26MIL3
VEL5 2020VI22PIM2
VEL206 2020IX17STB2
VEL64 2019XI05STB1
VEL175 2020V17PDN1
VEL111 2020III10MIL1
VEL88 2019X16PIM6
VEL61 2019XI03STB2
VEL73 2019XI21PIM1
VEL171 2020VII20STB4
VEL96 2019V26PIM1
VEL97 2019IV06PIM4
VEL14 2019XI01MIL5
CNIDC09514VelellaCalifornia


VEL108 2020II28MIL2
VEL31 2019X24MIL4
VEL125 2020V06MIL1
VEL143 2020VI22MIL3
VEL148 2020VII29MIL5
VEL140 2020VI28MIL1
VEL36 2020I14MIL5
VEL47 2019VI19STB2
VEL22 2019V08MIL1
VEL145 2020VII29MIL2
VEL204 2020X18STB1
VEL82 2019XI23PIM2
VEL78 2019IV10PIM9
VEL107 2020II28MIL1
VEL21 2019X30MIL3
VEL81 2019IX22PIM3
VEL146 2020VII29MIL3
VEL123 2020IV30MIL2
VEL139 2020V26MIL6
VEL30 2019X24MIL3
VEL9 2020VI14PDA5
VEL112 2020III10MIL2
VEL28 2019VIII22MIL3
VEL26 2019V11MIL1
VEL118 2020IV02MIL5
VEL190 2020XII15MIL4
VEL192 2020XII15MIL2
VEL7 2020V9PIM1
VEL95 2019VII19PIM1
VEL43 2019IV21STB1
VEL10 2019X24MIL5
VEL12 2019X24MIL6
VEL42 2019XI03STB1
VEL176 2020XII16CON1
VEL2 2020V5PIM1
VEL138 2020V26MIL5
VEL142 2020VI22MIL2
VEL15 2020I14MIL1
VEL169 2020VII20STB2
VEL150 2020VII30MIL2
VEL54 2019VII06STB1
VEL197 2020VIII17MIL2
VEL34 2020I14MIL4
VEL65 2020I15STB5
VEL172 2020VII20STB5
VEL131 2020V24MIL4
VEL77 2019IV10PIM10
VEL136 2020V26MIL2
VEL170 2020VII20STB3
VEL8 2020VI14PDA3
VEL67 2019X20STB1
VEL141 2020VI23MIL1
VEL119 2020IV02MIL6
VEL91 2019IX22PIM2
VEL184 2020X31MIL1
VEL166 2020VI30STB5
VEL174 2020VII21STB1
VEL13 2019VIII22MIL6
VEL24 2019V08MIL3
VEL210 2020VIII17MIL3
VEL110 2020III09MIL1
VEL130 2020V24MIL3
VEL23 2019V08MIL2
VEL44 2019IV21STB2
VEL48 2019XII16STB1
VEL11 2019VIII22MIL5
VEL56 2019XI02STB5
VEL188 2020VIII17MIL5
VEL205 2020IX17STB3
VEL16 2019X30MIL1
VEL63 2020I15STB4
VEL20 2020I14MIL3
VEL191 2020VIII17MIL1
VEL4 2020VI22PIM1
VEL207 2020IX17STB1
VEL152 2020V09STB1
VEL80 2019XI23PIM1
VEL200 2020XI07STB1
VEL59 2019VI18STB1
VEL202 2020X17STB2
VEL29 2019VIII22MIL4
VEL209 2020IX06STB1

These results exhibit that some species have low statistical support, such as the PPO14 and the group PPO64,58 and 50. However, there are suggested species with high support values, including some with maximum support. The PPO 23, PPO 28 and PPO 63 display a 100% probability of being different species.

For 16S a Maximum-likelihood partition exhibiting two different species: one with all the sequences from *Velella* and other that group all the sequences of *Porpita* together, with a support of 86%.

4. Discussion

4.1 Genetic diversity

From the sequencing results it was promptly observed that in these samples, all the selected genes were successfully amplified using the selected primers. As a result, COI and 16S presented a higher rate of sequencing, even thought 16S was more successful than COI. Despite 16S being often chosen as the most suitable gene for DNA barcoding in hydrozoans (Moura *et al.* 2008), several studies establish the successful use of more molecular markers, including COI and ITS (Peter 2020).

In a first approach regarding the genetic diversity, COI is the gene with the largest intraspecific variety, with greater values of polymorphic sites as well as the highest number of haplotypes (61 for *Porpita porpita* and 150 for *Velella velella*). The other mitochondrial gene (16S) also displays representative values of intraspecific variety. In opposition, the nuclear ITS gene has very low intraspecific divergence, with the haplotypic and nucleotidic diversity exhibiting minimum values. The main discrepancy on these results is due to the type of molecular marker. Contrary to COI and 16S that are mitochondrial markers, ITS is a non-transcribed region of the rRNA, located on the chromosome. Nuclear markers, although less conserved, diverge more slowly than mitochondrial markers (Hellberg *et al.* 2002). However, in some taxa this gene may show some significant nucleotide variations, including some intra-individual heterogeneity. The moon jellyfish, *Aurelia aurita*, displays an example of this variability in ITS (Schroth *et al.* 2002; Kim & Cho 2007).

The haplotype networks exhibited similar results with the phylogenetic tress built. In the COI analyses for *Porpita porpita,* a branch is highlighted in which many nucleotide differences were evidenced and where the samples PPO30 and PPO38 presented higher phylogeographical affinity with the specimens from Caribbean and Indo-Pacific, in relation to the other Azorean specimens. This branch displays a medium support, with an approximate

bootstrap of 750. The other Azorean sequences are grouped together along with the sequences from Mediterranean and NO Atlantic, displaying minimal genetic distance. The 16S analysis revealed some similar results, with a clear spatial segregation. Although all the sequences belong the same branch, the sequences PPO30 and PPO 38 are grouped together with the sequences from NE and W Pacific with strong bootstrap support. The ITS analysis exhibited a low genetic diversity. Even a sequence of *Porpita porpita* from the Caribbean is represented as the same haplotype as the Azorean sequences. As there is only one sequence from other place available, it is not possible to reassure that there is spatial segregation in this gene.

As the number of samples was considerably higher, *Velella velella* analysis allowed a better understanding of the phylogeographical relations. In COI, all Azorean sequences are grouped together. In this cluster is also possible to identify a sequence from NE Pacific. In other branch, with relevant genetic distance and bootstrap support it is possible to distinguish the sequences from Indo-Pacific. The 16S analysis resulted in a single clade. The sequence from Mediterranean share a haplotype with the ones from the Azores. This result may suggest that Mediterranean specimens are more genetically similar to those from the Azores. Finally, the ITS displayed no genetic variability within the analysed sequences, all from the Azores.

Thus, these analyses reinforce, for mitochondrial genes, the difference of PPO30 and PPO38 when compared with specimens from the Azores. At first, this result might suggest a correlation based on the geographical proximity, however there isn't enough data to support this statement.

4.2 Population connectivity

These studies seem to indicate that the geographical distance between species can be correlated to the genetic diversity between samples. However, this cannot be understood as an absolute certainty, as two Azorean specimens were found to be genetically closer to geographical distant populations. Also, there is evidence of haplotype sharing between the Azorean sequences and the Mediterranean and NO Atlantic.

Both species analysed have a worldwide distribution in tropical to temperate waters (Calder 1988). As this sampling was carried out in two islands of the Azores, located in the North Atlantic, it would be expected some genetic diversity, which was found on this study. This diversity may be explained by the fact that there are no geographical barriers on the Atlantic Ocean, which suggests the maintaince of a large flow of genetic information. This large flow occurs due to the high potential for dispersion and therefore random reproduction (Palumbi 1994; Patarnello *et al.* 2007).

The studied animals often produce blooms, originating massive aggregations on the coast. This suggests that these populations have fluctuations. When the population increases intensely, the tendency is for genetic diversity to be greater. In turn, when there are drastic reductions in the size of the population, diversity is also expected to be reduced (Nei *et al.* 1975; Kimura 1983). In the Azores these blooms also occur occasionally, and may corroborate some of the genetic diversity found. If there is an increase of the population size, there is more individuals who can reproduce and therefore more genetic diversity.

4.3 Cryptic species or overestimation?

In this work, two methods were applied to define the interspecific limit of the sequences obtained. The main purpose was to verify whether there were only the two previously identified species: *Vellela velella* and *Porpita porpita* or if there was some cryptic species. In hydrozoans the finding of cryptic species is often common (Govindarajan *et al.* 2005; Schuchert 2005; Moura *et al.* 2008; Miglietta *et al.* 2009; Moura *et al.* 2012b; Schuchert 2014; Maggioni *et al.* 2016; Moura *et al.* 2018; Maggioni *et al.* 2020), due to few morphological diagnostic characters, high phenological plasticity and many taxonomy errors. (Moura *et al.* 2018).

The most commonly used gene for DNA barcoding and identification of cryptic species hydrozoans is 16S (Moura *et al.* 2008; Miglietta *et al.* 2009; Moura *et al.* 2012b; Montano *et al.* 2015; Maggioni *et al.* 2016; Postaire *et al.* 2016b; Moura *et al.* 2018). In this study the delimitation methods were applied for both COI and 16S. The ASAP method displayed different results for the two analysed genes. For COI the best partition suggested three species, where the *Porpita* sequences are all grouped together and the *Velella* sequences are divided in two species: one with the sequences from Indo-Pacific and other with the remaining *Velella* sequences. The following partition suggested the presence of six species. In this hypothesis the Velella sequences from the Indo-Pacific now represents three different species and the PPO 30 and PPO 38 a single species. The other two species are composed with the remaining *Velella* and *Porpita* sequences. For 16S, as the number of sequences from databases was much smaller, the best partition suggested two species: one with all the *Porpita* sequences and other with the *Velella* sequences.

This data was not coincident with the obtained in the other method, PTP. This last method suggested the presence of sixteen different species in COI dataset. The *Porpita* sequences exhibit twelve distinct species, with some sequences representing a single species. In these cases, the probability was maximum. It is also important to mention two possible species described in this method: the species that include sequences from Indo-Pacific and Caribbean (with a

probability of 78%) and the species that contains the PPO 30 and PPO 38 (66%). The *Velella* sequences in this method, includes four distinct species. Three of them include sequences from Indo-Pacific (with probabilities around 90%) and the last one comprises all the sequences from the Azores and one from NE Pacific (50%). The 16S, with maximum likelihood analyses suggesting two different species: one with all the *Porpita* sequences and the other with all the *Velella* sequences.

In the specie delimitation analysis, the main difference is the number of species suggested for each gene. The COI analysis included nineteen databases sequences for Porpitidae. 16S displays only five sequences from databases, which led to a lower number of possible species. Thus, this suggests that if there are more sequences from other locations, more species will be suggested in the analysis.

It is also important to point out the differences in both applied methods. The ASAP is a simple method that uses single-locus data, only with pairwise genetic distances and avoiding phylogenetic constructions (Puillandre *et al.* 2021). The PTP method takes into account the evolutionary relationships, but tends to be slower when applied to a large date set and may not be sensitive to the intraspecific variation (Kapli *et al.* 2017). These differences seem to explain the results obtained for the sequences under study, since the ASAP method suggested fewer species for both genes, using only the pairwise distance. In opposition, the PTP method suggested a large number of species for the COI, which indicate that the method considered the evolutionary relationships between the sequences.

Although the results demonstrate that there is some probability of a cryptic species hidden within *Porpita porpita* there are other factors that should be highlighted. An important issue is the obtained result in the analysis of ITS sequences for this species. Although there are not many studies using this marker to reveal Hydrozoa crypticism (Postaire *et al.* 2016a) there is evidence of its successfully use on other cnidarians (Dawson & Jacobs 2001; Dawson 2003;

Pontin & Cruickshank 2012). Thus, it is not correct to ignore the results obtained for this gene, specifically the low genetic variability and low polymorphism.

5. Final conclusions and future prospects

The samples collected for this study were morphologically identified as two species: *Velella velella* and *Porpita porpita*. By the application of DNA barcoding techniques, using with a new method of sequencing, the correct identification was corroborated. A great difference was shown on the analysis by mitochondrial vs nuclear molecular markers. The mitochondrial genes exhibited greater intraspecific differences, allowing to distinguish genetic lineages between individuals of the same species.

In the Azores, the populations of *Velella velella* and *Porpita porpita* reveal genetic diversity. No pattern of spatial genetic segregation is shown between the two sampled islands. The samples from Azores seem to be more similar with specimens from nearby locations such as Mediterranean and the North-West Atlantic. The analysis further highlighted a distinctive lineage with two specimens from the Azorean population with some nucleotide differences: PPO 30 e PPO 38, possibly with more genetic affinity with an Indo-Pacific clade rather than the main Azorean lineage. Species delimitation methods were tested, in order to check if these samples could represent a different species. There is some evidence in these methods that suggest the presence of more than two species.

The ASAP analysis displayed different results for the two molecular markers, with COI suggesting a higher number of species. The PTP method also produced contrasting results for both genes, suggesting sixteen different species for COI and only two for 16S. In addition, these evidences are not enough sustained, producing unclear results, mainly for COI. The main reason for these distinct results is that there are few sequences available in the databases, and the COI is the one with the most sequences. It is also necessary to take into account the low diversity displayed in the studied sequences for the ITS gene. Consequently, these samples are more likely to be from the same species (*Porpita porpita*), being genetically similar to populations from different locations. In conclusion, these analyses indicate that there may be two to sixteen species, but that there

is a greater probability of the existence of only two species: *Porpita porpita* and *Velella velella*.

One of the factors that affected the results of this study was the reduced sampling area. Only two islands from Azores were sampled, so all samples were prevenient from the same oceanic region. It would be interesting to increase the number of sampling sites for a more complex analysis. To further test the possible cryptic diversity highlighted in this study the samples could be collected from all over the world, in order to obtain important results at the taxonomic and phylogeographic level. However, it is necessary to point out that the large number of samples studied in this research revealed a good portrait of the genetic lineages in the studied genes for the Azorean specimens of Porpitidae.

The question about the possible crypticism is also important. It would be noteworthy to apply an analysis with different molecular markers since the results obtained with the three studied molecular markers were distinct. Consequently, if the sequences available in the databases were increased, showing results from various sites of the world, it would probably be much easier to verify the presence of cryptic species.

Finally, the Porpitidae taxonomy requires more study. Although there are some researches dedicated to the taxonomy of this family, there are still many open questions. The current work on the Porpitidae taxonomy is more focused on the sub-order Capitata, without any study dedicated only to this family. This study offered some important conclusions, yet it can be considered as a previous work for further taxonomic and phylogeographic investigation of this family.

6. References

Addad S., Exposito J.-Y., Faye C., Ricard-Blum S. & Lethias C. (2011) Isolation, characterization and biological evaluation of jellyfish collagen for use in biomedical applications. *Marine drugs* **9**, 967-83.

Araya J.-F. & Aliaga J. (2018) El niño invaders: The occurrence of the by-the-wind sailor Velella velella (linnaeus, 1758) in the southeastern Pacific: (Cnidaria, hydrozoa). *Spixiana* **41**.

Aubert A., Antajan E., Lynam C., Pitois S., Pliru A., Vaz S. & Thibault D. (2018) No more reason for ignoring gelatinous zooplankton in ecosystem assessment and marine management: Concrete cost-effective methodology during routine fishery trawl surveys. *Marine Policy* **89**, 100-8.

Azinheiro S., Roumani F., Carvalho J., Prado M. & Garrido-Maestu A. (2021) Suitability of the MinION long read sequencer for semi-targeted detection of foodborne pathogens. *Analytica Chimica Acta* **1184**, 339051.

Bandelt H.J., Forster P. & Röhl A. (1999) Median-joining networks for inferring intraspecific phylogenies. *Mol Biol Evol* **16**, 37-48.

Bandyopadhyaya S., Ramakrishnan M., Kulkarni S. & Rajanna L. (2014) DNA barcoding and its applications – A critical review. *CMRIMS Journal of Research and Development* **2013**, 77-81.

Bates M., Polepole P., Kapata N., Loose M. & O'Grady J. (2016) Application of highly portable MinION nanopore sequencing technology for the monitoring of nosocomial tuberculosis infection. *International Journal of Mycobacteriology* **5**, S24.

Beckmann A. & Özbek S. (2012) The Nematocyst: a molecular map of the Cnidarian stinging organelle. *The International journal of developmental biology* **56**, 577-82.

Bedot M. (1904) *Siphonophores provenant des campagnes du yacht Princesse-Alice (1892-1902). Résultats des Campagnes Scientifiques accomplies sur son yacht par Albert Ier Prince Souverain de Monaco*.

Benítez-Páez A., Portune K.J. & Sanz Y. (2016) Species-level resolution of 16S rRNA gene amplicons sequenced through the MinION™ portable nanopore sequencer. *Gigascience* **5**, 4.

Betti F., Bo M., Enrichetti F., Manuele M., Cattaneo-Vietti R. & Bavestrello G. (2019) Massive strandings of Velella velella (Hydrozoa: Anthoathecata: Porpitidae) in the Ligurian Sea (North-western Mediterranean Sea). *The European Zoological Journal* **86**, 343-53.

Bickford D., Lohman D.J., Sodhi N.S., Ng P.K.L., Meier R., Winker K., Ingram K.K. & Das I. (2007) Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution* **22**, 148-55.

Bieri R. (1959) Dimorphism and Size Distribution in Velella and Physalia. *Nature* **184**, 1333-4.

Boero (1980) Life cycles of hydroids and hydromedusae: some cases of difficult interpretation. (ed. by AGRIS). Memorie di Biologia Marina e di Oceanografia.

Bosc L.A.G. (1801) Histoire naturelle des vers : contenant leur description et leurs moeurs, avec figures dessinées d'après nature. *Guilleminet, Paris*.

Bosch-Belmar M., Milisenda G., Basso L., Doyle T.K., Leone A. & Piraino S. (2021) Jellyfish Impacts on Marine Aquaculture and Fisheries. *Reviews in Fisheries Science & Aquaculture* **29**, 242-59.

Bosch-Belmar M., Milisenda G., Girons A., Taurisano V., Accoroni S., Totti C., Piraino S. & Fuentes V. (2017) Consequences of Stinging Plankton Blooms on Finfish Mariculture in the Mediterranean Sea. *Frontiers in Marine Science* **4**.

Böttger-Schnack R. & Machida R.J. (2011) Comparison of morphological and molecular traits for species identification and taxonomic grouping of oncaeid copepods. *Hydrobiologia* **666**, 111-25.

Bouillon (1999) Hydromedusae. In: *South Atlantic Zooplankton* (Backhuys Publishers.

Bouillon, Gravili C., F P., Gili J.-M. & Boero F. (2006) An Introduction to Hydrozoa. *Mémoires du Muséum national d'Histoire naturelle* **194**, 1-591.

Bouillon J. & Boero F. (2000) Synopsis of the families and genera of the Hydromedusae of the world, with a list of the worldwide species. *Thalassia Salentina* **24**.

Bouillon J.G., C; Pagès,F; Gili,J; Boero, F; (2006) An introduction to hydrozoa. In: *Mémoires du Muséum national d'Histoire naturelle* (

Boyra A E.F., Tuya F, Freitas M, Haroun RJ, Biscoito MJ, González JA (2008) *Guia de campo 365 espécies atlânticas*.

Brancaccio R.N., Robitaille A., Dutta S., Rollison D.E., Tommasino M. & Gheit T. (2021) MinION nanopore sequencing and assembly of a complete human papillomavirus genome. *Journal of Virological Methods* **294**, 114180.

Bridge D., Cunningham C., Schierwater B., Desalle R. & Buss L. (1992) Class-Level Relationships in the Phylum Cnidaria: Evidence from Mitochondrial Genome Structure. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 8750-3.

Brinckmann-Voss A. (1970) *Anthomedusae-Athecatae (Hydrozoa, Cnidaria) of the Mediterranean*. Stazione zoologica, Napoli.

Brodeur R., Link J., Smith B., Ford M., Kobayashi D. & Jones T. (2016) Ecological and Economic Consequences of Ignoring Jellyfish: A Plea for Increased Monitoring of Ecosystems. *Fisheries* **41**, 630-7.

Calder D. (1988) *Shallow-water hydroids of Bermuda: The Athecatae*.

Carrera M., Trujillo J.E. & Brandt M. (2019) First record of a by-the-wind-sailor (Velella velella Linnaeus, 1758) in the Galápagos Archipelago - Ecuador. *Biodiversity data journal* **7**, e35303-e.

Cartwright P. & Collins A. (2007) Fossils and phylogenies: Integrating multiple lines of evidence to investigate the origin of early major metazoan lineages. *Integrative and Comparative Biology* **47**, 744-51.

Cartwright P., Evans N., Dunn C., Marques A., Miglietta M., Schuchert P. & Collins A. (2008) Phylogenetics of Hydroidolina (Hydrozoa: Cnidaria). *Journal of the Marine Biological Association of the UK* **88**, 1663-72.

Cartwright P. & Nawrocki A. (2010) Character Evolution in Hydrozoa (phylum Cnidaria). *Integrative and Comparative Biology* **50**, 456-72.

Chamisso A.G., Eysenhardt Carolus (1821) De animalibus quibusdam e classe vermium Linneana, in circumnavigatione Terrae, auspicante Comite N. Romanoff, duce Ottone di Kotzebue, annis 1815-1818 peracta, observatis Fasciculus secundus, reliquos vermes continens. *Nova Acta physico-medica Academiae Cesareae Leopoldino-Carolinae* **10**, 343-73.

Collins A. (2002) Phylogeny of Medusozoa and the evolution of life cycles. *Journal of Evolutionary Biology* **15**, 418-32.

Collins A., Bentlage B., Lindner A., Lindsay D., Haddock S., Jarms G., Norenburg J., Jankowski T. & Cartwright P. (2008) Phylogenetics of Trachylina (Cnidaria: Hydrozoa) with new insights on the evolution of some problematical taxa. *Journal of the Marine Biological Association of the UK* **88**, 1673-85.

Collins A., Schuchert P., Marques A., Jankowski T., Medina M. & Schierwater B. (2006) Medusozoan Phylogeny and Character Evolution Clarified by New Large and Small Subunit rDNA Data and an Assessment of the Utility of Phylogenetic Mixture Models. *Systematic Biology* **55**, 97-115.

Collins A.G., Winkelmann S., Hadrys H. & Schierwater B. (2005) Phylogeny of Capitata and Corynidae (Cnidaria, Hydrozoa) in light of mitochondrial 16S rDNA data. *Zoologica Scripta* **34**, 91-9.

Condon R., Graham W., Duarte C., Pitt K., Lucas C., Haddock S., Sutherland K., Robinson K., Dawson M., Decker M., Mills C., Purcell J., Malej A., Mianzan H., Uye S.-i., Gelcich S. & Madin L. (2012) Questioning the rise of gelatinous zooplankton in the World's oceans. *BioScience* **62**.

Condon R., Steinberg D., Giorgio P., Bouvier T., Bronk D., Graham W. & Ducklow H. (2011) Jellyfish blooms result in a major microbial respiratory sink of carbon in marine systems. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 10225-30.

Conway D. (2012) Marine zooplankton of southern Britain. Part 1: Radiolaria, Heliozoa, Foraminifera, Ciliophora, Cnidaria, Ctenophora, Platyhelminthes, Nemertea, Rotifera and Mollusca. In: *Occasional Publications.* (ed. by Kingdom MBAotU), p. 138.

Corgnati L., Marini S., Mazzei L., Ottaviani E., Aliani S., Conversi A. & Griffa A. (2016) Looking inside the Ocean: Toward an Autonomous Imaging System for Monitoring Gelatinous Zooplankton. *Sensors (Basel, Switzerland)* **16**, 2124.

Cunha A.F., Genzano G.N. & Marques A.C. (2015) Reassessment of Morphological Diagnostic Characters and Species Boundaries Requires Taxonomical Changes for the Genus Orthopyxis L. Agassiz, 1862 (Campanulariidae, Hydrozoa) and Some Related Campanulariids. *PLOS ONE* **10**, e0117553.

Cunningham C.W. & Buss L.W. (1993) Molecular evidence for multiple episodes of paedomorphosis in the family Hydractiniidae. *Biochemical Systematics and Ecology* **21**, 57-69.

Daly M., Brugler M., Cartwright P., Collins A., Dawson M., Fautin D., France S., McFadden C., Opresko D., Rodriguez E., Romano S. & Stake J. (2006) The Phylum Cnidaria: A Review Of Phylogenetic Patterns And Diversity 300 Years After Linnaeus *. *Zootaxa* **1668**.

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

Dawson M.N. (2003) Macro-morphological variation among cryptic species of the moon jellyfish, Aurelia (Cnidaria: Scyphozoa). *Marine Biology* **143**, 369-79.

Dawson M.N. & Jacobs D.K. (2001) Molecular Evidence for Cryptic Species of Aurelia aurita (Cnidaria, Scyphozoa). *The Biological bulletin* **200**, 92-6.

Dayrat B. (2005) Towards integrative taxonomy. *Biological Journal of the Linnean Society* **85**, 407-15.

de Vere N., Rich T.C.G., Trinder S.A. & Long C. (2015) DNA Barcoding for Plants. In: *Plant Genotyping: Methods and Protocols* (ed. by Batley J), pp. 101-18. Springer New York, New York, NY.

Deutsche S.-E. & Drygalski E.v. (1912) *Deutsche Südpolar-Expedition, 1901-1903, im Auftrage des Reichsamtes des Innern*. G. Reimer, Berlin.

Doyle T., Houghton J., Buckley S., Hays G. & Davenport J. (2007) The broad-scale distribution of five jellyfish species across a temperate coastal environment. *Hydrobiologia* **579**, 29-39.

Dunn C., Pugh P. & Haddock S. (2006) Molecular Phylogenetics of the Siphonophora (Cnidaria), with Implications for the Evolution of Functional Specialization. *Systematic Biology* **54**, 916-35.

Eschscholtz F. (1825) Bericht über die zoologische Ausbeute während der Reise von Kronstadt bis St. *Peter und Paul. - Isis* **1825**, 733-47.

Felsenstein J. (1984) DISTANCE METHODS FOR INFERRING PHYLOGENIES: A JUSTIFICATION. *Evolution* **38**, 16-24.

Ferreira V. (2011) *Guia de campo da fauna e flora marinha de Portugal*. Gráfica Maiadouro, Leça da Palmeira - Portugal,.

Fleming N., Harrod C. & Houghton J. (2013) Identifying potentially harmful jellyfish blooms using shoreline surveys. *Aquaculture Environment Interactions* **4**, 263-72.

Flux J. (2008) First mass stranding of Velella velella in New Zealand. *Marine Biodiversity Records* **1**.

Folmer O., Black M., Hoeh W., Lutz R. & Vrijenhoek R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* **3**, 294-9.

France S.C. & Hoover L.L. (2002) DNA sequences of the mitochondrial COI gene have low levels of divergence among deep-sea octocorals (Cnidaria: Anthozoa). *Hydrobiologia* **471**, 149-55.

Furfaro G., Di Giulio A., Mantoni C. & Mariottini P. (2017) On the occurrence of Porpita porpita in the Tyrrhenian Sea: COI and ITS2 DNA barcoding identification (Cnidaria, Hydrozoa). *Spixiana* **40**, 138.

Gershwin L.-a., Zeidler W. & Davie P. (2010) Medusae (Cnidaria) of Moreton Bay, Queensland, Australia. *Memoirs of the Queensland Museum* **54**, 47-108.

Govindarajan A.F., Halanych K.M. & Cunningham C.W. (2005) Mitochondrial evolution and phylogeography in the hydrozoan Obelia geniculata (Cnidaria). *Marine Biology* **146**, 213-22.

Graham W.M., Pagès F. & Hamner W.M. (2001) A physical context for gelatinous zooplankton aggregations: a review. In: *Jellyfish Blooms: Ecological and Societal Importance* (eds. by Purcell JE, Graham WM & Dumont HJ), pp. 199-212. Springer Netherlands, Dordrecht.

Groen K., van Nieuwkoop S., Bestebroer T.M., Fraaij P.L., Fouchier R.A.M. & van den Hoogen B.G. (2021) Whole genome sequencing of human metapneumoviruses from clinical specimens using MinION nanopore technology. *Virus Research* **302**, 198490.

Guilding L. (1828) *Observations on the zoology of the Carribaean Sea*. Zoological Journal 3.

Guindon S., Dufayard J.-F., Lefort V., Anisimova M., Hordijk W. & Gascuel O. (2010) New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Systematic Biology* **59**, 307-21.

Gul S. & Gravili C. (2014) On the occurrence of Porpita porpita (Cnidaria: Hydrozoa) at Pakistan coast (North Arabian Sea). *Marine Biodiversity Records* **7**.

Haeckel E. (1888) Report on the Siphonophorae collected by HMS Challenger during the years 1873–1876. *Report of the Scientific Results of the voyage of H.M.S. Challenger* **28**, 1-380.

Hay S. (2006) Marine Ecology: Gelatinous Bells May Ring Change in Marine Ecosystems. *Current Biology* **16**, R679-R82.

Heath T., Hedtke S. & Hillis D. (2008) Taxon sampling and accuracy of phylogenetic analyses. *Journal of Systematics and Evolution* **46**.

Hebert P.D.N., Cywinska A., Ball S.L. & deWaard J.R. (2003a) Biological identifications through DNA barcodes. *Proceedings. Biological sciences* **270**, 313-21.

Hebert P.D.N., Ratnasingham S. & de Waard J.R. (2003b) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **270**, S96-S9.

Hellberg M., Burton R., Neigel J. & Palumbi S. (2002) Genetic assessment of marine population connectivity. *Bulletin of Marine Science* **70**, 273-90.

Ho J.K.I., Puniamoorthy J., Srivathsan A. & Meier R. (2020) MinION sequencing of seafood in Singapore reveals creatively labelled flatfishes, confused roe, pig DNA in squid balls, and phantom crustaceans. *Food Control* **112**, 107144.

Hosia A., Falkenhaug T., Baxter E.J. & Pagès F. (2017) Abundance, distribution and diversity of gelatinous predators along the northern Mid-Atlantic Ridge: A comparison of different sampling methodologies. *PLOS ONE* **12**, e0187491-e.

Houghton J., Doyle T., Davenport J., Lilley M., Wilson R. & Hays G. (2007) Stranding events provide indirect insights into the seasonality and persistence of Jellyfish Medusae (Cnidaria: Scyphozoa). *Hydrobiologia* **589**, 1-13.

Huang D., Meier R., Todd P.A. & Chou L.M. (2008) Slow Mitochondrial COI Sequence Evolution at the Base of the Metazoan Tree and Its Implications for DNA Barcoding. *Journal of Molecular Evolution* **66**, 167-74.

Ionescu M., Wilson S. & Evans E.J. (2016) Jellyfish stranding observations around the Isle of Anglesey in the summer of 2014. *Geo-Eco-Marina* **2016**, 109-18.

Jain M., Olsen H.E., Paten B. & Akeson M. (2016) The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. *Genome Biology* **17**, 239.

Kapli P., Lutteropp S., Zhang J., Kobert K., Pavlidis P., Stamatakis A. & Flouri T. (2017) Multi-rate Poisson tree processes for single-locus species delimitation under maximum likelihood and Markov chain Monte Carlo. *Bioinformatics* **33**, 1630-8.

Karst S.M., Dueholm M.S., McIlroy S.J., Kirkegaard R.H., Nielsen P.H. & Albertsen M. (2018) Retrieval of a million high-quality, full-length microbial 16S and 18S rRNA gene sequences without primer bias. *Nature Biotechnology* **36**, 190-5.

Katoh K., Misawa K., Kuma K. & Miyata T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* **30**, 3059-66.

Kayal E., Roure B., Philippe H., Collins A. & Lavrov D. (2013) Cnidarian phylogenetic relationships as revealed by mitogenomics. *BMC evolutionary biology* **13**, 5.

Khalturin K., Shinzato C., Khalturina M., Hamada M., Fujie M., Koyanagi R., Kanda M., Goto H., Anton-Erxleben F., Toyokawa M., Toshino S. & Satoh N. (2019) Medusozoan genomes inform the evolution of the jellyfish body plan. *Nature Ecology & Evolution* **3**, 811-22.

Kim S.-Y. & Cho E.-S. (2007) Molecular phylogeny of moon jellyfish Aurelia aurita Linnaeus collected from Yeosu waters in Korea based on nuclear and mitochondrial DNA sequences. *Journal of Life Science* **17**.

Kimura M. (1983) *The Neutral Theory of Molecular Evolution*. Cambridge University Press.

Kirkendale L. & Calder D. (2003) Hydroids (Cnidaria: Hydrozoa) from Guam and the Commonwealth of the Northern Marianas Islands (CNMI). *Micronesica* **3536**, 159-88.

Kirkpatrick & Pugh (1984) *Siphonophores and Velellids: Keys and notes for the identification of the species*. Brill, Leiden, The Netherlands.

Kumar S., Stecher G., Li M., Knyaz C. & Tamura K. (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol Biol Evol* **35**, 1547-9.

Lamarck J.-B.P.A.d.M.d. (1801a) *Systême des animaux sans vertèbres; ou, Tableau général des classes, des classes, des orres et des genres de ces animaux*. L'Auteur, Paris.

Lamarck J.B. (1801b) Système des animaux sans vertèbres, ou tableau général des classes, des ordres et des genres de ces animaux; Présentant leurs caractères essentiels et leur distribution, d'apres la considération de leurs rapports naturels et de leur organisation, et suivant l'arrangement établi dans les galeries du Muséum d'Histoire Naturelle, parmi leurs dépouilles conservées; Précédé du discours d'ouverture du Cours de Zoologie, donné dans le Muséum National d'Histoire Naturelle l'an 8 de la République. *Paris*.

Larson R.J. (1980) The Medusa of Velella velella (Linnaeus, 1758) (Hydrozoa, Chondrophorae). *Journal of Plankton Research* **2**, 183-6.

Leclère L., Schuchert P., Cruaud C., Couloux A. & Manuel M. (2009) Molecular Phylogenetics of Thecata (Hydrozoa, Cnidaria) Reveals Long-Term Maintenance of Life History Traits despite High Frequency of Recent Character Changes. *Systematic Biology* **58**, 509-26.

Leclère L., Schuchert P. & Manuel M. (2007) Phylogeny of the Plumularioidea (Hydrozoa, Leptothecata): evolution of Colonial Organisation and Life Cycle. *Zoologica Scripta* **36**, 371-94.

Leigh J.W. & Bryant D. (2015) popart: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* **6**, 1110-6.

Leray M., Yang J.Y., Meyer C.P., Mills S.C., Agudelo N., Ranwez V., Boehm J.T. & Machida R.J. (2013) A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Frontiers in Zoology* **10**, 34.

Licandro P., Fischer A. & Lindsay D.J. (2017) Cnidaria: Scyphozoa and Non-Colonial Hydrozoa. In: *Marine Plankton* (Oxford University Press, Oxford.

Lillo A., Tiralongo F. & Tondo E. (2019) New Records of Porpita porpita (Linnaeus, 1758) (Cnidaria: Hydrozoa) in the Mediterranean Sea. *Natural and Engineering Sciences* **4**, 293-8.

Linné C.v. & Salvius L. (1758) *Caroli Linnaei...Systema naturae per regna tria naturae :secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis*. Impensis Direct. Laurentii Salvii, Holmiae :.

Mackie G.O. (1962) Factors affecting the distribution of Velella (Chondrophora). *Internationale Revue der gesamten Hydrobiologie und Hydrographie* **47**, 26-32.

Madin L.P. & Harbison G.R. (2001) Gelatinous Zooplankton. In: *Encyclopedia of Ocean Sciences* (ed. by Steele JH), pp. 1120-30. Academic Press, Oxford.

Madkour F., Zaghloul W. & Mohammad S. (2019) First record of Porpita porpita (Linnaeus, 1758) (Cnidaria: Hydrozoa, Porpitidae) from the Red Sea of Egypt. *Journal of Aquatic Science and Marine Biology* **2**, 24-7.

Maggioni D., Montano S., Seveso D. & Galli P. (2016) Molecular evidence for cryptic species in Pteroclava krempfi (Hydrozoa, Cladocorynidae) living in association with alcyonaceans. *Systematics and Biodiversity* **14**, 484-93.

Maggioni D., Schiavo A., Ostrovsky A.N., Seveso D., Galli P., Arrigoni R., Berumen M.L., Benzoni F. & Montano S. (2020) Cryptic species and host specificity in the bryozoan-associated hydrozoan Zanclea divergens (Hydrozoa, Zancleidae). *Molecular Phylogenetics and Evolution* **151**, 106893.

Mann B.C., Bezuidenhout J.J., Swanevelder Z.H. & Grobler A.F. (2021) MinION 16S datasets of a commercially available microbial community enables the evaluation of DNA extractions and data analyses. *Data in Brief* **36**, 107036.

Maxam A.M. & Gilbert W. (1977) A new method for sequencing DNA. *Proc Natl Acad Sci U S A* **74**, 560-4.

Mayr E. (1985) *The Growth of Biological Thought*. Harvard University Press

Miglietta M.P., Faucci A. & Santini F. (2011) Speciation in the Sea: Overview of the Symposium and Discussion of Future Directions. *Integrative and Comparative Biology* **51**, 449-55.

Miglietta M.P., Schuchert P. & Cunningham C.W. (2009) Reconciling genealogical and morphological species in a worldwide study of the Family Hydractiniidae (Cnidaria, Hydrozoa). *Zoologica Scripta* **38**, 403-30.

Montano S., Maggioni D., Arrigoni R., Seveso D., Puce S. & Galli P. (2015) The Hidden Diversity of Zanclea Associated with Scleractinians Revealed by Molecular Data. *PLOS ONE* **10**.

Mora C., Tittensor D.P., Adl S., Simpson A.G.B. & Worm B. (2011) How Many Species Are There on Earth and in the Ocean? *PLOS Biology* **9**, e1001127.

Moura C., Cunha M., Porteiro F. & Rogers A. (2012a) A molecular phylogenetic appraisal of the systematics of the Aglaopheniidae (Cnidaria: Hydrozoa, Leptothecata) from the north-east Atlantic and west Mediterranean. *Zoological Journal of the Linnean Society* **164**, 717-27.

Moura C.J., Cunha M., Porteiro F. & Rogers A.J.Z.S. (2011) The use of the DNA barcode gene 16S mRNA for the clarification of taxonomic problems within the family Sertulariidae (Cnidaria, Hydrozoa). *Zoologica Scripta* **40**.

Moura C.J., Cunha M.R., Porteiro F.M. & Rogers A.D. (2012b) Polyphyly and cryptic diversity in the hydrozoan families Lafoeidae and Hebellidae (Cnidaria : Hydrozoa). *Invertebrate Systematics* **25**, 454-70.

Moura C.J., Harris D.J., Cunha M.R. & Rogers A.D. (2008) DNA barcoding reveals cryptic diversity in marine hydroids (Cnidaria, Hydrozoa) from coastal and deep-sea environments. *Zoologica Scripta* **37**, 93-108.

Moura C.J., Lessios H., Cortés J., Nizinski M.S., Reed J., Santos R.S. & Collins A.G. (2018) Hundreds of genetic barcodes of the species-rich hydroid superfamily Plumularioidea (Cnidaria, Medusozoa) provide a guide toward more reliable taxonomy. *Scientific Reports* **8**, 17986.

Msn C., Sharifuzzaman S., Chowdhury S., Nabi M.R.-U. & Hossain M.S. (2016) First Record of Porpita porpita (Cnidaria: Hydrozoa) from the coral reef ecosystem, Bangladesh. *Ocean Science Journal* **51**.

Müller O.F. (1776) Beschreibung zwoer Medusen. *Beschaftigungen der Berlinischen Gesellschaft Naturforschender Freunde*.

Munro C., Vue Z., Behringer R.R. & Dunn C.W. (2019) Morphology and development of the Portuguese man of war, Physalia physalis. *Scientific Reports* **9**, 15522.

Nawrocki A., Schuchert P., Cartwright P., Muséum & Naturelle H. (2010) Phylogenetics and evolution of Capitata (Cnidaria: Hydrozoa), and the systematics of Corynidae. *Zoologica Scripta - ZOOL SCR* **39**.

Nawrocki Annalise M. & Cartwright P. (2012) A Novel Mode of Colony Formation in a Hydrozoan through Fusion of Sexually Generated Individuals. *Current Biology* **22**, 825-9.

Nei M., Maruyama T. & Chakraborty R. (1975) The Bottleneck Effect and Genetic Variability in Populations. *Evolution* **29**, 1-10.

Ngo T.T.D., Lea R.A., Maksemous N., Eccles D.A., Smith R.A., Dunn P.J., Thao V.C., Ha T.M.T., Bùi C.B., Haupt L.M., Scott R. & Griffiths L.R. (2021) The MinION as a cost-effective technology for diagnostic screening of the SCN1A gene in epilepsy patients. *Epilepsy Research* **172**, 106593.

Omori M. & Nakano E. (2001) Jellyfish fisheries in southeast Asia. *Hydrobiologia* **451**, 19-26.

Ortman B., Bucklin A., Pagès F. & Youngbluth M. (2010) DNA barcoding the Medusozoa using mtCOI. *Deep Sea Research Part II: Topical Studies in Oceanography* **57**, 2148-56.

Osadchenko B.V. & Kraus Y.A. (2018) Trachylina: The Group That Remains Enigmatic Despite 150 Years of Investigations. *Russian Journal of Developmental Biology* **49**, 134-45.

Padial J.M. & De La Riva I. (2010) A response to recent proposals for integrative taxonomy. *Biological Journal of the Linnean Society* **101**, 747-56.

Palumbi S.R. (1994) GENETIC DIVERGENCE, REPRODUCTIVE ISOLATION, AND MARINE SPECIATION. *Annual Review of Ecology and Systematics* **25**, 547-72.

Patarnello T., Volckaert F.A. & Castilho R. (2007) Pillars of Hercules: is the Atlantic-Mediterranean transition a phylogeographical break? *Mol Ecol* **16**, 4426-44.

Peña Cantero Á., Sentandreu V. & Latorre A. (2010) Phylogenetic relationships of the endemic Antarctic benthic hydroids (Cnidaria, Hydrozoa): What does the mitochondrial 16S rRNA tell us about it? *Polar Biology* **33**, 41-57.

Peter S. (2020) DNA barcoding of some Pandeidae species (Cnidaria, Hydrozoa, Anthoathecata). *Revue suisse de Zoologie* **125**, 101-27.

Petersen K. (2008) Evolution and taxonomy in Capitate Hydroids and Medusae. *Zoological Journal of the Linnean Society* **100**, 101-231.

Peterson K.J. & Butterfield N.J. (2005) Origin of the Eumetazoa: Testing ecological predictions of molecular clocks against the Proterozoic fossil record. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 9547.

Piraino S.D., A; (2014) Guía de identificación y tratamento de picaduras de medusas y otros organismos gelatinosos. p. 42. MED-JELLYRISK, Project funded by the European Union within the framework of the ENPI CBC Cross-Border Cooperation in the Mediterranean and Program IEVA CT Cuente Mediterrânea.

Pontin D.R. & Cruickshank R.H. (2012) Molecular phylogenetics of the genus Physalia (Cnidaria: Siphonophora) in New Zealand coastal waters reveals cryptic diversity. *Hydrobiologia* **686**, 91-105.

Postaire B., Magalon H., Bourmaud C., Nicole G.-B. & Bruggemann H. (2016a) Phylogenetic relationships within Aglaopheniidae (Cnidaria, Hydrozoa) reveal unexpected generic diversity. *Zoologica Scripta* **15**, 103-14.

Postaire B., Magalon H., Bourmaud C.A.F. & Bruggemann J.H. (2016b) Molecular species delimitation methods and population genetics data reveal extensive lineage diversity and cryptic species in Aglaopheniidae (Hydrozoa). *Molecular Phylogenetics and Evolution* **105**, 36-49.

Pray L. (2008) Discovery of DNA Structure and Function: Watson and Crick. In: *Nature Education*.

Prieto L., Enrique-Navarro A., Li Volsi R. & Ortega M.J. (2018) The Large Jellyfish Rhizostoma luteum as Sustainable a Resource for Antioxidant Properties, Nutraceutical Value and Biomedical Applications. *Marine drugs* **16**.

Puillandre N., Brouillet S. & Achaz G. (2021) ASAP: assemble species by automatic partitioning. *Molecular Ecology Resources* **21**, 609-20.

Purcell J., Clarkin E. & Doyle T. (2012) Foods of Velella velella (Cnidaria: Hydrozoa) in algal rafts and its distribution in Irish seas. *Hydrobiologia* **690**.

Purty R.S. & Chatterjee S. (2016) DNA Barcoding: An Effective Technique in Molecular Taxonomy. *Austin Journal of Biotechnology & Bioengineering* **3**, 1059.

Raskoff K., Sommer F., Hamner W. & Cross K. (2003) Collection and Culture Techniques for Gelatinous Zooplankton. *The Biological bulletin* **204**, 68-80.

Risso A. (1826) Histoire naturelle des principales productions de l'Europe Méridionale et particulièrement de celles des environs de Nice et des Alpes Maritimes. *Paris, F.G. Levrault*.

Ronowicz M., Boissin E., Postaire B., Bourmaud C.A.-F., Gravier-Bonnet N. & Schuchert P. (2017) Modern alongside traditional taxonomy—Integrative systematics of the genera Gymnangium Hincks, 1874 and Taxella Allman, 1874 (Hydrozoa, Aglaopheniidae). *PLOS ONE* **12**, e0174244.

Rozas J., Ferrer-Mata A., Sánchez-DelBarrio J.C., Guirao-Rico S., Librado P., Ramos-Onsins S.E. & Sánchez-Gracia A. (2017) DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. *Mol Biol Evol* **34**, 3299-302.

Ruppert E.E., Fox R.S. & Barnes R.D. (2004) *Invertebrate zoology : a functional evolutionary approach*. Thomson-Brooks/Cole, Belmont, CA.

Sanger F. & Coulson A.R. (1975) A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J Mol Biol* **94**, 441-8.

Schoch C.L., Seifert K.A., Huhndorf S., Robert V., Spouge J.L., Levesque C.A., Chen W. & Fungal Barcoding C. (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences* **109**, 6241.

Schroth W., Jarms G., Streit B. & Schierwater B. (2002) Speciation and phylogeography in the cosmopolitan marine moon jelly, Aurelia sp. *BMC evolutionary biology* **2**, 1-.

Schuchert P. (2005) Species boundaries in the hydrozoan genus Coryne. *Molecular Phylogenetics and Evolution* **36**, 194-9.

Schuchert P. (2010) The European athecate hydroids and their medusae (Hydrozoa, Cnidaria): Capitata Part 2. *Revue suisse de zoologie; annales*

de la Société zoologique suisse et du Muséum d'histoire naturelle de Genève **117**, 337-555.

Schuchert P. (2014) High genetic diversity in the hydroid Plumularia setacea: a multitude of cryptic species or extensive population subdivision? *Molecular Phylogenetic Evolution* **76**, 1-9.

Schuchert P. (2018) DNA barcoding of some Pandeidae species (Cnidaria, Hydrozoa, Anthoathecata). *Revue suisse de zoologie; annales de la Société zoologique suisse et du Muséum d'histoire naturelle de Genève* **125**.

Schuchert P. (2021) World Hydrozoa Database.

Schuchert P., Hosia A. & Leclère L. (2017) Identification of the polyp stage of three leptomedusa species using DNA barcoding. *Revue suisse de zoologie; annales de la Société zoologique suisse et du Muséum d'histoire naturelle de Genève* **124**, 167-82.

Schwentner M. & Bosch T.C. (2015) Revisiting the age, evolutionary history and species level diversity of the genus Hydra (Cnidaria: Hydrozoa). *Molecular Phylogenetic Evolution* **91**, 41-55.

Schwentner M., Timms B.V. & Richter S. (2011) An integrative approach to species delineation incorporating different species concepts: a case study of Limnadopsis (Branchiopoda: Spinicaudata). *Biological Journal of the Linnean Society* **104**, 575-99.

Shikina S. & Chang C.-F. (2018) Cnidaria. In: *Encyclopedia of Reproduction (Second Edition)* (ed. by Skinner MK), pp. 491-7. Academic Press, Oxford.

Sibley A. (2007) Blooms of 'by-the-wind-sailors' (Velella velella) in summer 2004 and possible implications for rainfall and climate. *Weather* **62**, 134-6.

Sivalingam G. (2019) Notes on the occurrence of Porpita porpita (Blue button) from Pulicat Lagoon. *Journal of Research in Biology*.

Tan S., Dvorak C.M.T., Estrada A.A., Gebhart C., Marthaler D.G. & Murtaugh M.P. (2020) MinION sequencing of Streptococcus suis allows for functional characterization of bacteria by multilocus sequence typing and antimicrobial resistance profiling. *Journal of Microbiological Methods* **169**, 105817.

Tautz D., Arctander P., Minelli A., Thomas R.H. & Vogler A.P. (2002) DNA points the way ahead in taxonomy. *Nature* **418**, 479-.

Tautz D., Arctander P., Minelli A., Thomas R.H. & Vogler A.P. (2003) A plea for DNA taxonomy. *Trends in Ecology & Evolution* **18**, 70-4.

Technau U. & Steele R.E. (2011) Evolutionary crossroads in developmental biology: Cnidaria. *Development* **138**, 1447-58.

Theodoridis S., Nogués-Bravo D. & Conti E. (2019) The role of cryptic diversity and its environmental correlates in global conservation status assessments: Insights from the threatened bird's-eye primrose (Primula farinosa L.). *Diversity and Distributions* **25**.

Totton A. (1954) *Siphonophora of the Indian ocean*. University Press.

Tsugama D. & Fujino K. (2020) Data of whole genome sequencing of five garden asparagus (Asparagus officinalis) individuals with the MinION nanopore sequencer. *Data in Brief* **28**, 104838.

Tyler A.D., Mataseje L., Urfano C.J., Schmidt L., Antonation K.S., Mulvey M.R. & Corbett C.R. (2018) Evaluation of Oxford Nanopore's MinION Sequencing Device for Microbial Whole Genome Sequencing Applications. *Scientific Reports* **8**, 10931.

UCMP (2021) Hydrozoa: More on Morphology. URL https://ucmp.berkeley.edu/cnidaria/hydrozoamm.html.

Venter J.C.e.a. (2001) The sequence of the human genome. *Science* **291**, 1304-51.

Wang J., Ke Y.H., Zhang Y., Huang K.Q., Wang L., Shen X.X., Dong X.P., Xu W.B. & Ma X.J. (2017) Rapid and Accurate Sequencing of Enterovirus Genomes Using MinION Nanopore Sequencer. *Biomedical and Environmental Sciences* **30**, 718-26.

Wiens J.J. (2007) Species Delimitation: New Approaches for Discovering Diversity. *Systematic Biology* **56**, 875-8.

Will K.W., Mishler B.D. & Wheeler Q.D. (2005) The Perils of DNA Barcoding and the Need for Integrative Taxonomy. *Systematic Biology* **54**, 844-51.

WoRMS (2021) Cnidaria.

Zheng L., He J., Yuanshao L., Wenqing C. & Zhang W. (2014) 16S rRNA is a better choice than COI for DNA barcoding hydrozoans in the coastal waters of China. *Acta Oceanologica Sinica* **33**.

Appendix A – Synonymized names of *Porpita porpita.* Adapted from World Hydrozoa Database (Schuchert 2021)

Name	Type	Reference
Acies palpebrans Lesson, 1830	synonym	
Chrysomitra striata Gegenbaur, 1857	synonym	
Discalia primordialis Haeckel, 1888	synonym	
Disconalia gastroblasta Haeckel, 1888	synonym	
Disconalia pectyllis Haeckel, 1888	synonym	
Disconalia ramifera Haeckel, 1888	synonym	
Medusa porpita Linnaeus, 1758	basionym	(Linné & Salvius 1758)
Medusa umbella Müller, 1776	synonym	(Müller 1776)
Polybrachionia linnaeana Guilding, 1828	synonym	(Guilding 1828)
Porpita appendiculata Bosc, 1802	synonym	(Bosc 1801)
Porpita atlantica Lesson, 1826	synonym	
Porpita chrysocoma Lesson, 1826	synonym	
Porpita coerulea Eschscholtz, 1825	synonym	(Eschscholtz 1825)
Porpita forskahli de Haan, 1827	synonym	
Porpita forskalea Oken, 1815	name rejected	
Porpita fungia Haeckel, 1888	synonym	
Porpita gigantea Péron & Lesueur, 1807	synonym	
Porpita glandifera Lamarck, 1816	synonym	
Porpita globosa Eschscholtz, 1825	synonym	
Porpita granulata Cranch, 1818	synonym	
Porpita indica Lamarck, 1801	synonym	(Lamarck 1801b)
Porpita kuhlii de Haan, 1827	synonym	
Porpita lutkeana Brandt, 1835	synonym	
Porpita mediterranea Eschscholtz, 1829	synonym	
Porpita moneta Risso, 1827	synonym	(Risso 1826)
Porpita pacifica Lesson, 1826	synonym	
Porpita radiata Bory de St Vincent, 1804	synonym	
Porpita ramifera Eschscholtz, 1825	synonym	(Eschscholtz 1825)
Porpita reinwardtii de Haan, 1827	synonym	
Porpita umbella Müller, 1776	synonym	(Müller 1776)
Porpitella pectanthis Haeckel, 1888	synonym	
Ratis medusae Lesson, 1830	synonym	

Appendix B – Synonymized names of *Velella velella.* Adapted from World Hydrozoa Database (Schuchert 2021)

Name	Type	Reference
Armenista sigmoides Haeckel, 1888	synonym	
Holothuria spirans Forsskål, 1775	synonym	
Medusa pocillum Montagu, 1815	synonym	
Medusa velella Linnaeus, 1758	basionym	(Linné & Salvius 1758)
Rataria cordata Eschscholtz, 1829	synonym	
Rataria mitrata Eschscholtz, 1829	synonym	
Velella antarctica Eschscholtz, 1829	synonym	
Velella aurora Eschscholtz, 1829	synonym	
Velella australis de Haan, 1827	synonym	
Velella caurina Eschscholtz, 1829	synonym	
Velella cyanea Lesson, 1826	synonym	
Velella emarginata Quoy & Gaimard, 1824	synonym	
Velella indica Eschscholtz, 1829	synonym	
Velella lata Chamisso & Eysenhardt, 1821	synonym	(Chamisso 1821)
Velella limbosa Lamarck, 1816	synonym	
Velella meridionalis Fewkes, 1889	synonym	
Velella mutica Lamarck, 1801	synonym	
Velella oblonga Chamisso & Eysenhardt, 1821	synonym	(Chamisso 1821)
Velella oxyothone Brandt, 1835	synonym	
Velella oxyothone var. brachyothone Brandt, 1835	synonym	
Velella oxyothone var. oxyothone Brandt, 1835	synonym	
Velella pacifica de Haan, 1827	synonym	
Velella patellaris Brandt, 1835	synonym	
Velella pyramidalis Cranch, 1818	synonym	
Velella radackiana de Haan, 1827	synonym	
Velella sandwichiana de Haan, 1827	synonym	
Velella scaphidia Peron & Lesueur, 1807	synonym	
Velella septentrionalis Eschscholtz, 1829	synonym	
Velella sinistra Chamisso & Eysenhardt, 1821	synonym	(Chamisso 1821)
Velella tentaculata Lamarck, 1801	synonym	
Velella tropica Eschscholtz, 1829	synonym	
Velella vella, Linnaeus, 1758	misspelling	(Linné & Salvius 1758)

Appendix C – Sequences retrieved from GenBank and BOLD databases.

Specie	Specimen	Local	Oceanic Region	Database	Acession number	Gene	Collection date	Reference
Porpita porpita	07ASMP-027	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP027-08	COI	28/12/2007	Hebert (2008) – direct submission
Porpita porpita	07ASMP-028	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP028-08	COI	28/12/2007	Hebert (2008) – direct submission
Porpita porpita	07ASMP-029	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP029-08	COI	28/12/2007	Hebert (2008) – direct submission
Porpita porpita	07ASMP-030	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP030-08	COI	28/12/2007	Hebert (2008) – direct submission
Porpita porpita	07ASMP-031	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP031-08	COI	28/12/2007	Hebert (2008) – direct submission
Porpita porpita	07ASMP-032	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP032-08	COI	28/12/2007	Hebert (2008) – direct submission
Porpita porpita	07ASMP-033	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP033-08	COI	28/12/2007	Hebert (2008) – direct submission
Porpita porpita	HY019.1	Sargasso Sea North Atlantic;	NO Atlantic	GENBANK	GQ120060	COI		(Ortman *et al.* 2010)
Porpita porpita	RM3_747	Sabaudia; Tyrrhenian Sea; Italy	Mediterranean	GENBANK	LT795124	COI		(Furfaro *et al.* 2017)
Porpita porpita	CB_POP1	Colombia; Caribbean Sea	W Atlantic	GENBANK	MT576016	COI	4/8/2019	Umar (2020) – direct submission
Porpita porpita	CB_POP1	Colombia; Caribbean Sea	W Atlantic	GENBANK	MT569977	ITS	4/8/2019	Umar (2020) – direct submission
Porpita porpita	-	Gulf of California; Mexico	NE Pacific	GENBANK	AY935322	16S		(Dunn *et al.* 2006)
Porpita porpita	AGC-2001	Guam; Pacific Oceanic Region	W Pacific	GENBANK	AY512529	16S		(Collins *et al.* 2005)
Velella velella	07ASMP-34	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP034	COI	28/12/2007	Hebert (2008) – direct submission
Velella velella	07ASMP-35	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP035	COI	28/12/2007	Hebert (2008) – direct submission
Velella velella	07ASMP-36	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP036	COI	28/12/2007	Hebert (2008) – direct submission
Velella velella	07ASMP-37	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP037	COI	28/12/2007	Hebert (2008) – direct submission
Velella velella	07ASMP-38	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP038	COI	28/12/2007	Hebert (2008) – direct submission)
Velella velella	07ASMP-39	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP039	COI	28/12/2007	Hebert (2008) – direct submission)
Velella velella	07ASMP-40	Tallow Beach; New South Wales; Australia	SO Pacific	BOLD	ASMP040	COI	28/12/2007	Hebert (2008) – direct submission
Velella velella	BIOUG01213	San Clemente; California; USA	NE Pacific	BOLD	CNIDC095-14	COI	22/8/2014	Bryant (2014) – direct submission
Velella velella	BMOO03092	French Polynesia; Pacific Ocean	S Pacific	GENBANK	KC706685	COI	17/11/2008	(Leray *et al.* 2013)
Velella velella	Sch71	Villefranche-sur-Mer; France	Mediterranean	GENBANK	EU305487	16S	3/5/2001	(Cartwright *et al.* 2008)
Velella velella	-	California; USA	NE Pacific	GENBANK	AY935323	16S		(Dunn *et al.* 2006)
Velella velella	-	Coast of California	NE Pacific	GENBANK	AY512528	16S	1999	(Collins *et al.* 2005)
Velella velella	65SK	Pacific Ocean	Pacific	GENBANK	AB377541	ITS		Chow (2008) – direct submission