DNA BARCODING OF SEVEN HERMIT CRAB SPECIES (CRUSTACEA : DECAPODA) FROM TURKEY WITH PHYLOGEOGRAPHIC COMPARISONS

by

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ABSTRACT

Identification of species in a correct, fast and accurate way is essential for understanding species biodiversity and undertaking species conservation action. Due to significant limitations, identification of species with routine identification methods is not always easy for taxonomists. To relieve the hard work on taxonomists, to accelerate the identification process and to make it more accurate, new methodologies are needed. DNA barcoding, as the main methodology used in this study, is a fast and reliable method for species identification and for determining interspecific and intraspecific relations. This study aims to undertake the DNA barcoding of hermit crab species living in the Turkish waters and to determine the interspecific and intraspecific relations of these species. In this study, DNA from 46 samples belonging to seven different species were barcoded, and the genetic diversity of these species were evaluated. Also, global comparisons were made with samples taken from genetic databases when available. Three of seven hermit crab species analyzed in this study had unique barcode sequences that are distinct from those found in any other species in the databases. Rest of the species had shared or overlapping barcode sequences. Using the sequences obtained in this study, global phylogeographic comparisons were made.

ÖZET

Türlerin doğru, hızlı ve kesin bir şekilde tanımlanması, tür koruması ve biyolojik çeşitliliğin anlaşılması açısından çok büyük önem taşımaktadır. Fakat bazı sınırlamalar nedeniyle rutin tür tanımlaması yöntemleri ile tanımlama yapmak taksonomistler için her zaman kolay olmamaktadır. Taksonomistlere düşen yükün hafifletilmesi, tür tanımlama işlemlerinin hızlanması ve bu konuda kesinlik sağlanması için yeni yöntemlere gün geçtikçe daha fazla ihtiyaç duyulmaktadır. Bu tezde başlıca çalışılan metod olarak DNA barkodlama, tür tanımlanması ve tür içi ve türler arası genetik ilişkilerin belirlenmesinde hızlı ve güvenilir bir metottur. Bu çalışmada Türkiye sularında bulunan keşiş yengeci türlerinin barkodlaması ve bu yolla genetik çeşitliliğinin ve türler arası ile tür içi ilişkilerinin belirlenmesi hedeflenmektedir. DNA barkodlama yöntemi ile Türkiye sularında bulunan yedi farklı keşiş yengeci türünden 46 bireyin genetik çeşitliliği incelenmiş ve mümkün olduğunda genetik veri tabanları ile karşılaştırmalar yapılarak hem Türkiye suları dahilinde hem de dünya çapında karşılaştırılmalar yapılmıştır. İncelenen yedi farklı keşiş yengeci türünden üç tanesinin veri tabanları ile karşılaştırıldığında, kendi türüne özgü bir barkoda sahip olduğu, kalan türlerin ise başka türlerle kesişen veya paylaşılan barkodları olduğu gözlemlenmiştir. Bu türlerin dizileri kullanılarak global filocoğrafi karşılaştırılmalar da yapılmıştır.

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LIST OF SYMBOLS/ABBREVIATIONS

BOLD	Barcode of Life Database
Bp	Base pair
CBOL	Consortium for the Barcode of Life
Hap	Haplotype
Cytochrome c oxidase I	COI, CO1, cox1
DNA	Deoxyribonucleic Acid
DNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
IUCN	World Conservation Union
mtDNA	Mitochondrial deoxyribonucleic acid
PCR	Polymerase chain reaction
TBE	Tris base boric acid, EDTA
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid

1. INTRODUCTION

1.1 Species Identification and DNA Barcoding

Species are the foremost currency of biodiversity and usually the focal taxonomic unit of conservation biology. The majority of laws and conservation programs are focused on protecting species (U.S. Endangered Species Act [ESA], World Conservation Union [IUCN], Red List of Threatened Species, Convention on International Trade of Endangered Species [CITES]). To undertake reliable and consistent conservation management, accurate, unambiguous, and robust species identifications are needed. Accurate, dependable and detailed species identifications are vital for viable and consistent conservation action (Rubinoff, 2005). In total 1.7 million species have been described by taxonomists until today (about 10% of the estimated 10-15 million species), however this is a small portion of the true biological diversity on Earth (Savolainen et al., 2005; Costa et al., 2007). There are an estimated 230,000 described species of marine metazoans and perhaps another 1,000,000 or more species awaiting discovery and description (Bucklin et al., 2010).

Due to significant limitations, delimiting all species with routine species identification is not easy. New methodologies are needed to accelerate and simplify the work of documenting current biodiversity levels and distribution patterns (Bucklin et al., 2010). Hebert et al. (2003) proposed that these limitations require a new approach to taxon recognition, like microgenomic identification systems. Microgenomic identification systems contribute to the understanding of biological diversity by using small segments of the genomes of species to discriminate them. This method was named “DNA barcoding” (Hebert et al., 2003).

1.2. Conservation Ecology and the DNA Barcoding Process

Species identification is essential for large-scale biodiversity monitoring and conservation (Hajibabei et al., 2007a). In DNA-based identification systems, DNA sequences are used to identify and classify the world's biodiversity. DNA sequences can also explain evolutionary and genetic relationships among species (Hajibabei et al., 2007b). Subsequently DNA barcoding can be used for conservation purposes, as the main principle of conservation ecology is preservation and management of global biodiversity. Preservation and management of global biodiversity has inherent difficulties because there are millions of unidentified and unknown species living in the world. To focus on the conservation work and help to identify species, DNA barcode approach can be a fast, efficient, and inexpensive way to catalogue all biodiversity (Rubinoff, 2005; Hebert et al., 2003). DNA barcoding can be used to rapidly sort specimens and highlight divergent taxa that may represent new species. As a result of this approach, DNA barcoding expedites the rate of species discovery and can provide a greatly expanded, and eventually completed, global inventory of life's diversity (Hebert, 2005). Additionally, for groups that are already relatively well known, such as birds and mammals, molecular studies based on barcode-sized sequences have revealed cryptic DNA lineages (Rubinoff, 2005). DNA barcoding is also in use for phylogenetic research on species. Phylogenies of species help to understand the evolutionary process better, also assisting to comprehend overall adaptation and divergence dynamics. Molecular systematic and molecular characters are both useful for phylogenetic research. Information on gene phylogeny also leads to phylogeographic analyses on species. Because of high mutational rate, conservative role in gene content and lower effective population size compared to the nuclear DNA, mitochondrial DNA (mtDNA) analyses have been frequently used in evolutionary studies for more than 30 years (Moritz, 2011; Hickerson, 2000; Kamaruzzaman, 2011).

DNA barcoding enables species identification, recognition and discovery in a particular domain of life by derivation of short standardized gene regions (DNA sequences) as internal species tags named DNA barcodes. Species identification by DNA barcoding is based on sequencing a short standardized genomic region of the target specimen and comparing this information to a sequence library from known species

(Hajibabei et al., 2007a). DNA barcoding uses short standardized DNA sequences, in other words specific gene regions to identify genetic variability, as genetic variation within species occurs in that standard part of the genome. Selection of the best-suited DNA marker for barcoding is essential to avoid misleading and uninformative results (Rubinoff, 2005). Specimens classification methods based on DNA markers were first proposed for least morphologically distinguished species like archaea, bacteria, protists and viruses, and then extended to higher organisms. Barcoding has subsequently been extended to other groups such as fungi, plants and protists, and the Barcode of Life Initiative has gained international momentum by the establishment of the Consortium for the Barcode of Life (CBOL), which plans to assemble DNA barcode libraries for all fish and birds (Hajibabei et al., 2007a), among other taxa.

DNA barcoding primarily aims to identify species in terms of known classification; therefore it is possible to think DNA barcoding and DNA taxonomy as being similar to each other. In fact DNA taxonomy involves the delimitation and definition of species, under evolutionary species concepts and uses DNA sequences as the primary and diagnostic characters to identify and classify species. On the other hand DNA barcoding focuses on identifying known species by sequence similarity. DNA barcoding provides assistance to detect overlooked species and makes DNA taxonomy more accessible for ecologists, conservationists, and the diversity of agencies charged with the control of pests, invasive species, and food safety. In addition DNA barcoding evokes collection of additional genetic, morphological, ecological, geographical, and behavioral data and complements existing taxonomic practices (Hebert et al., 2005; Bucklin et al., 2010).

The benefits of DNA barcoding can be briefly summarized as follows; DNA barcoding:

(i) Provides a standardized, fast and economic identification regime, such as for parasites and vectors (biomedicine), for pests (agriculture) and for endangered species;

(ii) DNA barcoding speeds up identification of known organisms and facilitates rapid recognition of new species, thereby it relieves the time-consuming work of identification from taxonomists, so they can focus on more relevant work such as delimiting taxa, resolving their relationships and discovering and describing new species;

(iii) Pair-up various life stages of the same species, barcoding can identify a species in its many forms. Hence barcoding can distinguish among species that look alike and enable a more accurate view of biodiversity.

(iv) Help to delimit species boundaries for conservation purposes (Savolainen et al., 2005).

1.3. DNA Barcoding – Methodology & Examples

Methodologically speaking, the barcoding approach uses universal polymerase chain reaction (PCR) primers to amplify and sequence an approximately 600-base-pair fragment of the 5' end of the mitochondrial gene, cytochrome oxidase subunit I (COI) to be used as a taxon "barcode". COI differs by several percent, even in closely related species, and contains enough information to identify the species of an individual (Hebert et al., 2003; Rubinoff, 2005). Mitochondrial DNA can be a powerful tool in the effort to identify species, their relationships to each other, and threatened or endangered populations with divergent haplotypes worthy of conservation attention. The mitochondrial genome of metazoans have numerous advantages over the nuclear genome: limited exposure to recombination, high copy numbers in every cell, haploid character, lack of introns (non-coding section of DNA inside a gene), and generally strict mode of inheritance. mtDNA is especially informative for species-level questions because it is haploid with a maternal inheritance pattern. For animals, adoption of a 650-base fragment of the COI gene is the global standard as the barcode region (Hajibabei et al., 2007b, Rubinoff, 2005, Bucklin et al., 2010). That portion of sequence is then compared using distance-based algorithms with an existing database of "known" sequences from specimens previously identified by taxonomists. If the new sequence meets an arbitrary similarity criterion, recently set at 3% for all insects, but 2% for birds and mammals, then the sample is "identified." If the dissimilarity is >3%, the sample remains unidentified and must be examined by a taxonomist (Savolainen et al., 2005). This barcode region has been shown to exhibit a marked genetic divergence between metazoan species (typically <3 %) versus that between species (typically 10-25%) (Bucklin et al., 2010).

However in some cases, like incomplete lineage sorting, the application of the 3% gene cut–off requires high levels of attention and caution. Radulovici (2009) studied crustaceans from the Estuary and Gulf of the St. Lawrence River by DNA barcoding. They chose 507 crustacean species for this study, mainly amphipods and decapods. They found out that the intraspecific variation is much smaller than the interspecific variation, and this suggests that DNA barcoding is a proper tool for identifying species in these marine crustaceans. In addition they found out that in four morphological species barcodes grouped into two-three clusters that diverged by at least 3%. They found the smallest divergence (2.81 %) for two species, which morphologically show different characteristics in larval and adult life stages. This is an important finding that suggests application of the 3% cut-off in sequence divergence requires high levels of attention and caution (Radulovici et al., 2009).

DNA barcoding is in general an effective way to identify species for marine crustaceans. Although diversity in the marine fauna is much smaller than the terrestrial fauna, taxonomists identified only %66 percent of the whole species estimated in the sea (Radulovici, et al., 2009). Morphological identification of crustaceans is very time consuming and difficult and needs highly specified taxonomists and DNA barcoding was found to be effective for identification for crustaceans (Bucklin et al., 2010; Costa et al., 2007).

Hirose et al. (2010), studied Clibanarius, a group of hermit crabs that currently includes 59 species worldwide and 37 species from the Indo-West pacific. Clibanarius play significant roles in seashore ecosystems, and accurate identification of species is important in ecological studies. Hermit crab species are identified mainly by the external morphology and coloration in life. Clibanarius species have distinct color patterns, which can be treated as primary species-specific characters, however preservation techniques of species may result in colorless specimens. Therefore, as some species are very similar in morphology, identification of preserved species can sometimes be very difficult. To enable identification of preserved specimens without special knowledge of the crustaceans, Hirose et al. determined partial COI gene sequences for 11 Clibanarius species from the interdial zones of Okinawa-jima and Iriomote-jima islands, and Ryukyu Archipelago in southwestern Japan. In total they obtained 15 haplotypes from sequencing COI gene of 11 Clibanarius species. As a result, they found that the intraspecific variations of four species (C.

corallines, C. englaucus, C. striolatus, and C. virescens) among two different collection sites were much smaller than the interspecific variations of the total of 11 Clibanarius species. This suggests that DNA barcodes are useful for species identification and DNA barcodes can be used to identify specimens that have lost their coloration due to preservation techniques that use ethanol (Hirose et al., 2010).

1.4. Taxonomy and Characteristics of Hermit Crabs

The order Decapoda is composed of more than 8000 species of crustaceans (phylum Arthropoda) that include shrimp, lobsters, crayfish, hermit crabs and crabs. Until now, a total number of 220 decapod species have been identified in Turkey (Balkıs, 2001). This thesis focuses on the hermit crabs, which belong to the phylum Arthropoda, sub-phylum Crustacea, order Decapoda, and infraorder Anomura. Anomura, an infraorder of the decapod crustaceans, are existent all over the world. Nearly 1100 species are currently recognized worldwide and many species continue to be described from intertidal or subtidal shallow waters (Hirose et al., 2010). In Turkish Seas 37 Anomura species have been recorded up to date. 23 of these species have been recorded in the Levantin Sea, 35 in the Aegean Sea, six in the Dardanelles, 24 in the Sea of Marmara, eight in the Bosphorus and three in the Black Sea (Balkıs and Kurun, 2008). There are around 600 species of hermit crabs all over the world. In Turkish Seas 21 hermit crab species (seven in the genus Pagurus, six in Anapagurus, one in Cestopagurus, one in Clibanarius, one in Calcinus, one in Diogenes, two in Paguristes, two in Dardanus) have been identified (Kocatas and Katagan, 2003).

1.5. Hermit Crabs – Basic Characteristics

The species inhabiting the intertidal region, which can be considered as a high risk zone, must be able to evade or endure the stress induced by both abiotic (wave action, dehydration, variations in salinity and thermal conditions) and biotic factors (predators of marine and terrestrial environment). Hermit crabs adapt their behavioral characteristics and biology to fit habitat changes that makes them one of the most conquering groups in intertidal regions (Benvenuto and Gherardi, 2001). They are invertebrates, with an exoskeleton, which makes them vulnerable to predators, and hence they need shelter for protection from predators and also partial containment of the body. Due to limited calcification, they are not able to produce a shell themselves. On the other hand, limited calcification makes their abdominal exoskeletons soft and able to fit into a shell, which makes them adapted for occupation of empty snail shells and a few other types of cavities. Hermit crabs must inhabit empty gastropod shells such as those of sponges, bryozoans, polychaete or vermetid tubes, and cavities in corals, scaphopod and bivalve shells, pieces of bamboo or coral, barnacles, or stones, for shelter and protection from predation, desiccation and osmotic stress (Gherardi, 2004). They move progressively to larger shells as they grow. In addition, shells also start to weaken over time and need to be replaced. The effective shell selection can strongly affect their fitness (Giannelli, 2004).

Many animals utilize exogenous shelters, but almost all 800 species of hermit crabs are mobile while sheltered (Hazlett, 1981). With the mobility and protection gained from the exogenous shelters, hermit crabs have a worldwide influence on whole communities of species in a range of habitats, from the terrestrial to the deep sea. Hermit crabs also play an important role in the food chain, in many littoral and moderately deep benthic marine communities worldwide. They play a significant role in environmental care because they can be used as indicators of ecological health. Recent research suggest that they can be used as indicators of changes in heavy metals (iron and manganese), and as test organisms for detecting environmental impact. For instance, some species were found to be useful indicators of freshwater inundation on tropical shores (El-Wakeil et al., 2009).

Hermit crabs are omnivores that eat worms, plankton, and organic debris, which means that their limiting factor is not dependent upon nutrient availability, but abundant supply of shells, especially snail shells (Hazlett, 1981). Optimally sized, intact, empty shells are the limiting factor in most habitats suitable for hermit crabs. If there are not enough shells, the hermit crabs can become endangered (Giannelli, 2004).

1.6. The Hermit Crab of Turkey

In this project, out of the 21 species living in Turkish coastal waters, seven species are investigated. These species are given below, with a discussion of their distribution in Turkey and their basic taxonomy.

1.6.1. *Calcinus tubularis*

Calcinus tubularis is frequently known as Calcinus ornatus, however the oldest name for it is Cancer tubularis. It is an infrequent species inhabiting tide pools on rocky shores and subtidally shallow water (3 to 10 m) of the Mediterranean, west Africa, Ascension Island and is rarely found along the coasts of the Azores, Canary Islands and Cyprus (Gherardi, 2004; Lewinsohn and Holthuis, 1986; Manning and Chase, 1990). Holthuis (1961) recorded this species from the Turkish coasts of the Mediterranean and Kocataş (1981) reported it from the Aegean coast (Koçak et al., 2001). Calcinus tubularis (Linnaeus, 1767) belongs to the family: Coenobitidae (Ortmann, 1892) and genus: Calcinus (Dana, 1851).

1.6.2. *Clibanarius erythropus*

Clibanarius erythropus is a common intertidal Diogenidae, found in intertidal pools and on sand, gravel and algae in the shallow sublittoral zone, up to 40 m depth. They form large aggregations (Benvenuto and Gherardi, 2001), in the Mediterranean rocky shores, Black Sea and along the Atlantic coast from Brittany to southern Morocco and the Azores (Southward and Southward, 1977; Gherardi, 1991; Benvenuto and Gherardi, 2001; Benvenuto et al., 2003). Clibanarius erythropus inhabits the coasts of the Sea of Marmara, Aegean, Mediterranean and Black Sea in Turkey (Koçak et al., 2001). Clibanarius erythropus (Latreille, 1818) belongs to the family: Diogenidae (Ortmann, 1892) and genus: Clibanarius (Dana, 1852) (Balkıs, 2001).

1.6.3. *Pagurus anachoretus and Pagurus cuanensis*

Pagurus anachoretus is known from the Atlantic coast of Portugal, and from the entire Mediterranean (Holtuis, 1961). Additionally, another species in the genus, Pagurus cuanensis, is widely distributed in the Mediterranean and has been reported from several localities in its eastern parts (Greece, Turkey, Israel, Egypt)(Lewinsohn and Holthuis, 1986). Pagurus anachoretus and P. cuanensis species have also been reported from the Sea of Marmara and Aegean coasts of Turkey (Koçak et al., 2001). Pagurus anachoretus and Pagurus cuanensis (Risso, 1826) belong to the family: Paguridae (Latreille, 1803) and genus: Pagurus (Fabricius, 1775) (Ingle, 1985).

1.6.4. *Diogenes pugilator and Diogenes avarus*

Diogenes pugilator is a common species inhabiting fine sandy beach habitats (Lewinsohn and Holthuis, 1986). It is found in the eastern Atlantic from the southern North Sea south to Angola, the entire Mediterranean, and the Black Sea; it has also been reported from the Red Sea and Singapore (Tirelli, 2000). Diogenes pugilator has also been reported in the Sea of Marmara, the Aegean, the Mediterranean and the Black Sea coasts of Turkey (Koçak et al., 2001). Diogenes avarus, on the other hand, is found predominantly in the Indo-West Pacific, from East Africa to the Philippines (Davie, 2002). Diogenes pugilator (Roux, 1829) and Diogenes avarus (Heller, 1865) belong to the family: Diogenidae (Ortmann, 1892), and genus: Diogenes (Dana, 1851) (Balkıs, 2001).

1.6.5. *Cestopagurus timidus*

Cestopagurus timidus, a synonym of Catapaguroides timidus, is a species known from the eastern coast of the Atlantic ocean, from the British Isles to the Moroccan shores, and from the entire Mediterranean (Greece, Israel and Cyprus)(Holthuis, 1961; Lewinsohn and Holthuis, 1986; Pessani and Premoli, 1993). This species has also been reported from the coasts of the Mediterranean Sea, the Turkish coasts of the Aegean Sea and the coasts of the Sea of Marmara (Koçak et al., 2001). Cestopagurus timidus (Roux, 1830) belongs to the family: Paguridae (Latreille, 1803) and genus: Cestopagurus (Bouvier, 1897) (Balkıs, 2001).

2. THESIS OBJECTIVES

The proposed thesis focuses on the DNA barcoding of seven hermit crab species in Turkey. There are many studies on hermit crabs such as on population structure and growth and reproductive aspects, (Manjon-Cabeza and Garcia-Raso, 1998, 2000; Bertness 1981a, Bertness 1981b; Gherardi 1991; Garduno and Aguilar, 2006; Ziegler and Forward Jr., 2006), larval development (Pike and Williamson, 1960), taxonomy (Ingle, 1993, Mantelatto et al., 2006; El-Wakail et al., 2009), distribution and ecology (Southward and Southward, 1977; Barnes, 2003; Hazlett, 1981; Biagi et al., 2006), foraging behavior (Benvenuto et al., 2003), parental behaviour (Calado et al., 2006), shell utilization, population structure, population biology and population dynamics (Bell, 2009; Gherardi, 1991; Manjon-Cabeza and Garcia Raso, 1999; Benvenuto and Gherardi, 2001; Turra and Leite, 2000; Garcia and Mantelatto, 2001; Hazlett, 1989; Hazlett and Baron, 1989; Bertness, 1980; Pessani and Premoli, 1993; Botelho and Costa, 2000; Caruso and Chemello, 2009; Hahn, 1988, Bach and Hazlett, 2009, Domaniciano et al., 2009; Garcia and Mantelatto, 2001; Angel, 2000; Gianelli, 2004), relationships with other organisms (McDermott et al., 2010; Williams and McDermott, 2004; Bach et al., 2006; Damiani, 2003), gene rearrangements (Hickerson and Cunningham, 2000), including DNA barcoding (Hirose et al. 2010). However, genetic cataloging through DNA has not yet been done for hermit crabs in Turkey, and here we represent the application of this approach for the first time in Turkey for seven species from six genera, namely Calcinus tubularis, Pagurus anachoretus, Pagurus cuanensis, Diogenes pugilator, Diogenes avarus, Clibanarius erythropus and Cestopagurus timidus. With the data at hand, when possible, we also make global comparisons for these species, helping us gain insights into the phylogeography and evolutionary history of these species in a broader geographical context.

3. MATERIALS and METHODS

3.1. Field Methods

A total of 46 hermit crab samples, including one individual of Cestopagurus timidus, one Diogenes avarus, two Pagurus cuanensis, eight Calcinus tubularis, six Clibanarius erythropus, 19 Diogenes pugilator, and nine Pagurus anachoretus were collected and analyzed from eight locations, including 16 (Kaş, Kalkan, Antakya station 01 and Antakya station 11) from the Mediterranean Sea, 13 (Saros, Bodrum and Çanakkale Strait) from the Aegean Sea, and 17 (Yalova - Çınarcık) from the Sea of Marmara (Figure 3.1).

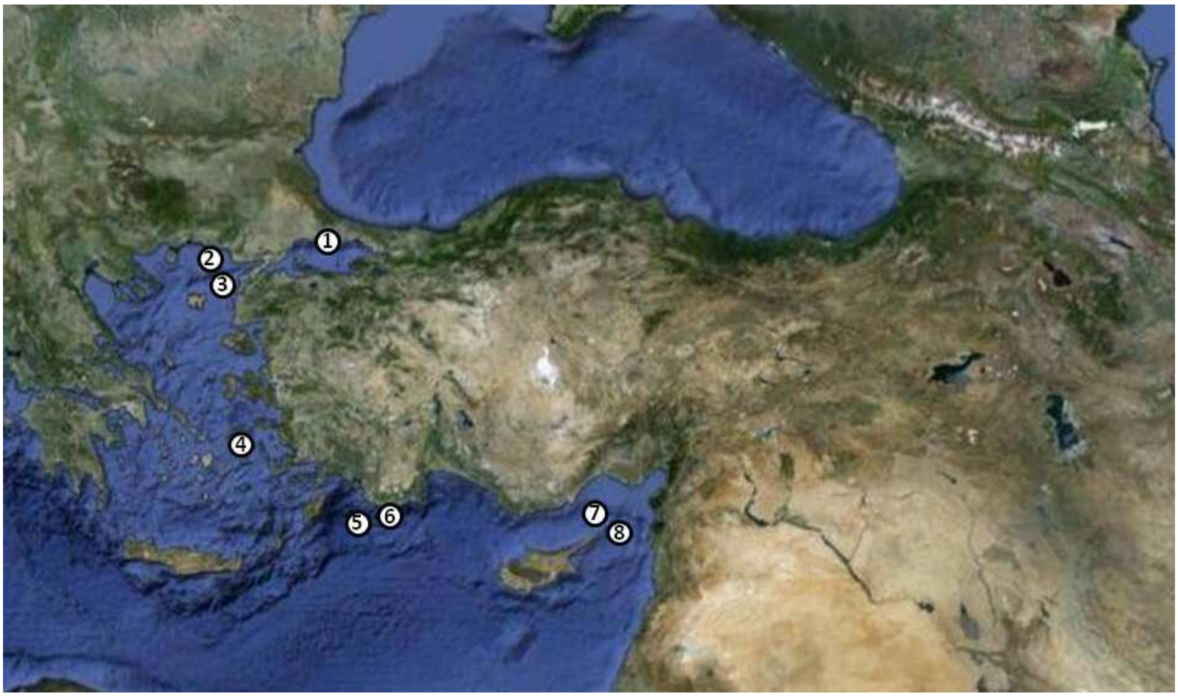

Figure 3.1. The map of sample collection sites. (Numbers indicate the codes of the sampling sites as given in Table 3.1).

Table 3.1. List of sampling location, sample size and geographic coordinates of the sites.

Code	Sampling Site	N	Geographic coordinates (N, E)
1	Çınarcık	17	38.9637o , 35.2433o
2	Saroz	10	40.6018o , 26.5420o
3	Çanakkale	2	40.2315o , 26.5368o
4	Bodrum	1	36.9943o , 27,5332o
5	Kalkan	4	36.1476o, 27.2401o
6	Kaş	5	30.7383o , 78.8378o
7	Antakya 01	3	36.0034o , 35.5840o
8	Antakya 11	4	35.5705o, 35.5510o

3.2. Laboratory Methods

The hermit crab samples were collected with scuba diving and free style diving in the different locations stated above. Hermit crab samples were preserved in 75% ethanol until DNA extraction. Morphological identification was done for hermit crab species using the keys in Ingle (1993) and a stereomicroscope. Total genomic DNA was extracted from tissues (soft abdominals and legs) of hermit crabs by using Roche DNA extraction kit, with slight modifications in the DNA binding and DNA elution steps. In the DNA binding step, the optimum incubation time suggested by the default protocol is one hour at 55 oC, however in this study the incubation was done for two days at 55 oC to enhance the lyses of the cell membranes. Additionally in the final DNA elution step, 100 µl of elution buffer was added instead of 200 µl, in order to have a higher final total DNA concentration. The eluted DNA was either used directly or stored at -20oC for later analysis. The isolated DNA was run on 1% agarose gels, prepared in 1X TBE (Tris base, boric acid, EDTA) buffer with Ethidium bromide. For each reaction, 3 µl of the isolated DNA was mixed with 3 µl of 2X loading dye (Fermantas) and loaded on the gel. Samples were run at 90 V for 45 minutes. The band images were taken under ultraviolet light with the Biorad Gel Doc Imaging System.

The CO1 barcode region was amplified and sequenced in both directions using six alternative sets of primers depending on the reaction success. Primers that have been used, the PCR conditions and the reaction mixture prepared for the PCR process are shown in Table 3.2. The names and actual sequences for all of the primers used for the amplification of the CO1 region are given in Table 3.3. Also, the mitochondrial DNA from the large subunit rRNA (16S) gene was amplified in some samples, for which the PCR of the CO1 region was not successful. The 16S region was amplified and sequenced in both directions using five different sets of primers. Primers that have been used in the 16S region amplification process are shown in Table 3.4. The names and actual sequences for all of the primers used for the amplification of the 16S region are given in Table 3.5.

Table 3.2. Primers, PCR conditions and the content of reaction mixture used in the CO1 region amplification.

Primers	PCR Conditions	Reaction mixture (50 µL)
LCO 1490 - HCO 2198	Denaturation step of 60 s. at 94 oC followed by five cycles of 30 s. at 94 oC, 90 s. at 45 oC and 60 s. at 72 oC, 35 cycles of 30 s. at 94 oC, 90 s at 51 oC, and 60 s. at 72 oC, and a final extension step of 5 min. at 72 oC (Costa et al., 2007).	2 µL of DNA, 5µL 10x high fidelity buffer, 4,4 µL MgCl2 (25mM), 1 µL of 10mM deoxyribonucleotide triphosphate (dNTPs), 0,5 µL of each primer (20µM) , 37,7 µL H2O and 0,3 µL of high fidelity Taq DNA polymerase.
CrustF1 - CO1R	Denaturation step of 60 s. at 94 oC followed by five cycles of 30 s. at 94 oC, 90 s. at 45 oC and 60 s. at 72 oC, 30 s. at 94 oC, 90 s at 51 oC, and 60 s. at 72 oC, and a final extension step of 5 min. at 72 oC (Costa et al., 2007).	3 µL of DNA, 5µL 10x KCL buffer, 4,4 µL MgCl2 (25mM), 1 µL of 10mM deoxyribonucleotide triphosphate (dNTPs), 0,5 µL of each primer (20µM) , 37,3 µL H2O and 0,3 µL of Taq DNA polymerase.
CrustF2 - CO1R	Denaturation step of 60 s. at 94 oC; followed by 35 cycles of 30 s. at 94 oC, 90 s. at 42 oC, 60 s at 72 oC; and a final extension step of 5 min. at 72 oC (Costa, et al., 2007).	3 µL of DNA, 5µL 10x KCL buffer, 4,4 µL MgCl2 (25mM), 1 µL of 10mM deoxyribonucleotide triphosphate (dNTPs), 0,5 µL of each primer (20µM) , 37,3 µL H2O and 0,3 µL of Taq DNA polymerase.
COL6a - HCO 2198 COL6b - HCO 2198	Denaturation step of 4 min. at 94 oC, followed by 30 cycles of 45 s. at 94 oC, 1 min. at 48 oC, 1 min. at 72 oC, followed by final extension step 10 min. at 72 oC.	3 µL of DNA, 5µL 10x of High Fidelity KCL buffer, 4,4 µL MgCl2 (25mM), 1 µL of 10mM deoxyribonucleotide triphosphate (dNTPs), 0,5 µL of each primer (20µM) , 37,7 µL H2O and 0,3 µL of High Fidelity Taq DNA polymerase.

Table 3.3. Primers used in the PCR amplification (CO1).

Primers	Actual names
HCO2198	5’-TAAACTTCAGGGTGACCAAAAAATCA-3’
LCO1490	5’-GGTCAACAAATCATAAAGATATTGG-3’
CrustF1	5'-TTTTCTACAAATCATAAAGACATTGG-3'
CrustF2	5'-GGTTCTTCTCCACCAACCACAARGAYATHGG-3'
COL6a	5’-TCWACAAATCATAAAGAYATTGG-3’
COL6b	5’-TYTCHACAAAYCATAAAGAYATYGG-3’

Table 3.4. Primers, PCR conditions and the content of reaction mixture used in the 16S region amplification.

Primers	PCR Conditions	Reaction mixture (50 µL)
16H3 - 16L2	Denaturation step of 4min at 94 oC, followed by 30 cycles of 45 s. at 94 oC, 30 s. at 47 oC, 1 min. 72 oC, and a final extension step for 10 min. at 72 oC.	2 µL of DNA, 5µL 10x of High Fidelity KCL buffer, 4,4 µL MgCl2 (25mM), 1 µL of 10mM deoxyribonucleotide triphosphate (dNTPs), 0,5 µL of each primer (20µM) , 36,25 µL H2O and 0,35 µL of High Fidelity Taq DNA polymerase.
16H11 - 16L29 16H11 - 16SarL 16H11 - 16L2 16H37 - 16SarL	Denaturation step of 4 min. at 94 oC, followed by 40 cycles of 45 s. at 94 oC, 1 min. at 48.5 oC, 1 min 72 oC, and a final extension step for 10 min. at 72 oC.	1,5 µL of DNA, 5µL 10x of High Fidelity KCL buffer, 4,4 µL MgCl2 (25mM), 1 µL of 10mM deoxyribonucleotide triphosphate (dNTPs), 0,5 µL of each primer (20µM) , 36,8 µL H2O and 0,3 µL of High Fidelity Taq DNA polymerase.

Table 3.5. Primers used in the PCR amplification (16S).

Primers	Actual names
16H3	5’–CCGGTTTGAACTCAAATCATGT–3’
16L2	5’–TGCCTGTTTATCAAAAACAT–3’
16H11	5'–AGATAGAAACCRACCTGG–3'
16L29	5'–YGCCTGTTTATCAAAAACAT–3'
16SarL	5’–CGCCTGTTTATCAAAAACAT–3’
16H37	5’–CCGGTYTGAACTCAAATCATGT–3’

The PCR products were run on 1% agarose gels, prepared in 1X TBE (Tris base, boric acid, EDTA) buffer with Ethidium bromide. For each reaction, 3 µl of the PCR product, mixed with 3 µl of 2X loading dye (Fermantas), was loaded on the gel. Samples were run at 95 V for 45 minutes and finally, the band images were checked under ultraviolet light with the Biorad Gel Doc Imaging System.

Successful PCR reactions were cleaned using the Roche PCR clean-up kit, and subsequently sent to the Macrogen Inc. in South Korea for base sequencing. The obtained sequences were cleaned manually with Sequencher v. 4.1 (Gene Codes Corp.), and also aligned using the same program. Subsequently these sequences will be deposited to GenBank and CBOL databases (BOLD).

3.3. Tree and Haplotype Network Construction

A tree is a mathematical structure, which is used to model the actual evolutionary history of a group of sequences or organisms. The evolutionary history of a group of species is called its phylogeny and that phylogenetic tree is a graphical summary of this history. There are many methods for constructing phylogenetic trees from molecular data (Saitou and Imanishi, 1989). Neighbor-joining method was used for constructing the intraspecific trees in this study.

The main principle of neighbor-joining method is to find pairs of operational taxonomic units (OTUs [=neighbors]) that minimize the total branch length at each stage of clustering of OTUs standing with a starlike tree (Saitou and Nei, 1987). In this study, the MEGA (Molecular Evolutionary Genetics Analysis Software for Microcomputers) program was used for constructing the trees. The MEGA program was developed for estimating evolutionary distances, reconstructing phylogenetic trees and computing basic statistical quantities from molecular data (Kumar et al., 1993). Intraspecific and interspecific distances were calculated by MEGA 4.0 using Kimura 2-parameter distances. The samples were combined with sequences downloaded from BOLD and Genbank, when available, for a comprehensive analysis. DnaSP (Rozas et al., 2003) was utilized to build haplotype data files and TCS 1.21 was used to prepare haplotype networks. In TSC 1.21, gaps were treated as missing data. The obtained sequences were also analyzed by using BOLD. The specimens were identified by comparing the sequences in BOLD under the “identify specimen” option of BOLD. Only one individual was compared for the species whose sequences fell within one clade in the neighbor-joining tree, whereas two or more individuals were compared in the species whose barcodes fell within different clades.

4. RESULTS

42 COI sequences from seven different species were generated for this study. Two CO1 sequences were added from Genbank. Additionally, nine 16S sequences from four different species were generated for this study. Two 16S sequences were also added from Genbank.

The mean intraspecific divergence for the CO1 sequences was 4.186%. The mean interspecific distance, on the other hand, was 21.07%. This interspecific value was seen to be less than 10 times the mean intraspecific distance, which suggested that the differentiation did not fit the barcoding gap (Hebert et al., 2003). The minimum intraspecific distance was 0.17% for Clibanarius erythropus, and the maximum intraspecific distance was 8.75% for Pagurus anachoretus. The minimum interspecific distance was 12.4%, between Diogenes avarus and Diogenes pugilator, and the maximum interspecific distance was 30.6% between Diogenes avarus and Clibanarius erythropus (Figure 4.1).

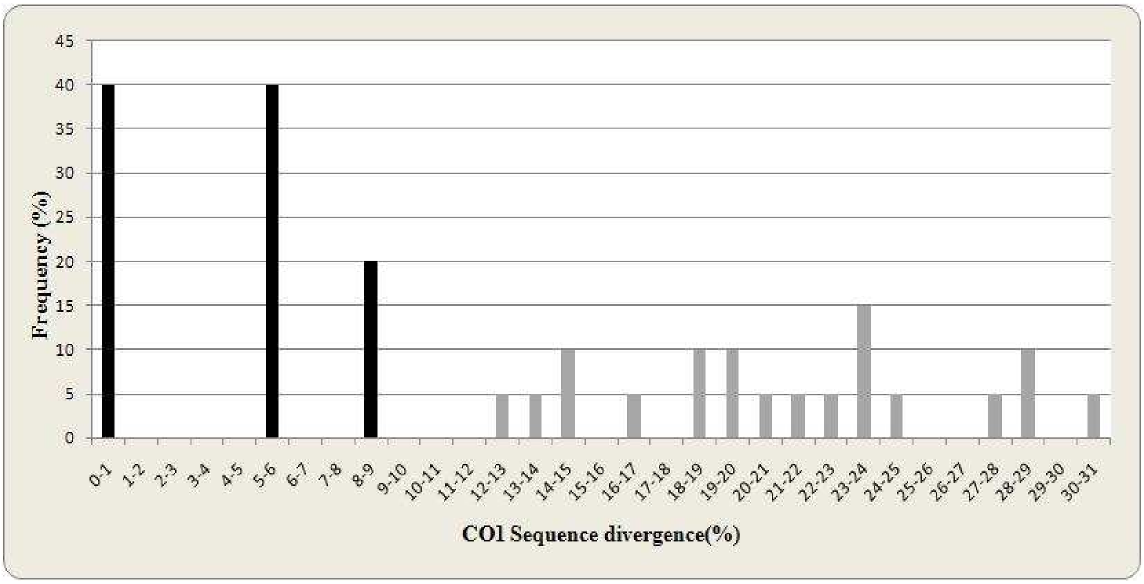

Figure 4.1. Frequency distribution of mean divergences for COI sequences (Kimura 2-parameter model) for 46 samples (1). (Two taxonomic levels are represented: within species, dark bars and between species, gray bars).

Due to the problem with the barcoding gap, as a second step, we repeated the analysis above by separately grouping individuals within a species that were different from each other, based on group designations in the haplotypes networks, discussed below. According to the new analyses, the mean intraspecific divergence for the CO1 sequences was 0.61% and the mean interspecific distance was 24%. The minimum intraspecific distance was 0% for Calcinus tubularis group 1 which contains samples 155, 163, FJ620332 and FJ620337 and the maximum intraspecific distance was 1% for Diogenes pugilator group 1 which contains all Diogenes pugilator species except 170 and 171 which were differentiated from the rest of the Diogenes pugilator species by 60 and 48 base pairs (bps) respectively (see Figure 4.31 below). The maximum interspecific distance was 34% for Clibanarius erythropus and Calcinus tubularis group 1.

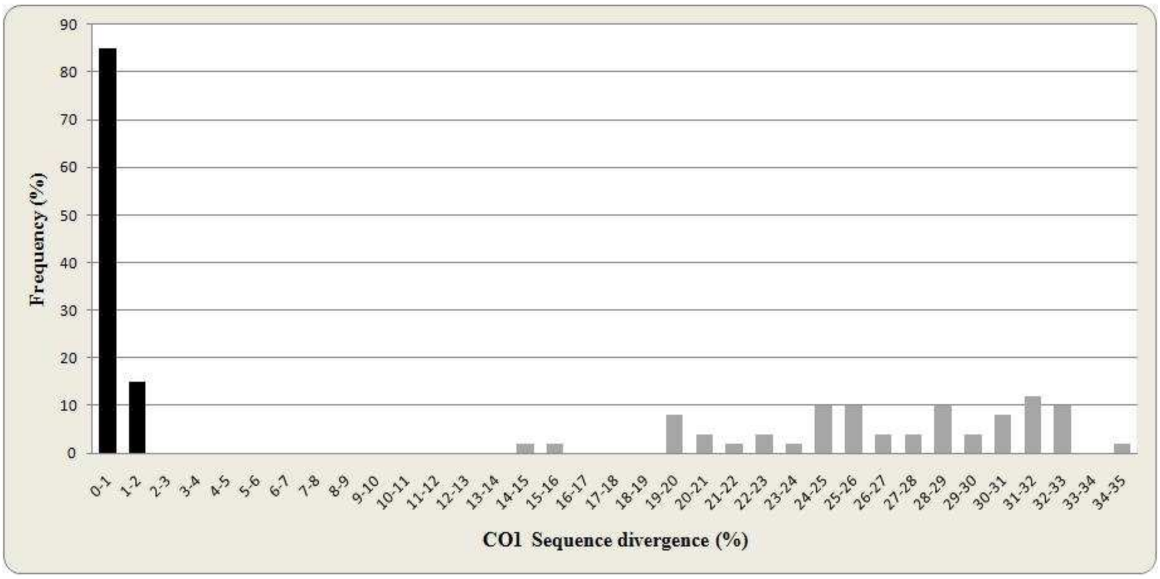

Figure 4.2. Frequency distribution of mean divergences for COI sequences (Kimura 2-parameter model) for 46 samples (2). (Two taxonomic levels are represented: within species, dark bars and between species, gray bars).

Next, for each species a map that shows the locations of samples, an intraspecific tree, a haplotype network, a table of the haplotypes and a neighbor-joining tree was prepared. In the haplotype networks and neighbor-joining trees, colors which were used to indicate different geographic regions are shown in Table 4.1.

Table 4.1. Colors that were used to indicate different locations in haplotype networks

Region	Color
East Atlantic	Purple
The Sea of Marmara	Blue
Mediterranean	Red
Aegean	Yellow

4.1. *Calcinus tubularis* (Linnaeus, 1767)

4.1.1. CO1 Sequences

Six different samples from three different locations (East Atlantic, the Aegean and the Mediterranean) are analyzed for this species (Figure 4.3). These included two sequences from BOLD (Madeira Island and the East Atlantic) (*C tubularis* CO1 FJ620332 and *C tubularis* CO1 FJ620337 on Figure 4.3).

Figure 4.3. Sampling locations for *Calcinus tubularis* (CO1). (The circles are proportional to the number of individuals. The green circles indicate sampling locations for samples belonging to Clade 1 in the neighbor-joining tree, the orange circles indicate sampling locations for samples belonging to Clade 2. The green circle within a black square indicates the collection sites for samples for which sequence data were available from BOLD (East Atlantic). The green-orange circle within a black square indicates the collection sites for samples for which sequence data were not publicly available from BOLD, Portugal).

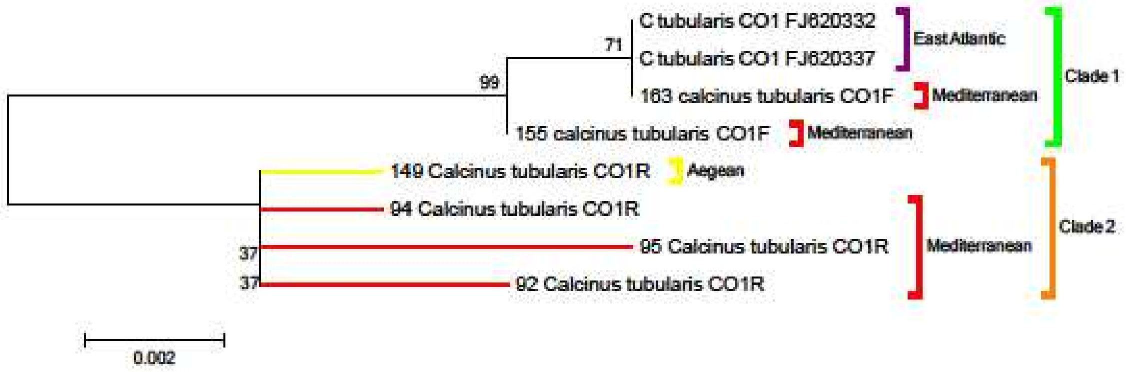

Figure 4.4. The neighbor-joining tree for *Calcinus tubularis* (CO1).

The neighbor-joining tree analysis showed two clades (Figure 4.4). The sample with the code 149 (149 *Calcinus tubularis* CO1R), collected from the Aegean, clustered with samples 92, 94 and 95, which were collected from the Mediterranean. Samples 155 and 163, which were collected from Mediterranean Sea clustered with the individuals from East Atlantic (FJ620332, FJ620337).

The haplotype network showed six different haplotypes for *Calcinus tubularis* (Figure 4.5). Four of the seven haplotypes were only observed in the Mediterranean (CH1, CH3, CH4, CH5), one only in the Aegean (CH6), and one was observed both in the East Atlantic and the Mediterranean (CH2). Three of the samples, 163, FJ620337, and FJ620332, have the same haplotype. Each haplotype except CH2 was seen in one individual only. CH2 was seen in three individuals, sample 163 from the Mediterranean and the two samples from the East Atlantic. Haplotype CH1 was found in sample 155 and is different by a single base pair from the haplotype CH2. These two haplotypes are different by six base pairs from the haplotypes CH3, CH4, CH5 and CH6, which assemble together on the haplotype network. The haplotypes CH1-CH2, and CH3-CH6 correspond to clade 1 and clade 2, in the neighbor-joining tree, showing that the results inferred from the network and the tree verify each other. The haplotype network table for *Calcinus tubularis* is given in Table 4.3.

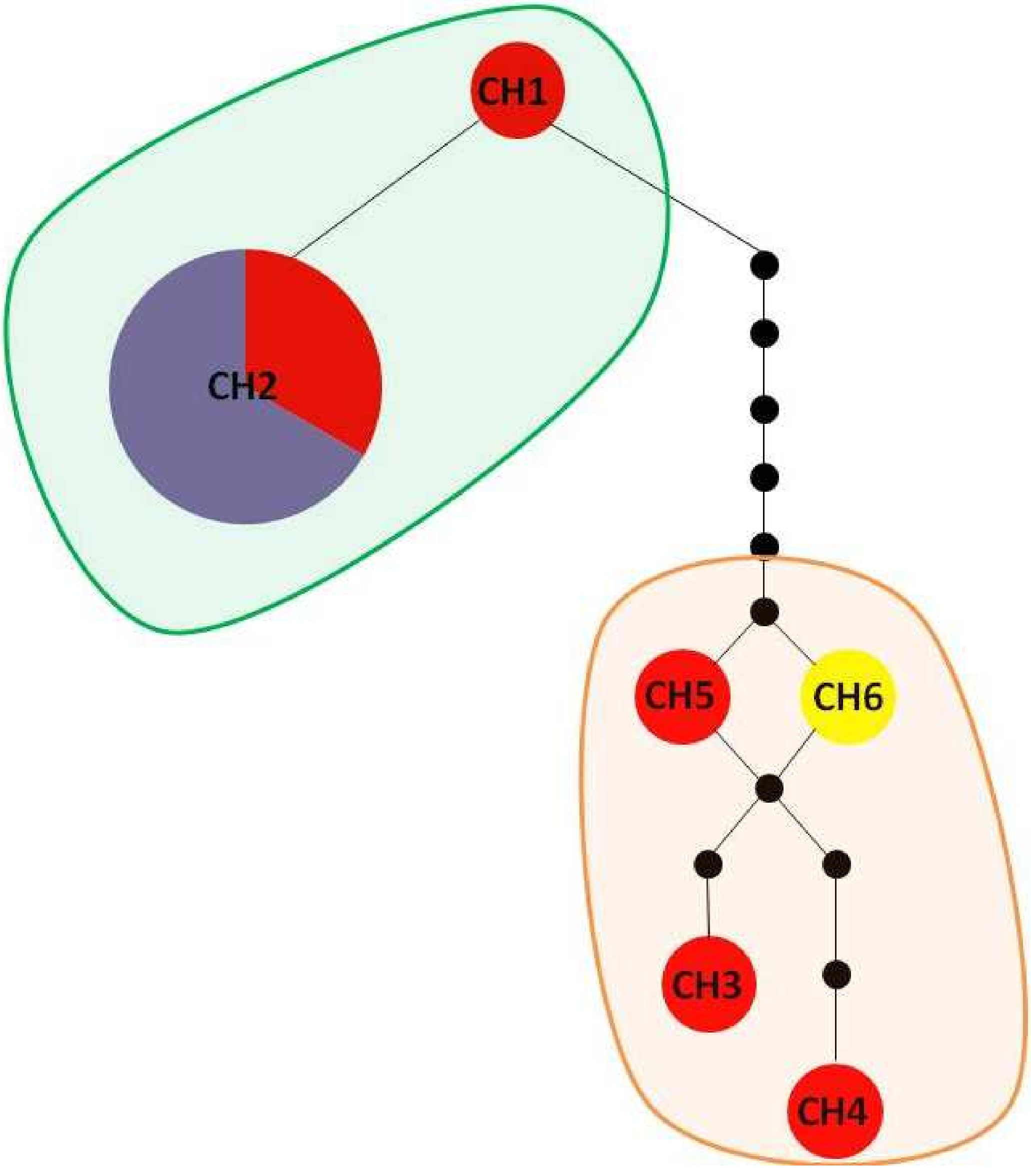

Figure 4.5. Haplotype network for *Calcinus tubularis* (CO1). (The size of the circles is proportional to the number of individuals. See Table 3.1. for the key to the color-codes of the geographic positioning of the haplotypes).

Table 4.2. Haplotype network table for Calcinus tubularis (CO1).

Haplotype	Number of individuals	Codes for the individuals as shown in the neighbor–joining tree
CH1	1	155
CH2	3	163, FJ620332, FJ620337
CH3	1	92
CH4	1	95
CH5	1	94
CH6	1	149

As a next step, we wanted to compare the individuals from each clade to those available in BOLD. A comparison with the BOLD of a representative individual from clade 1 (155) shows first, second and third closest matches to *Calcinus tubularis* with 99.84%, 99.83% and 98.86% similarity, respectively. A comparison with the BOLD database of a representative individual from clade 2 (94) shows first, second and third closest matches to *Calcinus tubularis* with 99.82%, 99.68% and 99.66% similarity, respectively.

The neighbor-joining tree for *Calcinus tubularis* created via BOLD, using one individual belonging to clade 1, 155, is shown in figure 4.6. In the tree, the barcoded individuals from Turkey clustered closely with the barcodes in BOLD, from East Atlantic (Madeira Island) and Portugal, showing that the results inferred from our intraspecific tree and the BOLD tree verify each other for this species. The neighbor-joining tree from BOLD for sample 94 is shown in figure 4.7. In the tree the barcoded individuals from Turkey clustered closely with barcodes in BOLD from Portugal, and into the same general *Calcinus tubularis* clade with other *Calcinus tubularis* CO1 sequences. Briefly, Clade 1 in the neighbor-joining tree corresponds to the BOLD Clade 1 composed of samples from Portugal and East Atlantic, and Clade 2 in the neighbor-joining tree corresponds to the BOLD Clade 2 composed of samples from Portugal only.

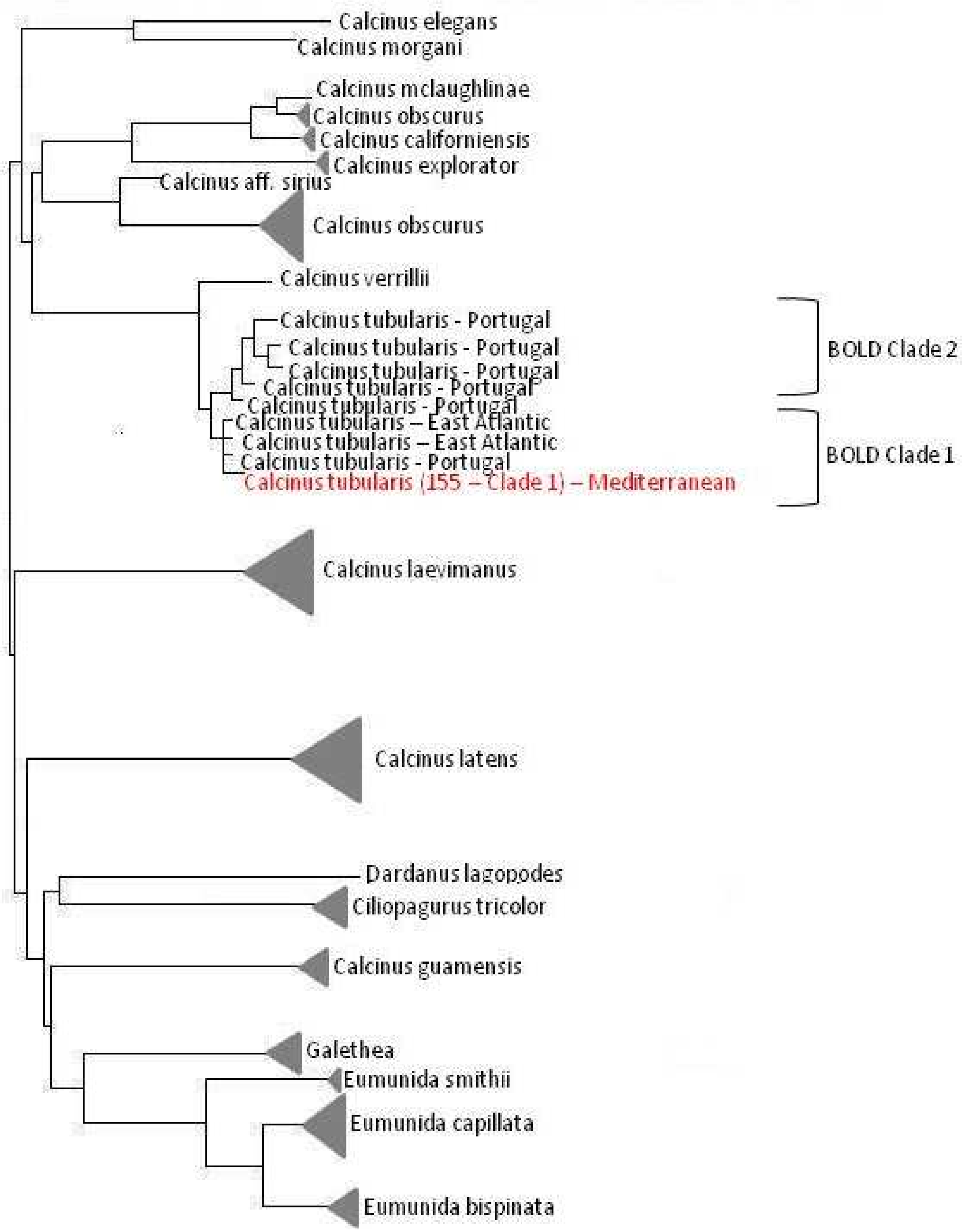

Figure 4.6. Neighbor-joining tree for our *Calcinus tubularis* – 155 (CO1) sequence (shown in red) constructed based on BOLD. (The size of the triangles are proportional to the number of sequences in BOLD for that species).

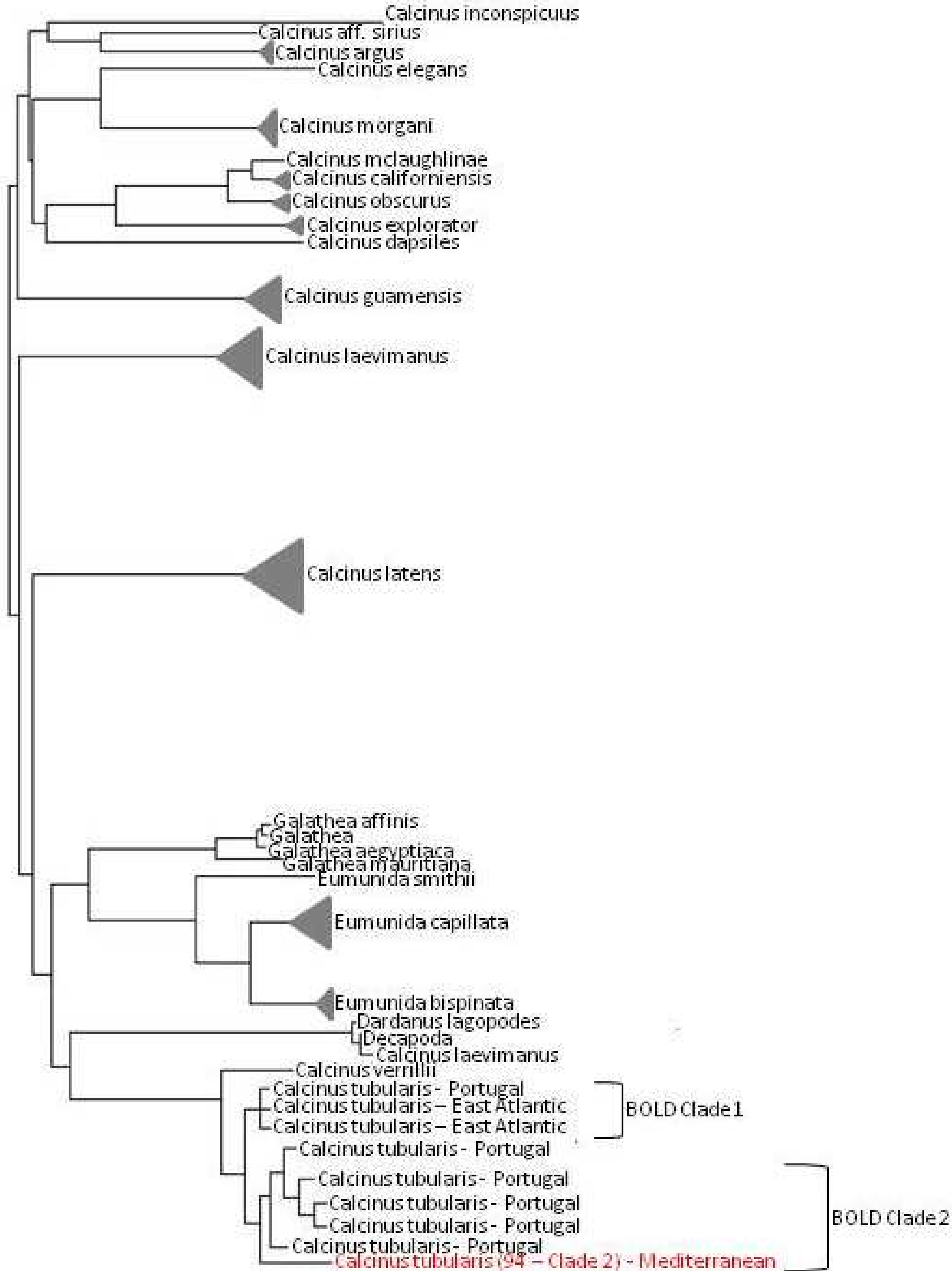

Figure 4.7. Neighbor-joining tree for our *Calcinus tubularis* – 94 (CO1) sequence (shown in red) constructed based on BOLD. (The size of the triangles is proportional to the number of sequences in BOLD for that species).

4.1.2. 16S Sequences

Four different samples from three different locations (East Atlantic, the Mediterranean Sea, Aegean Sea) were analyzed for this species. Two of the analyzed samples were taken from Genbank (C tubularis 16S FJ620233 and C tubularis 16S FJ620234)(Figure 4.8).

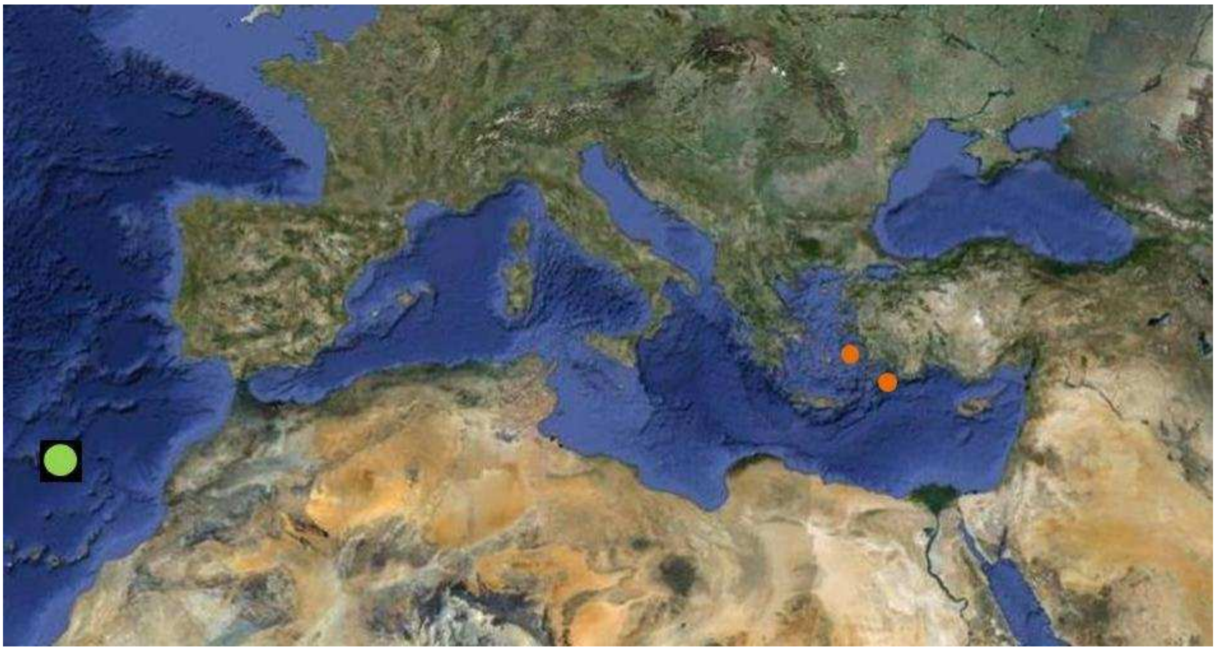

Figure 4.8. Sampling locations for *Calcinus tubularis* (16S). (The sizes of the circles are proportional the number of individuals. The orange circles indicates sampling locations for samples belonging to Clade 2 in the neighbor -joining tree, the green circle within a black square indicates sampling locations for sequences that were retrieved for BOLD, Clade 2 in the neighbor-joining tree).

The samples 99 and 133 were collected from the Aegean Sea and the Mediterranean Sea, respectively, and clustered into a different clade from the samples collected from East Atlantic. This pattern in 16S obtained from the neighbor-joining tree corresponds to the pattern from the neighbor-joining tree (Figure 4.4, above) for the CO1 region.

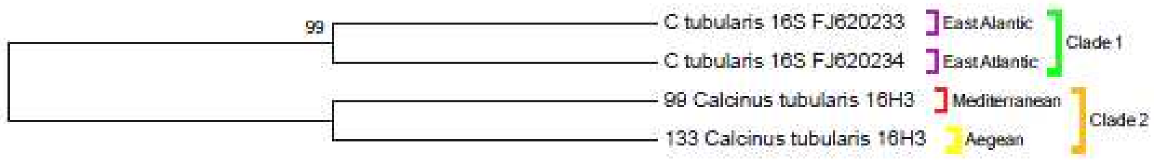

Figure 4.9. Neighbor-joining tree for *Calcinus tubularis* (16S).

Focusing on the haplotype network (Figure 4.10), there are two different haplotypes in the 16S region. Haplotype 16SH1 was found in the samples 99 and 133, which were differentiated from the haplotype 16SH2 by four base pairs. Haplotype 16SH2 was found in the samples collected from East Atlantic. These data also corresponded to those obtained from the haplotype network for the CO1 region (Figure 4.5). The haplotype network table for *Calcinus tubularis* (16S) is given in table 4.3.

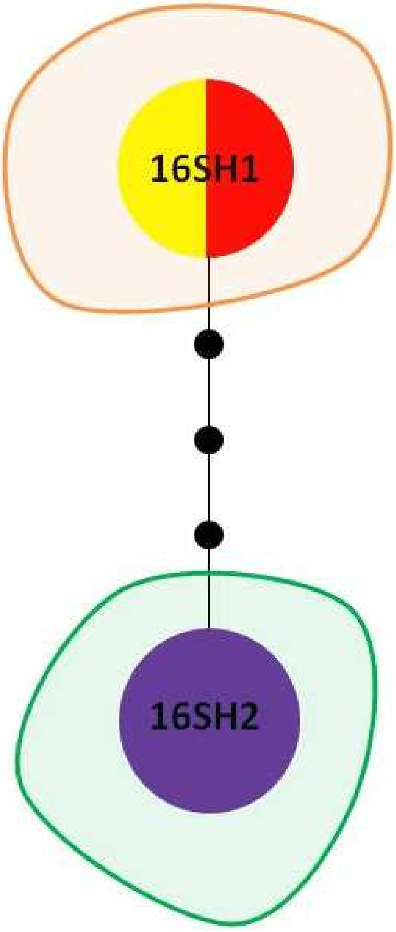

Figure 4.10. Haplotype network for *Calcinus tubularis* (16S). (The size of the circles is proportional to the number of individuals. See Table 3.1. for the key to the color-codes of the geographic positioning of the haplotypes).

Table 4.3. Haplotype network table for *Calcinus tubularis* (16S).

Haplotype	Number of individuals	Code for the individuals as shown in the neighbor-joining tree
16SH1	2	99, 133
16SH2	2	FJ620233, FJ620234

4.2. *Clibanarius erythropus* (Latreille, 1818)

4.2.1. CO1 Sequences

There are five different samples (four from the Aegean and one from the Sea of Marmara) analyzed for this species. CO1 sequence data were not publicly available in BOLD or GenBank for this species (Figure 4.11).

Figure 4.11. Sampling locations for *Clibanarius erythropus* (CO1). (The circles are proportional the number of individuals. The green color indicates sampling locations for samples 120, 121, 142, and 144, which belong to Clade 1, the orange color indicates sampling location for sample 123, which belongs to Clade 2 in the neighbor-joining tree).

The neighbor-joining tree showed two clades (Figure 4.12). The sample 120 was collected from the Sea of Marmara, and the rest of the samples were collected from the Aegean. Sample 120 clustered with the samples 121, 144 and 142, whereas sample 123 was found on a different branch.

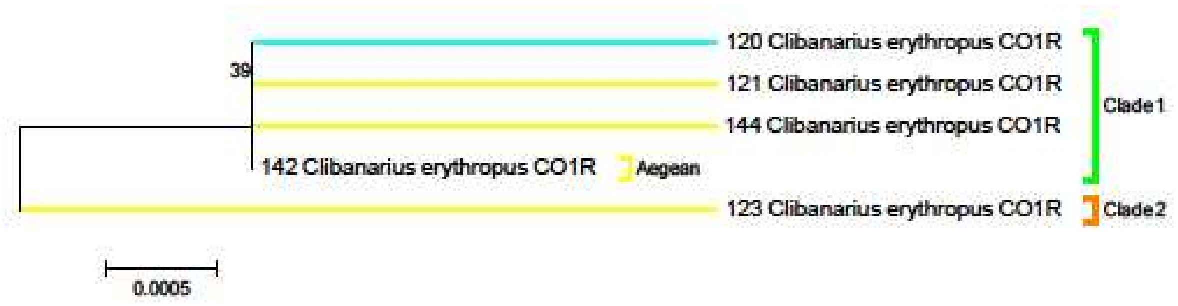

Figure 4.12. Neighbor-joining tree for *Clibanarius erythropus* (CO1).

There are five different haplotypes in *Clibanarius erythropus*, a different haplotype in each individual. Haplotype CH1 was found in the sample 144, CH2 in 142, CH3 in 123, CH4 in 121, and CH5 in the sample 120. The genetic differentiation was very low in the *Clibanarius erythropus* haplotype network (Figure 4.13), with the most divergent haplotypes, CH3 and CH4, differentiated by five base pairs. The haplotype network table for *Clibanarius erythropus* (CO1) is given in table 4.4.

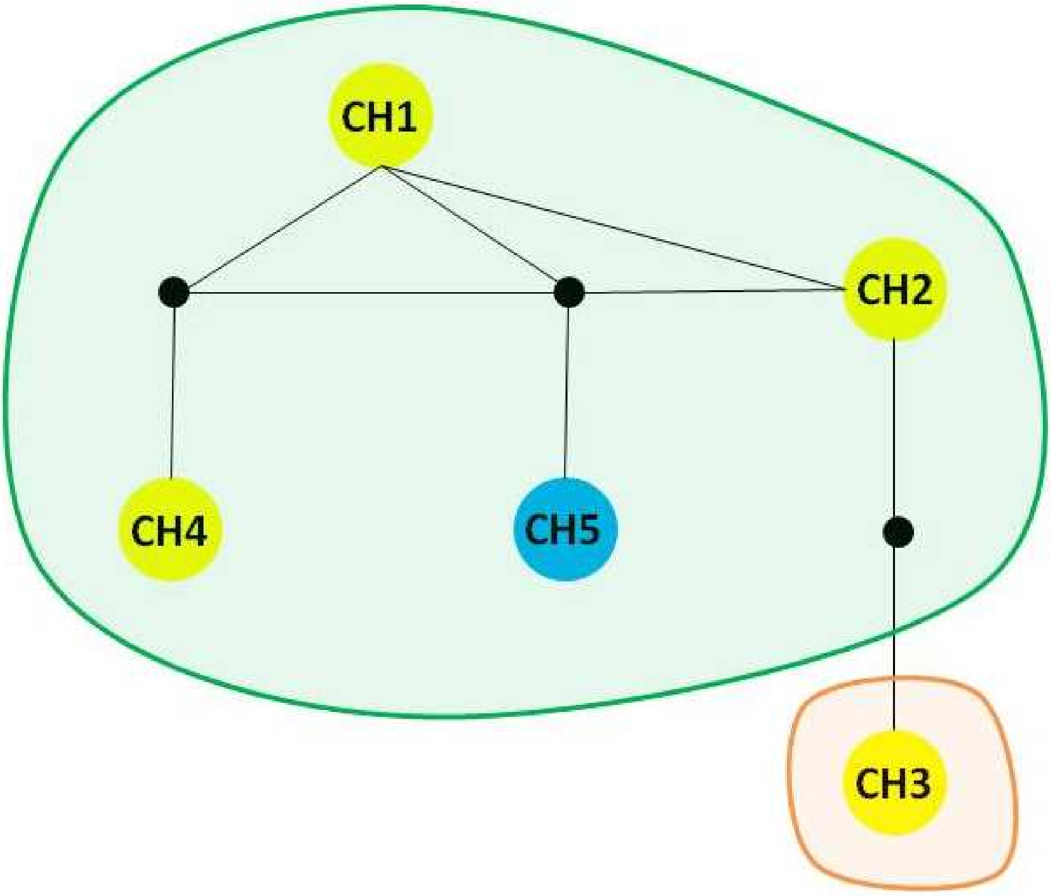

Figure 4.13. Haplotype network for *Clibanarius erythropus* (CO1). (The size of the circles is proportional to the number of individuals. See Table 3.1. for the key to the color-codes of the geographic positioning of the haplotypes).

Table 4.4. Haplotype network table for *Clibanarius erythropus* (CO1).

Haplotype	Number of individuals	Code for the individuals as shown in the neighbor-joining tree
CH1	1	144
CH2	1	142
CH3	1	123
CH4	1	121
CH5	1	120

Comparing a representative individual from clade 1 (144) with BOLD, shows the first, second and third closest matches to *Clibanarius erythropus* with 99.79%, 99.58% and 99.37% similarity, respectively. As mentioned above, the sample 123 was seen as a different lineage in the neighbor-joining tree. Comparing with the BOLD database, the first, second and third closest match to sample 123 was *Clibanarius erythropus* with 99.67%, 99.27% and 99.18% similarity, respectively.

The neighbor-joining tree for 144 (clade 1) constructed via BOLD is shown in figure 4.14. The sample that is barcoded from Turkey (shown in red), fell into the same clade with the *Clibanarius erythropus* CO1 sequences from BOLD, for which locality information were not available. The neighbor-joining tree for 123 (clade 2) constructed via BOLD is shown in figure 4.15. Sample 123 also clustered closely into the same clade with other *Clibanarius erythropus* CO1 sequences from BOLD. Our *Clibanarius erythropus* sample (144 – Clade 1) formed a basal branch to all of the four other *Clibanarius erythropus* sequences that were in BOLD Clade 2. On the other hand *Clibanarius erythropus* (123 – Clade 2) was also in BOLD Clade 2, but instead of being on a basal branch, it was sister to one of the *Clibanarius erythropus* sequences.

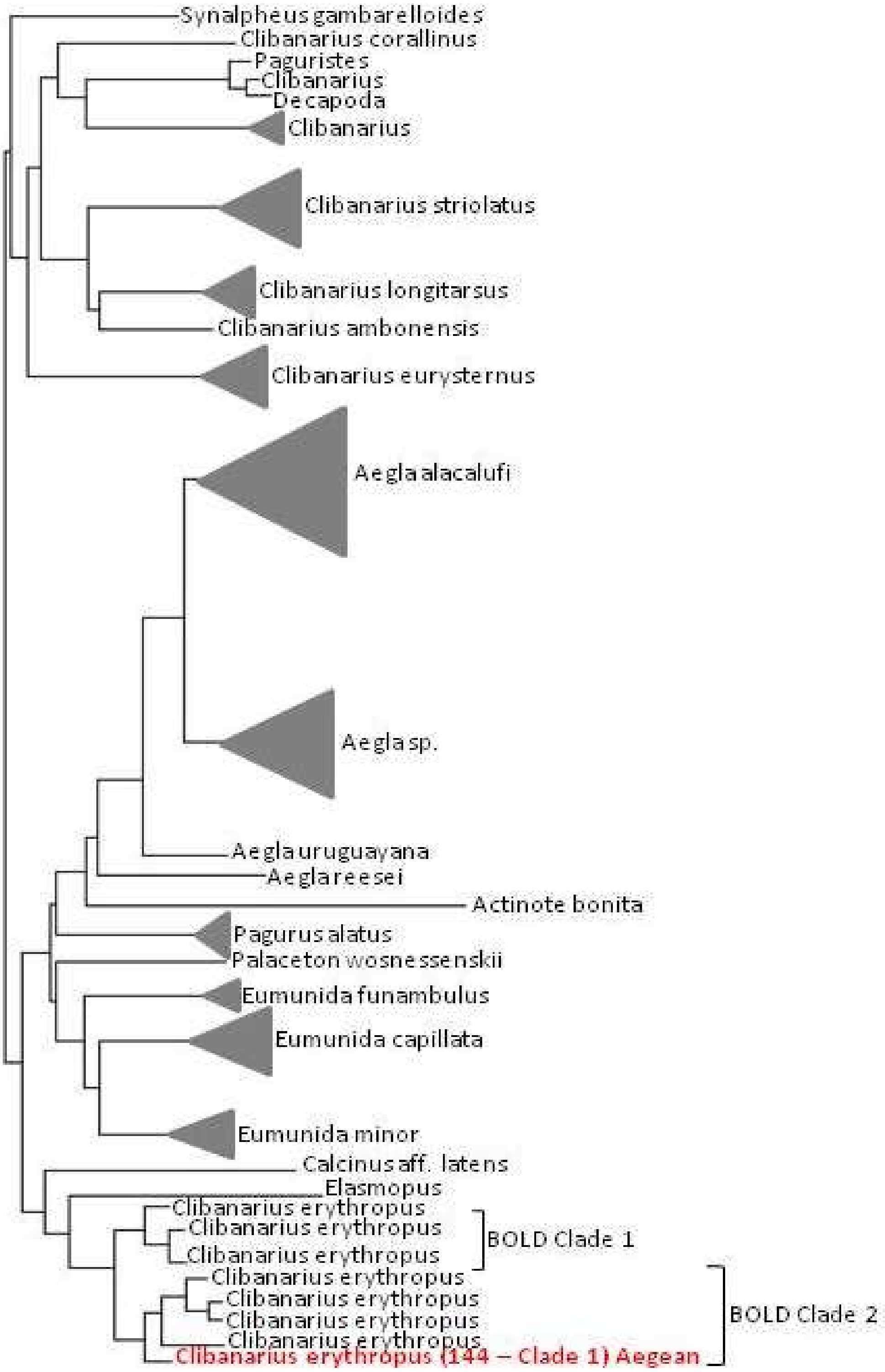

Figure 4.14. Neighbor-joining tree for our *Clibanarius erythropus* – 144 (CO1) sequence (shown in red) constructed based on BOLD. (The size of the triangles is proportional to the number of sequences in BOLD for that species).

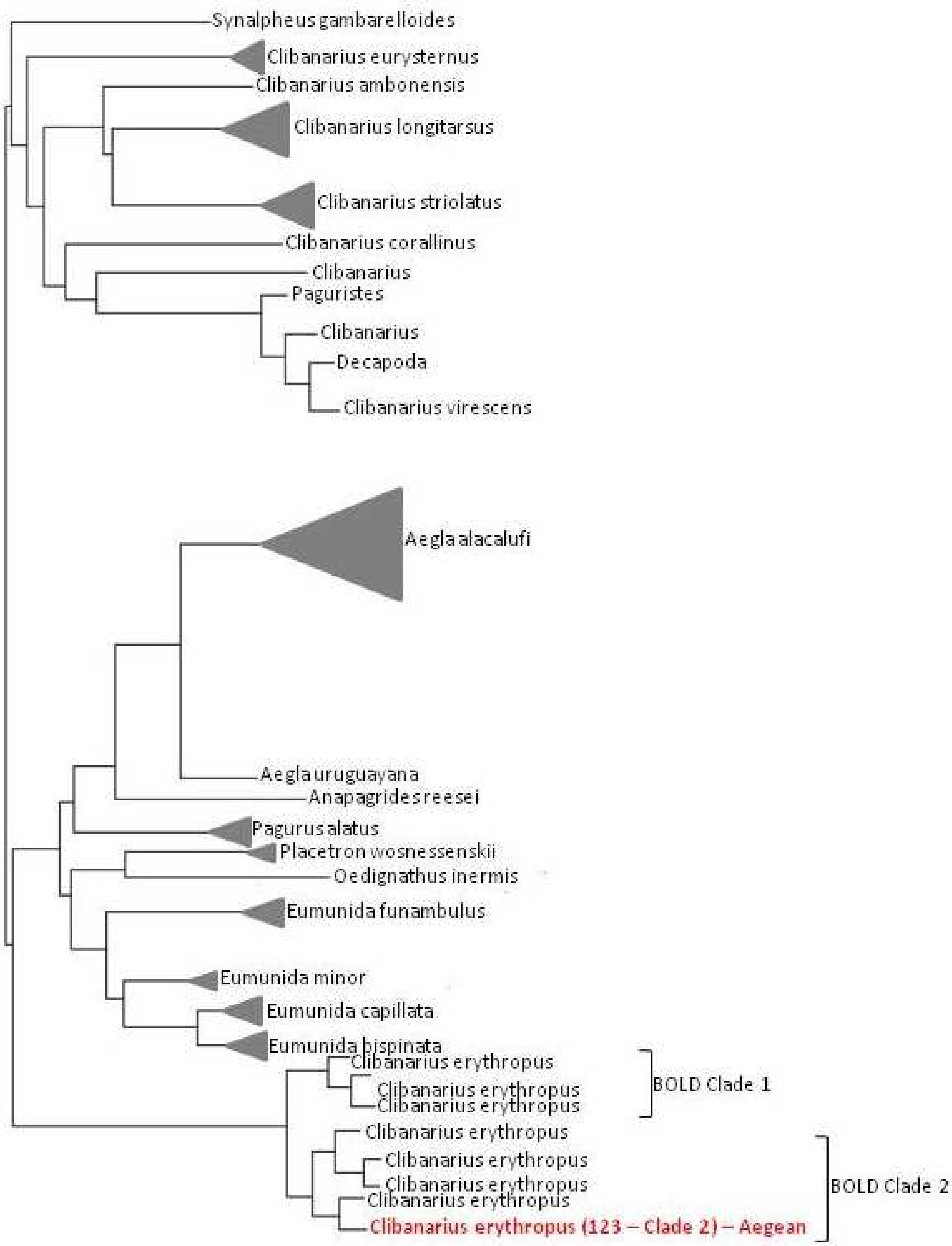

Figure 4.15. Neighbor-joining tree for our *Clibanarius erythropus* – 123 (CO1) sequence (shown in red) constructed based on BOLD. (The size of the triangles is proportional to the number of sequences in BOLD for that species).

4.2.2. 16S Sequences

Data was obtained for two individuals for the 16S region for this species. All of the samples were collected from the Aegean (one from the Saros Bay and the other one from the Çanakkale Strait) (Figure 4.16).

Figure 4.16. Sampling locations for *Clibanarius erythropus* (16S). (The sizes of the circles are proportional the number of individuals. The orange circle indicates sampling locations for sample 140 and the green indicates sampling location for sample 121).

The neighbor-joining tree could not be prepared in this case because a minimum of four samples are required to prepare a neighbor-joining tree. There were two different haplotypes in *Clibanarius erythropus* in the haplotype network for the 16S region (Figure 4.17). Each haplotype was seen in one individual only. Haplotype 16SH1 was found in the sample 140, and 16SH2 was seen in the sample 121. All haplotypes were observed in the Aegean Sea. Haplotype 1 and 2 were differentiated from each other by two base pairs. For sample 121, we sequenced both CO1 region and 16S region, but for sample 140 we only had 16S region sequence, therefore a comparison for CO1 region and 16S region sequences could not be done for this species. The haplotype network table for *Clibanarius erythropus* (16S) is given in table 4.5.

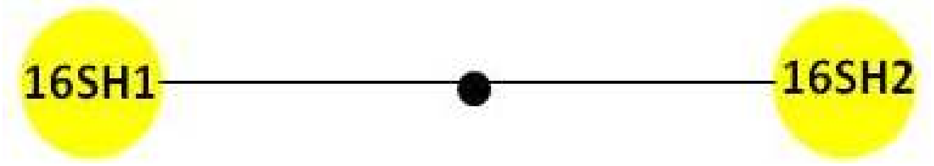

Figure 4.17. Haplotype network for *Clibanarius erythropus* (16S). (The size of circles is proportional to the number of individuals. See Table 3.1. for the key to the color-codes of the geographic positioning of the haplotypes).

Table 4.5. Haplotype nework table for *Clibanarius erythropus* (16S)

Haplotype	Number of individuals	Code for the individuals as shown in the haplotype network
16SH1	1	140
16SH2	1	121

4.3. *Pagurus anachoretus* (Risso, 1826)

4.3.1. CO1 Sequences

Nine different individuals were sequenced for this analysis. Three of the samples (124, 125, 148) were collected from the Aegean (Saros Bay) and six of the samples were collected from the Mediterranean (165, 167 and 164 from Antakya, 153, 154, 156 from Kas). CO1 sequence data were not available in BOLD or GenBank for this species (Figure 4.18).

Figure 4.18. Sampling locations for *Pagurus anachoretus* (CO1). (The size of the circles is proportional the number of individuals from a particular location. The orange color indicates sampling locations for individuals from Clade 2 in the neighbor-joining tree, and the green color indicates sampling locations for individuals from Clade 1).

The neighbor-joining tree analysis showed two clades. The samples with the codes 148, 124 and 125 which were collected from the Aegean Sea, clustered with the samples coded 167, 165, 153 and 164 from the Mediterranean, and the samples with the codes 154 and 156 from Mediterranean clustered together in a different clade (Figure 4.19).

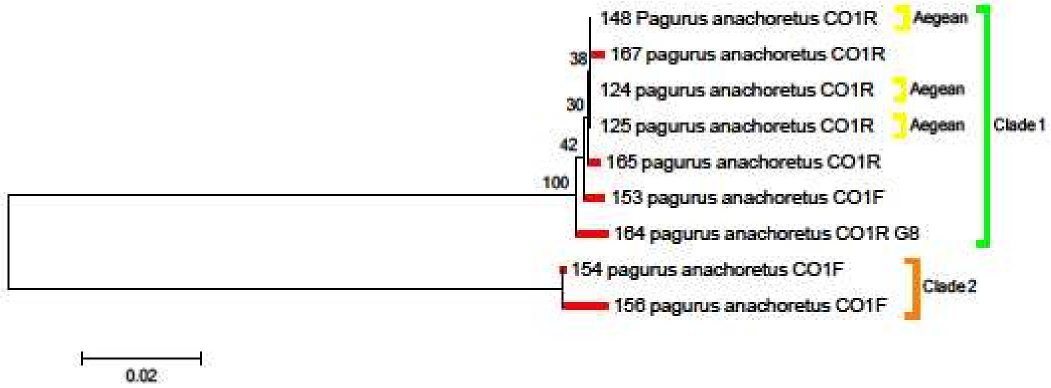

Figure 4.19. Neighbor-joining tree for *Pagurus anachoretus* (CO1).

The haplotype network showed seven different haplotypes for *Pagurus anachoretus* for the CO1 region (Figure 4.20). Haplotype CH1 was seen in the samples 124, 125 and 148, and this haplotype was only observed in the Aegean Sea. The other haplotypes (CH2, CH3, CH4, CH5, CH6 and CH7) were seen in one individual each and were observed in Mediterranean. Haplotypes CH2, CH3, CH4, CH5, CH6 and CH7 were seen in individuals 153, 165, 164, 167, 154 and 156, respectively. In the network two clusters were observed, one consisting of CH1, CH2, CH3, CH4 and CH5, and the other consisting of CH6 and CH7 and the genetic differentiation between these two clusters was very high (94 base pairs). The haplotype network table for *Pagurus anachoretus* (CO1) is given in table 4.6.

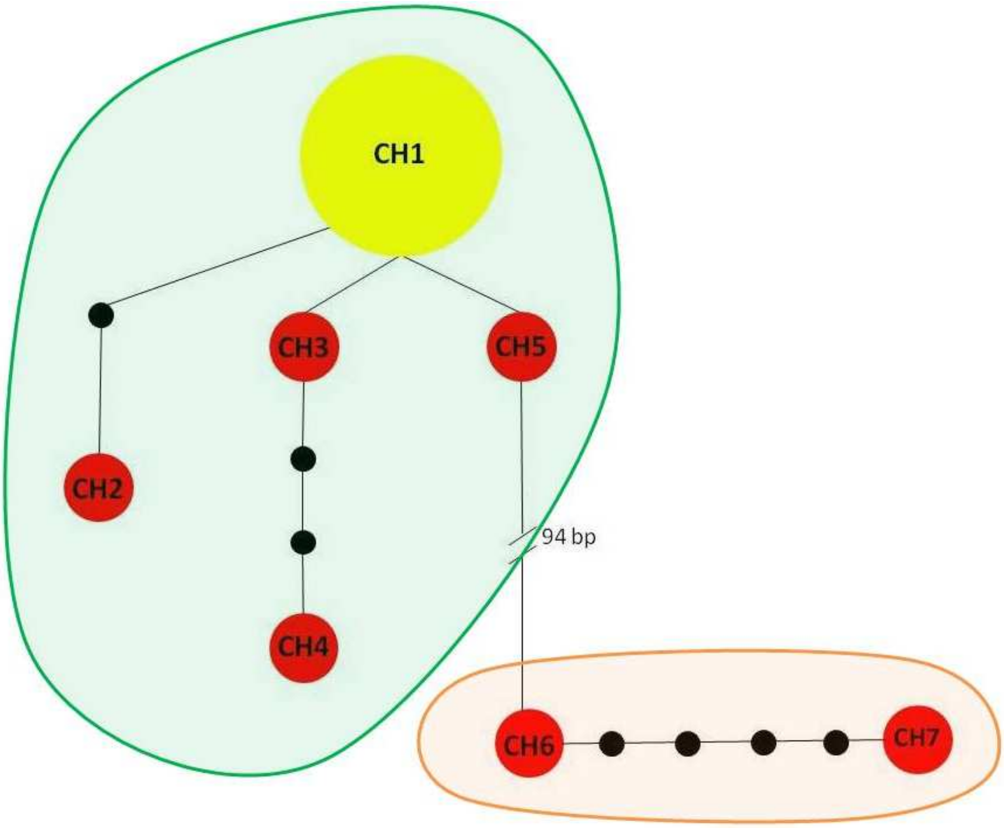

Figure 4.20. Haplotype network for *Pagurus anachoretus* (CO1). (The size of circles is proportional to the number of individuals. See Table 3.1. for the key to the color-codes of the geographic positioning of the haplotypes).

Table 4.6. The haplotype network table for *Pagurus anachoretus* (CO1).

Haplotype	Number of individuals	Code for the individuals as shown in the neighbor-joining tree
CH1	3	124, 125, 148
CH2	1	153
CH3	1	165
CH4	1	164
CH5	1	167
CH6	1	154
CH7	1	156

A comparison with BOLD of a representative individual from Clade 1 (124) showed first and second closest matches to *Pagurus cuanensis* with 85.24% and 85.07% similarity, respectively. A comparison with BOLD of a representative individual from Clade 2 (154) showed first closest match to *Munida taenia* with 84.82% similarity, and second closest matches to *Panamunida luminata* and *Munida roshanei* with 84.51% similarity. These results indicated that our sequences are the first DNA barcodes in BOLD for this species. The neighbor-joining tree for 124 (clade 1) constructed *via* BOLD is shown in figure 4.21. The sample that was barcoded from Turkey (written in red) fell within a complex clade with species from two other genera; *Darnaudus petterdi* and *Nematopagurus gardineri*. The neighbor-joining tree for 154 (clade 2) constructed *via* BOLD is shown in figure 4.22. The sample barcoded from Turkey (written in red) fell within the *Pagurus* clade and on a sister branch to *Pagurus pollicarsis*. As seen in figure 4.21 and figure 4.22, species in the genus *Pagurus* clustered with different species, therefore genus *Pagurus* does not seem to form a monophyletic clade.

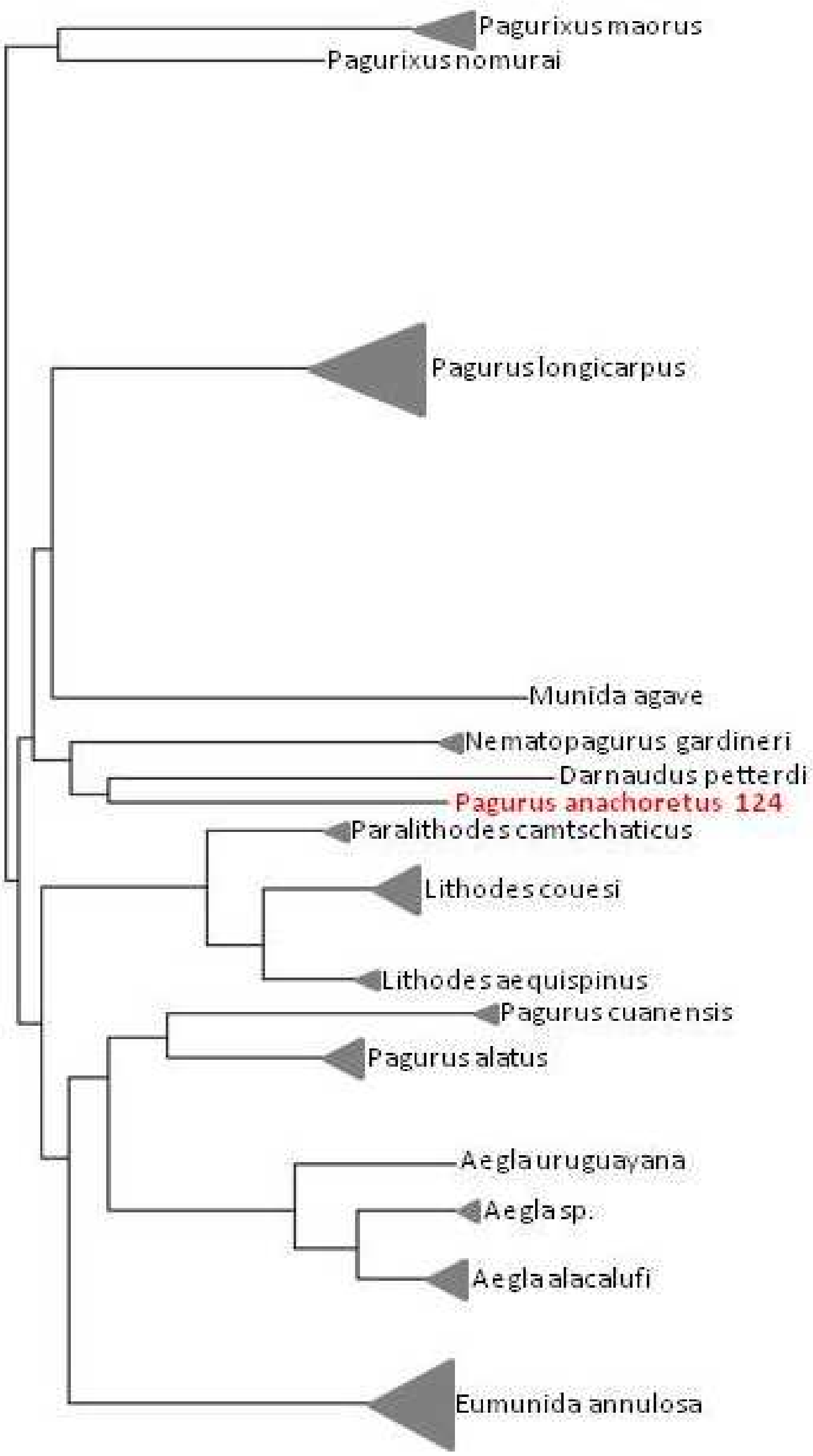

Figure 4.21. Neighbor-joining tree for our *Pagurus anachoretus* – 124 (CO1) sequence (shown in red) constructed based on BOLD. (The size of the triangles is proportional to the number of sequences in BOLD for that species).

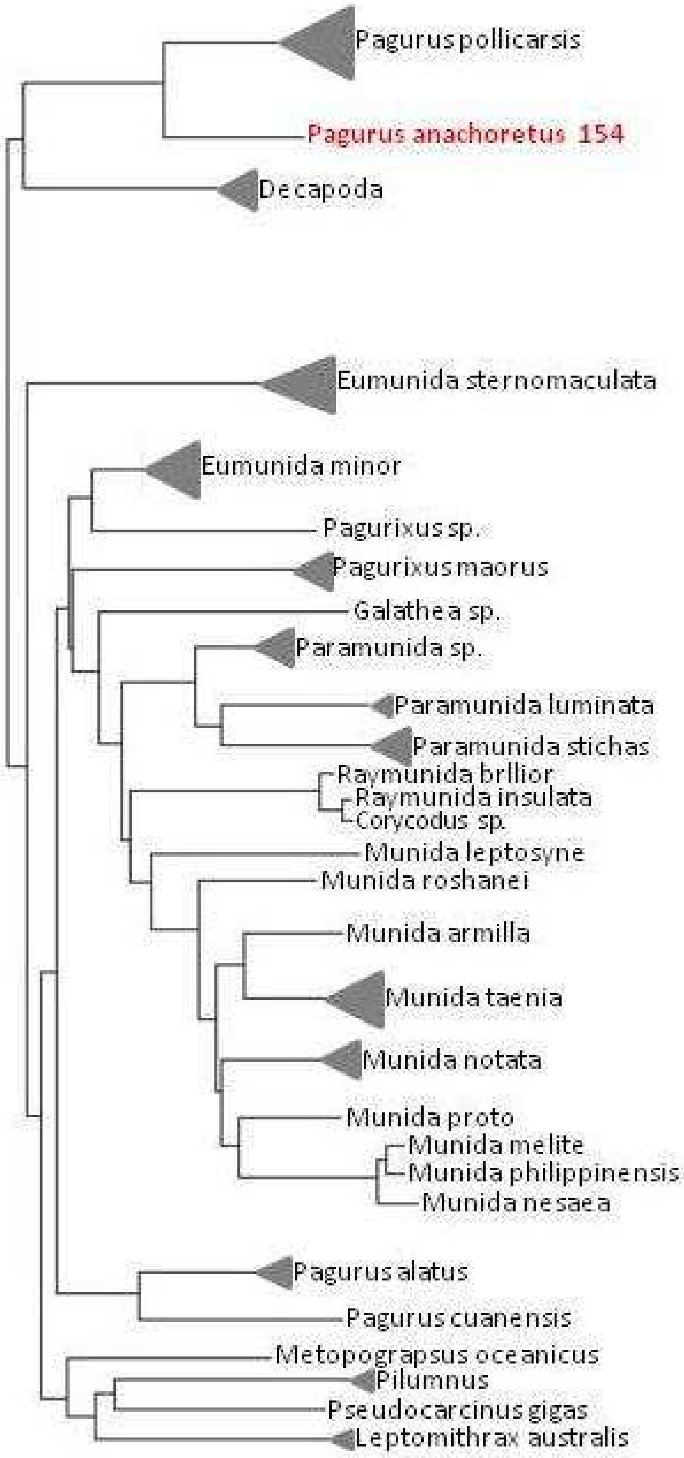

Figure 4.22. Neighbor-joining tree for our *Pagurus anachoretus* – 154 (CO1) sequence (shown in red) constructed based on BOLD. (The size of the triangles is proportional to the number of sequences in BOLD for that species).

4.3.2. 16S Sequences

16S region for two samples from one location (Saros Bay, the Aegean) was sequenced for this analysis (Figure 4.23).

Figure 4.23. The sampling location for *Pagurus anachoretus* (16S). (The pink circle indicates sampling location, Saros Bay - the Aegean, that the samples were collected from for this species).

An intraspecific tree could not be prepared as there were only two samples for this species. There was one haplotype (16SH1) in the haplotype network. Samples 124 and 125 also shared the same haplotypes for CO1 region. In that respect, the data from 16S region haplotype network corresponded to that from CO1. The haplotype network table for *Pagurus anachoretus* (16S) is given in table 4.7.

Figure 4.24. Haplotype network for *Pagurus anachoretus* (16S). (See Table 3.1. for the key to the color-codes of the geographic positioning of the haplotypes).

Table 4.7. Haplotype network table for *Pagurus anachoretus* (16S).

Haplotype	Number of individuals	Code for the individuals as shown in the haplotype network
16H1	2	124, 125

4.4. *Pagurus cuanensis* (Risso, 1826)

Two CO1 sequences were analyzed for *Pagurus cuanensis* and the samples were collected from the Aegean (Saros Bay) (Figure 4.25).

Figure 4.25. The sampling location for *Pagurus cuanensis* (CO1). (The brown circle indicates sampling location in Saros Bay, the Aegean that the samples were collected from for this species).

CO1 sequence data were not available in BOLD or GenBank for this species. An intraspecific tree also could not be prepared as there were only two samples for this species. The haplotype CH1 was found in the sample 146 and CH2 was found in 147 (Figure 4.26). The haplotype CH1 differentiates from CH2 by two base pairs. The haplotype network table for *Pagurus cuanensis* is given in table 4.8.

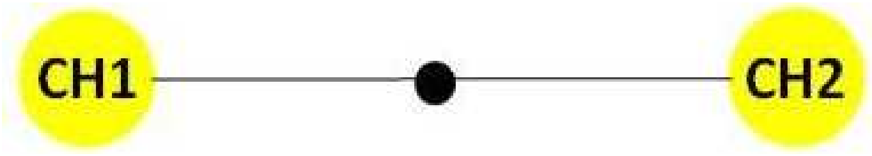

Figure 4.26. The haplotype network *Pagurus cuanensis* (CO1). (The size of the circles is proportional to the number of individuals. See Table 3.1. for the key to the color-codes of the geographic positioning of the haplotypes).

Table 4.8. Haplotype network table for *Pagurus cuanensis* (CO1).

Haplotype	Number of individuals	Code for the individuals as shown in the haplotype network
CH1	1	146
CH2	1	147

A comparison with BOLD showed that the first closest match with sample 146 was *Paramunida stichas* with 87.34% similarity, second closest match was *Munida proto* with 87.21% similarity and third closest match was *Pagurixus maorus* with 87.21% similarity. The other comparison with BOLD showed that the first closest match with sample 147 was *Munida proto* with 87.10% similarity, the second closest match was *Pagurixus maorus* with 86.83% similarity and the third closest match was to *Metopagurus oceanicus* with 86.38% similarity. The neighbor-joining trees for 146 (Haplotype 1) and 147 (Haplotype 2) constructed *via* BOLD is shown in Figure 4.27 and Figure 4.28, respectively. The sample 146 fell within a clade which was composed of *genus Pilumnus* and *Leptomithrax garricki*. In addition, sample 147, like sample 146, fell within a clade which was composed of a species in the genus *Dolichopodidae* and *Rheocricolopus effuses*.

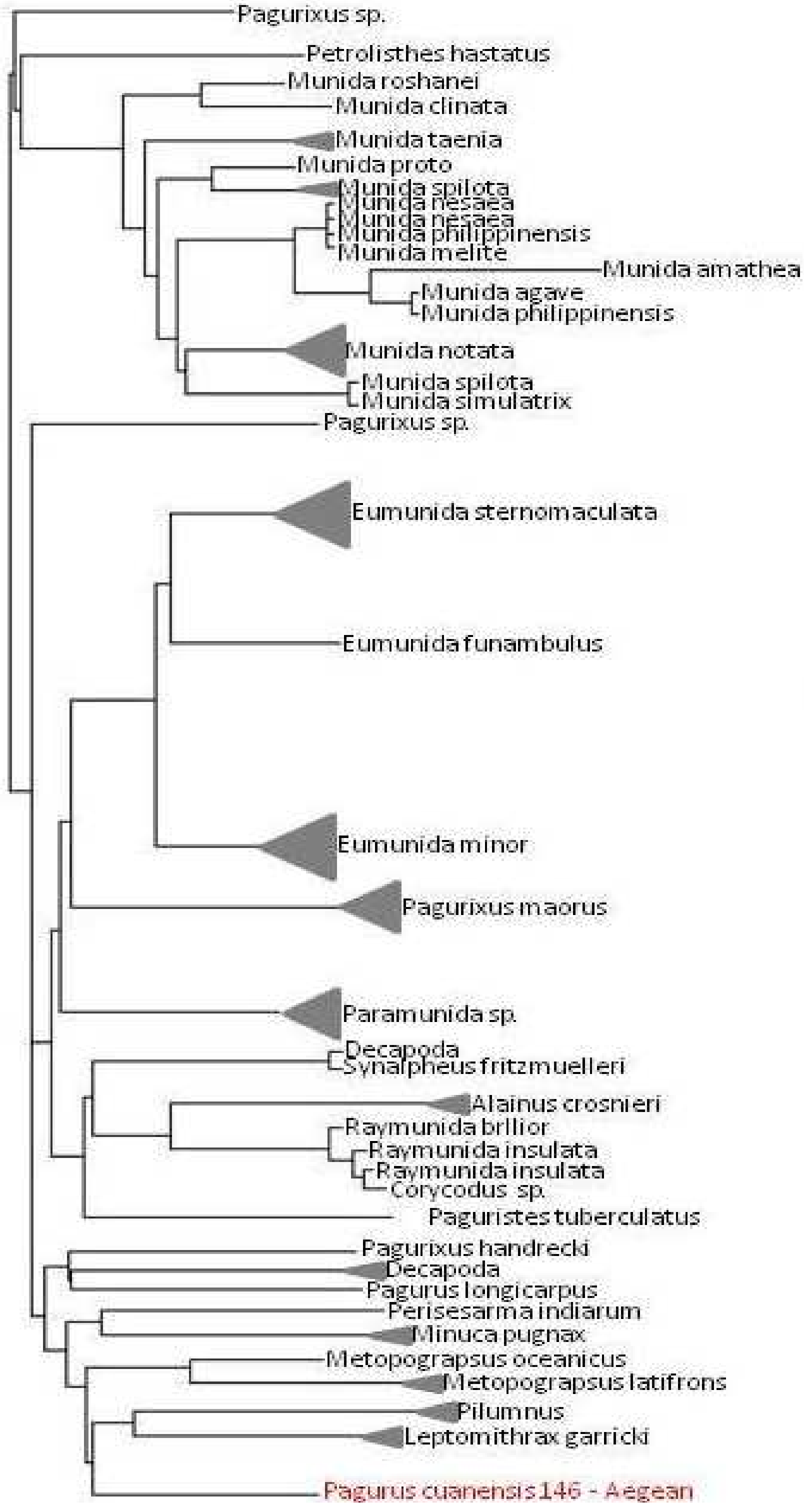

Figure 4.27. Neighbor-joining tree for our *Pagurus cuanensis* – 146 (CO1) sequence (shown in red) constructed based on BOLD. (The size of the triangles is proportional to the number of sequences in BOLD for that species).

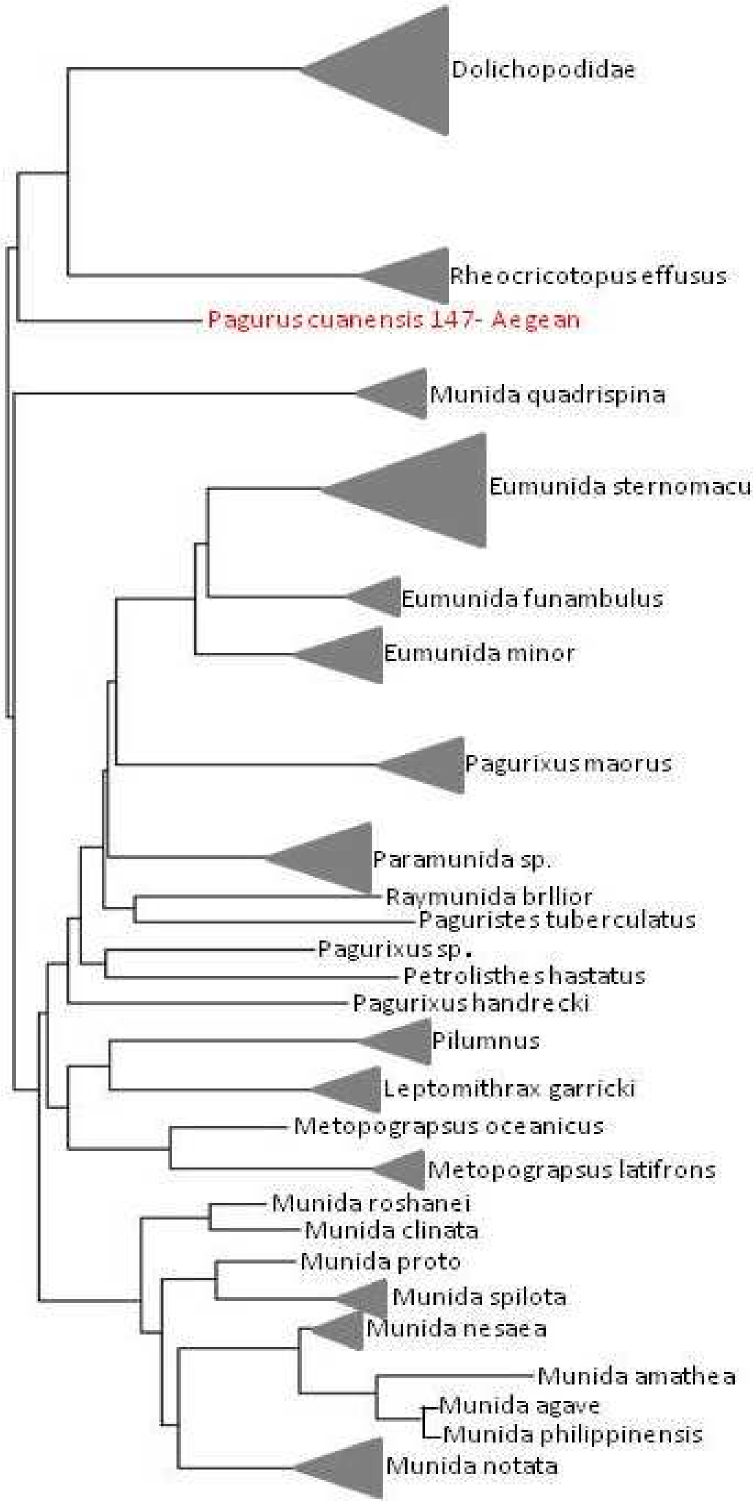

Figure 4.28. Neighbor-joining tree for our *Pagurus cuanensis* – 147 (CO1) sequence (shown in red) constructed based on BOLD. (The size of the triangles is proportional to the number of sequences in BOLD for that species).

4.5 *Diogenes pugilator* (Roux, 1829):

4.5.1. CO1 Sequences:

18 different samples from two different locations (16 from the Sea of Marmara and two from the Mediterranean) were collected and sequenced for this species. CO1 sequence data were not available in BOLD or GenBank for this species (Figure 4.29).

Figure 4.29. Sampling locations for *Pagurus anachoretus* (CO1). (The size of the circles is proportional the number of individuals. The orange color indicates sampling locations for samples from Clade 2 in the neigbor-joining tree, and the green color indicates sampling locations for samples from Clade 1).

The neighbor-joining tree for *Diogenes pugilator* CO1 region is shown in figure 4.30. All CO1 sequences generated two clades. All barcoded individuals from the Sea of Marmara (100, 101, 102, 103, 104, 105, 108, 110, 111, 113, 114, 116, 117, 118, 119) and the sample 171 from the Meditarranean formed one clade, whereas the sample 170 from the Meditarranean fell within a different branch.

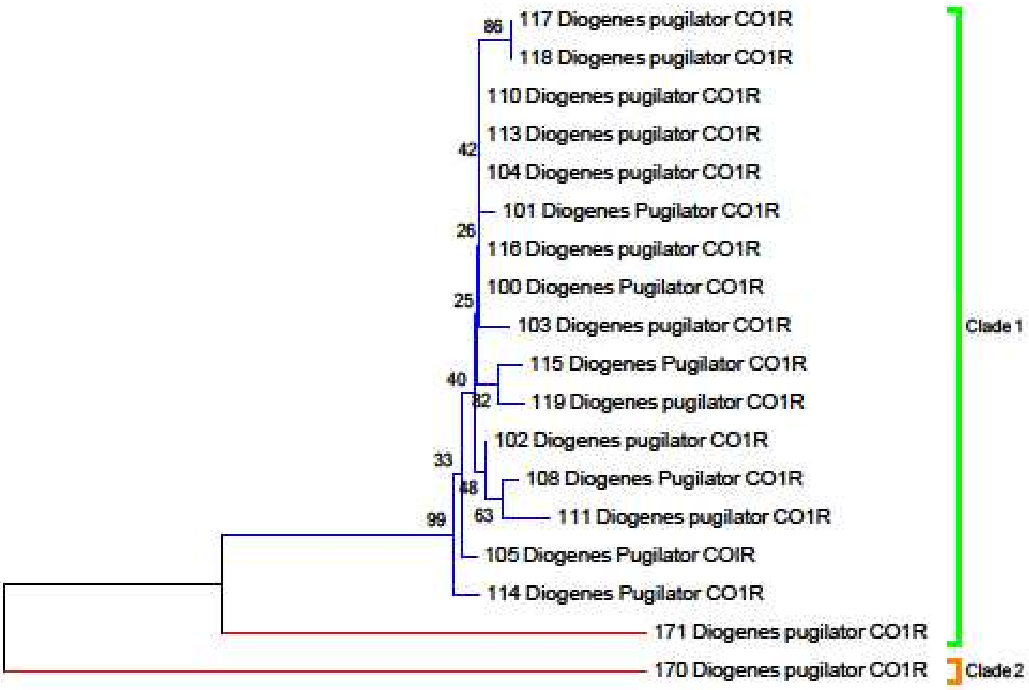

Figure 4.30. Neighbor-joining tree for *Diogenes pugilator* (CO1).

The haplotype network (Figure 4.31) showed 13 different haplotypes for *Diogenes pugilator* in the CO1 region. Haplotype CH1 was the most common haplotype found in 18 individuals. CH1 was seen in the samples 100, 104, 110, 113, 116 and this haplotype was only observed in the Sea of Marmara. Haplotype CH5 was the second most common haplotype found in 18 individuals and it was also observed only in the Sea of Marmara. CH5 was seen in the samples 117 and 118. Each haplotype except CH1 and CH5 were seen in one individual only. Haplotypes CH2, CH3, CH4, CH6, CH7, CH8, CH9, CH10, CH11, CH12 and CH13 were seen in the individuals 105, 119, 115, 114, 103, 111, 108, 101, 102, 171 and 170, respectively. In the haplotype network, the samples from the Sea of Marmara clustered together as seen previously in the neighbor-joining tree (Figure 4.30); also the genetic differentiation was very low within the samples from the Sea of Marmara. Again in a similar manner, the haplotypes CH12 and CH13 were differentiated from the other haplotypes from the Sea of Marmara by, 48 and 60 bp, respectively.These two results show that the haplotype network and the neighbor-joining tree corresponds each other. The haplotype network table for *Diogenes pugilator* is given in the table 4.9.

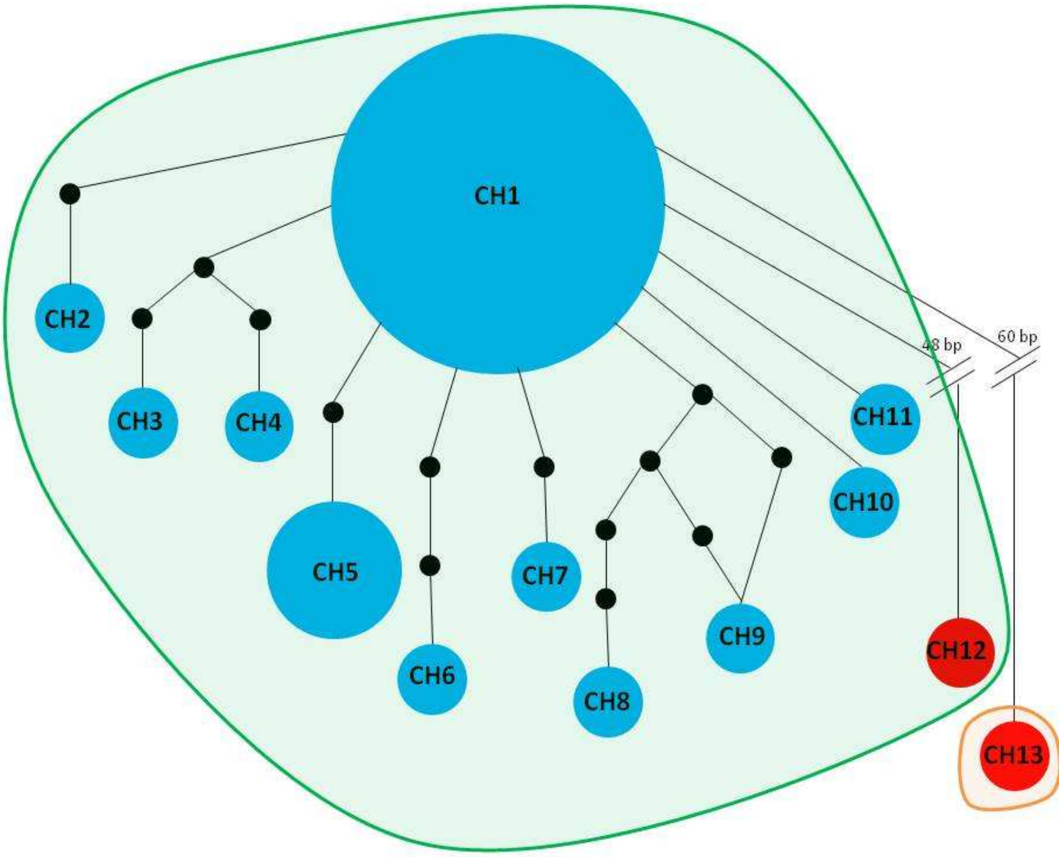

Figure 4.31. Haplotype network for *Diogenes pugilator* (CO1). (The sizes the of circles are proportional to the number of individuals. See Table 3.1. for the key to the color-codes of the geographic positioning of the haplotypes).

Table 4.9. Haploy
pe network table for *Diogenes pugilator* (CO1).

Haplotype	Number of individuals	Code for the individuals as shown in the neighbor-joining tree
CH1	5	100, 104, 110, 113, 116
CH2	1	105
CH3	1	119
CH4	1	115
CH5	2	117,118
CH6	1	114
CH7	1	103
CH8	1	111
CH9	1	108
CH10	1	101
CH11	1	102
CH12	1	171
CH13	1	170

A comparison with BOLD of a representative individual from Clade 1 (116) showed first closest match to *Diogenes pugilator* with 84.75% similarity, and the second and third closest matches to *Diogenes spinicarpus* with 83.42% and 83.25% similarity, respectively. Another comparison with BOLD of sample 171 showed first closest mach to *Diogenes pugilator* with 84.95% similarity, second closest match to *Anapagurides reesei* with 84.28% similarity, and third closest match to genus *Pagurixus* with 84.27% similarity. The third comparison with BOLD of sample 170 showed first closest match to *Pagurus cuanensis* with 84.95% similarity, and second and third closest matches to *Pagurus longicarpus* with 84.86% and 84.77% similarity, respectively.

The neighbor-joining trees for 116 (clade 1), 170 (clade 2) and 171 (clade 1) constructed *via* BOLD are shown in Figure 4.32, Figure 4.33 and Figure 4.34, respectively. In the intraspecific tree, the sample 171 clustered with the samples from the Sea of Marmara despite the 48 bp difference. On the other hand the sample 170 formed an individual branch with a 60 bp difference. The samples 116 and 171 fell within a *Diogenes* genus clade and as a sister species to *Diogenes pugilator* in the BOLD tree, however the sample 170 did not fall within a *Diogenes* clade.

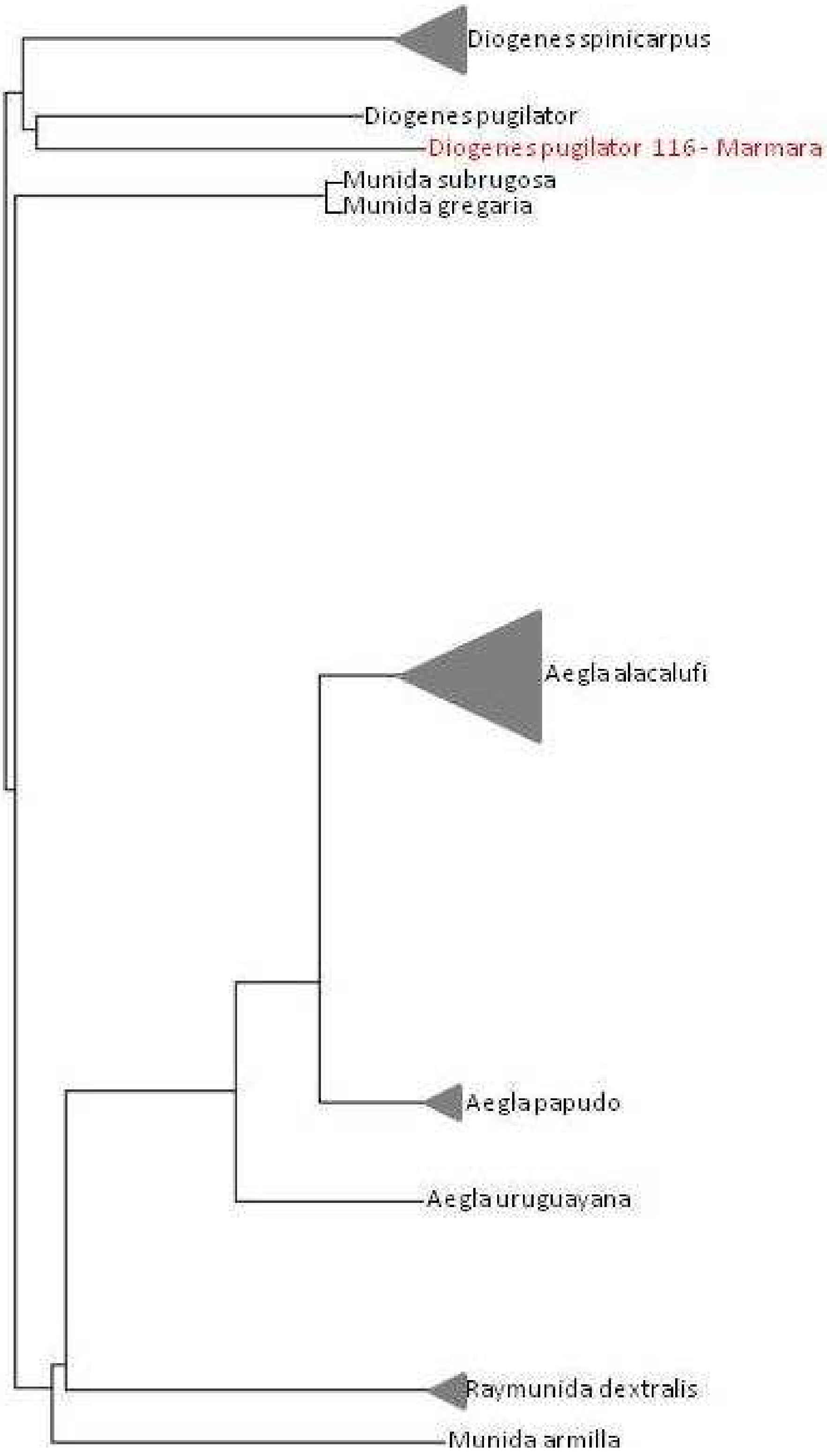

Figure 4.32. Neighbor-joining tree for our *Diogenes pugilator* – 116 (CO1) sequence (shown in red) constructed based on BOLD. (The size of the triangles is proportional to the number of sequences in BOLD for that species).

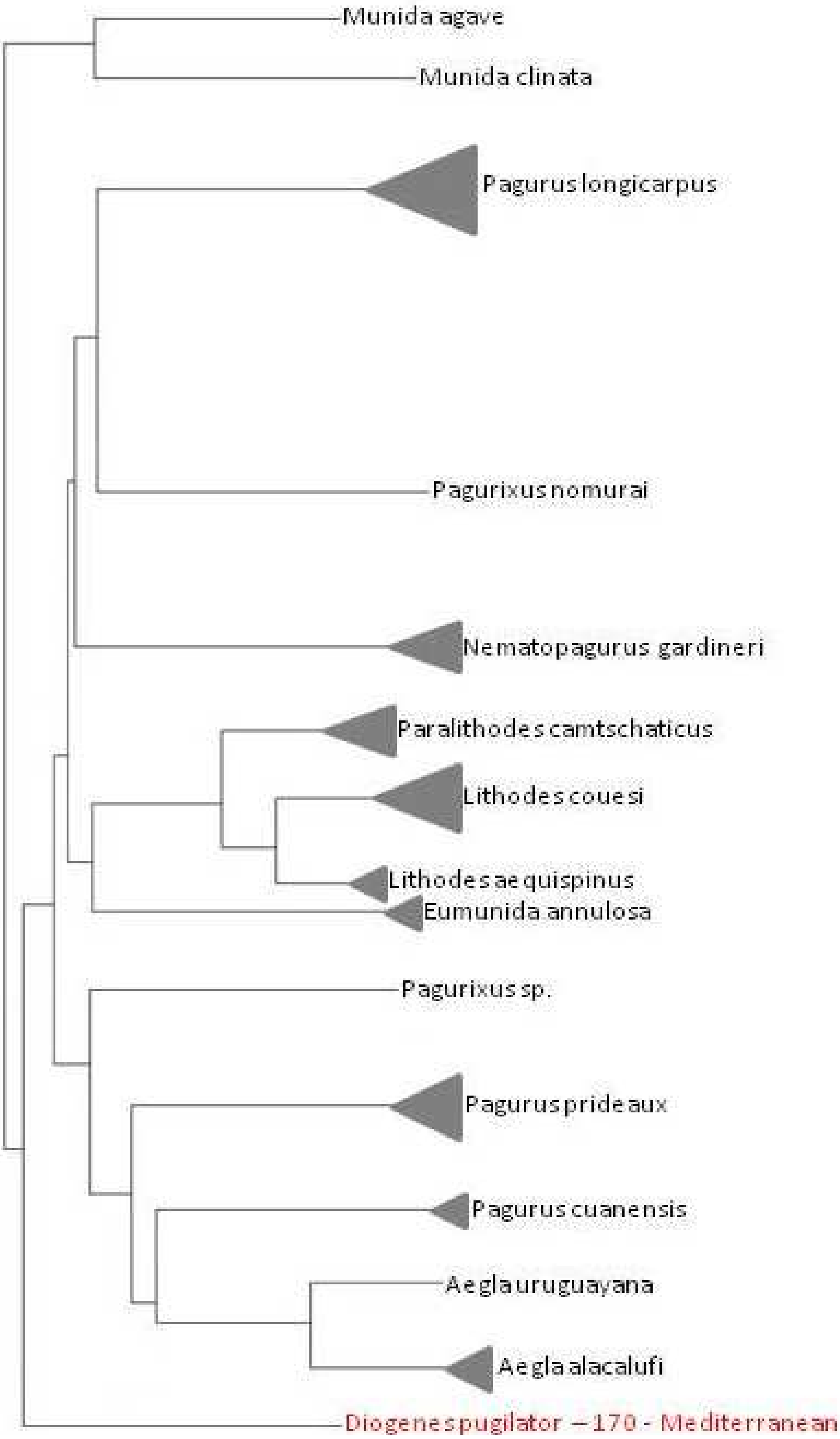

Figure 4.33. Neighbor-joining tree for our *Diogenes pugilator* – 170 (CO1) sequence (shown in red) constructed based on BOLD. (The size of the triangles is proportional to the number of sequences in BOLD for that species).

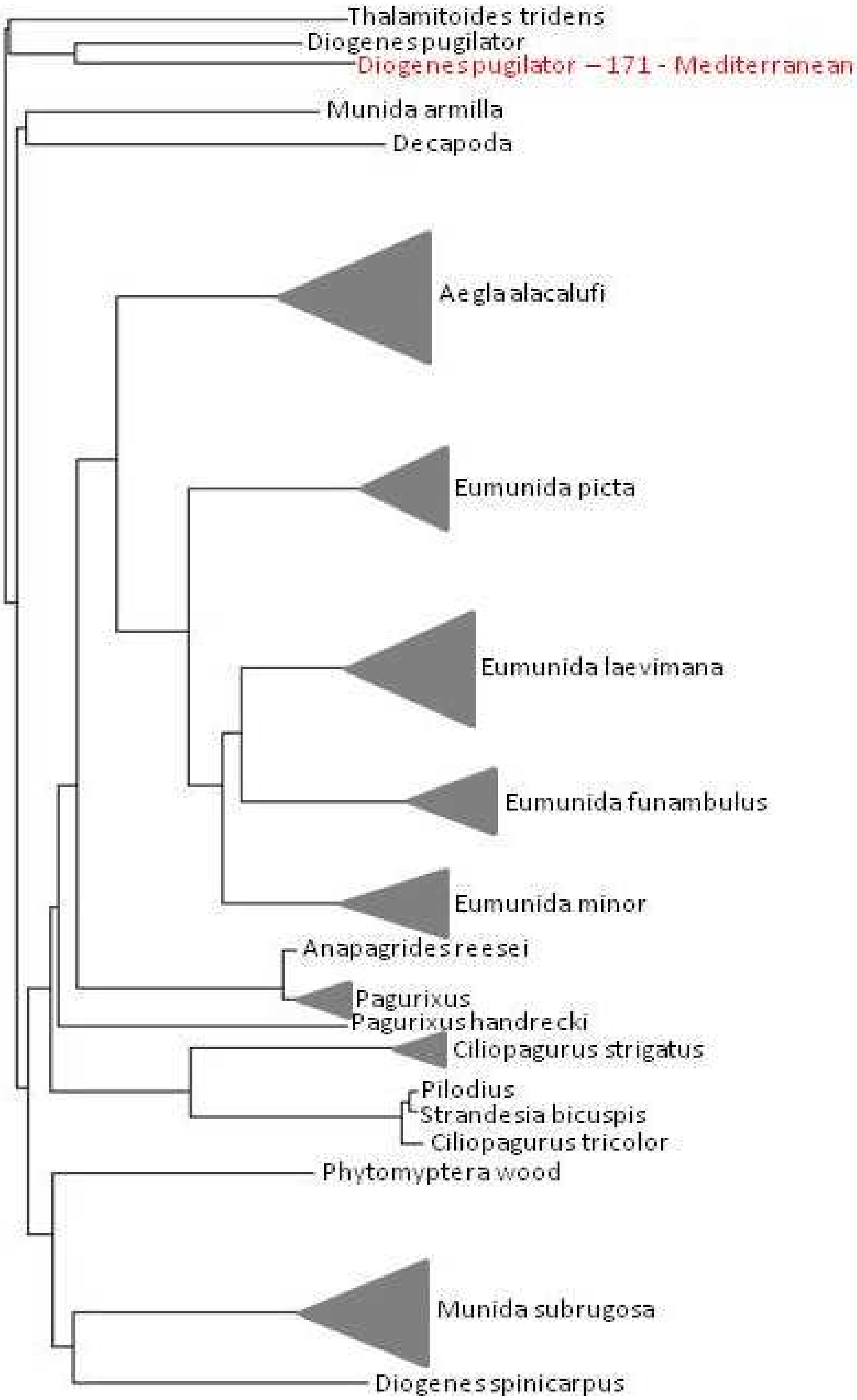

Figure 4.34. Neighbor-joining tree for our *Diogenes pugilator* – 171 (CO1) sequence (shown in red) constructed based on BOLD. (The size of the triangles is proportional to the number of sequences in BOLD for that species).

4.5.2. 16S Sequences

Three samples from two different locations (one from the Aegean and two from the Sea of Marmara) were collected and sequenced for this analysis (Figure 4.35).

Figure 4.35. The sampling location for *Diogenes pugilator* (16S). (The circles are proportional to the number of individuals. The gray circle indicates sampling location Dardanelle strait, the Aegean , the pink circle indicates sampling location, Çınarcık, the Sea of Marmara).

The haplotype network for 16S region showed two haplotypes. Haplotype 16SH1 was found in the samples 103 and 138. The haplotype 16SH2 was found in the sample 108. Geographically speaking the haplotype 16SH1 was found in the Sea of Marmara and the Aegean, and the haplotype 16SH2 was found in the Aegean. The haplotype 16SH1 differentiated from 16SH2 by one base pair (Figure 4.36). The haplotype network table for *Diogenes pugilator* (16S) is given in the table 4.10.

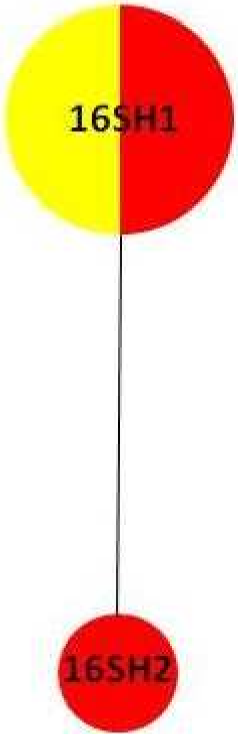

Figure 4.36. Haplotype network for *Diogenes pugilator* (16S). (The size of circles is proportional to the number of individuals. See Table 3.1. for the key to the color-codes of the geographic positioning of the haplotypes).

Table 4.10. Haplotype network table for *Diogenes pugilator* (16S).

Haplotype	Number of individuals	Code for the individuals as seen in the haplotype network
16SH1	2	103, 138
16SH2	1	108

4.6. *Diogenes avarus* (Heller , 1865)

One CO1 region sequence was analyzed for this species. The sample was collected from the Mediterranean (Antakya) (Figure 4.37). The haplotype network and the intraspecific tree could not be prepared as there was only one sample for this species.

Figure 4.37. The sampling location for *Diogenes avarus* (CO1). (The pink circle indicates sampling location in Antakya, the Mediterranean that the samples were collected from for this species).

Comparing with the three closest matches that were taken from BOLD, first closest match to this sample was *Diogenes pugilator* with a specimen similarity of 85.39%, the second was *Anapagurides reesei* with a specimen similarity of 84.77% and the third was genus *Pagurixus* with a similarity of 84.62%. These results indicate that our sequence is the first DNA barcode in BOLD for this species. The neighbor-joining tree for *Diogenes avarus*, constructed via BOLD is shown in Fig 4.38. The sample that was barcoded from Turkey (written in red) fell within the clade for the genus *Diogenes* and was sister to *Diogenes pugilator*.

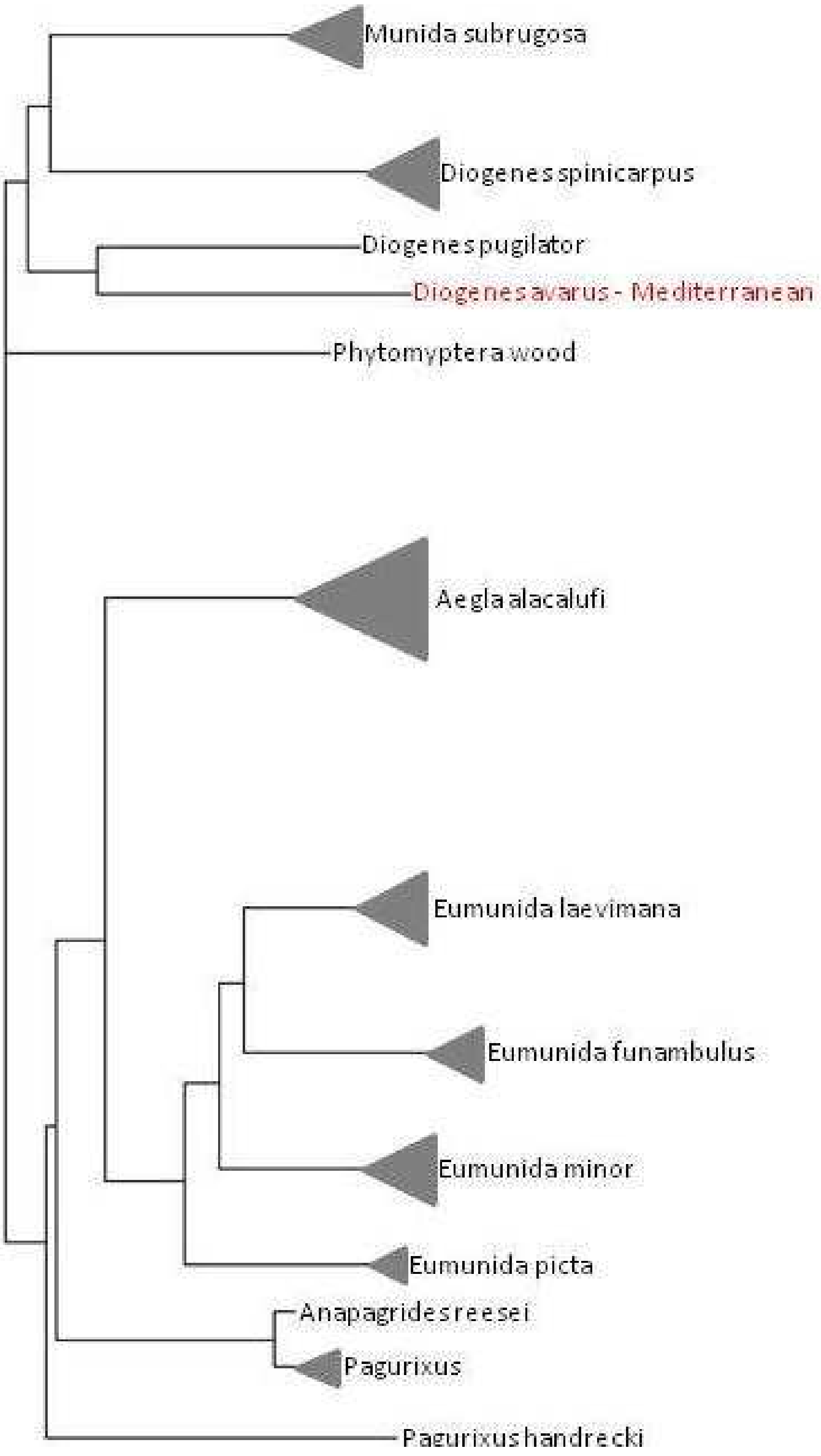

Figure 4.38. Neighbor-joining tree for our *Diogenes avarus* (CO1) sequence (shown in red) constructed based on BOLD. (The size of the triangles is proportional to the number of sequences in BOLD for that species).

4.7. *Cestopagurus timidus* (Roux, 1830)

One CO1 region was analyzed for this species. The sample was collected from the Mediterranean (Antakya) (Figure 4.39). The haplotype network and the intraspecific tree could not be prepared as there was only one sample for this species.

Figure 4.39. The sampling location for *Cestopagurus timidus* (CO1). (The pink circle indicates sampling location Antakya, the Mediterranean that the samples was collected from for this species).

Comparing with the three matches that were taken from BOLD, first closest match to this sample was *Munida roshanei* with a specimen similarity of 85.69%, the second was *Munida clinata* with a specimen similarity of 85.34% and the third was *Munida proto* with a specimen similarity of 85.20%. These results indicate that our sequence is the first DNA barcode in BOLD for this species. The neighbor-joining tree for *Cestopagurus timidus* constructed via BOLD is shown in Fig 4.40. The sample that is barcoded from Turkey (shown in red) did not fall within any clade in the BOLD tree.

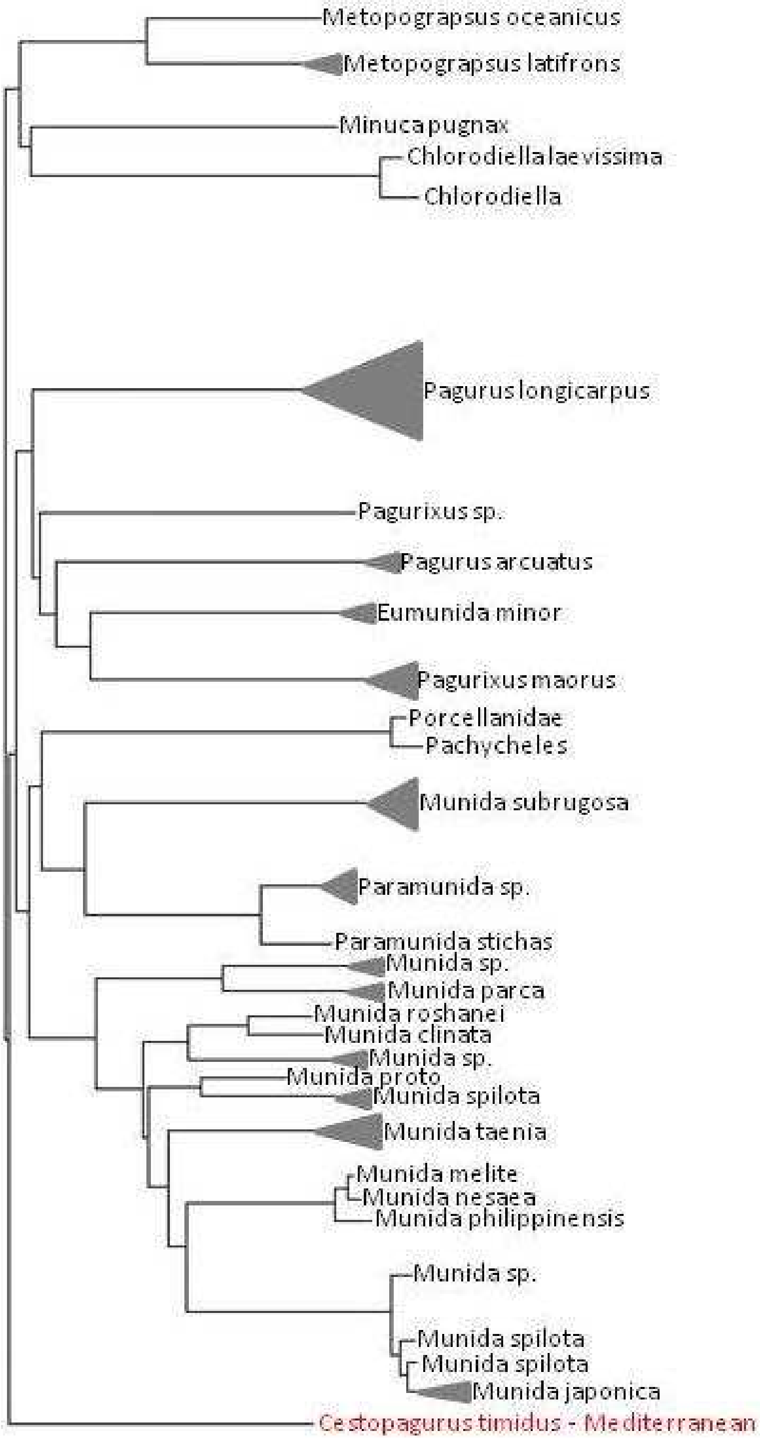

Figure 4.40. Neighbor-joining tree for our *Cestopagurus timidus* CO1 sequence (shown in red) constructed based on BOLD. (The size of the triangles is proportional to the number of sequences in BOLD for that species).

5. DISCUSSION

DNA barcoding is effectively in use for fast identification of unknown species, as well as defining new species. DNA barcoding should also be accurate to separate intraspecific variation within species and divergence in the barcode region (Meyer and Paulay, 2005). A threshold was suggested to be necessary to provide this accuracy and Hebert (2004) suggested a benchmark that the mean interspecific variation should be ten times larger than the mean intraspecific variation in order for DNA barcoding be considered successful (Hebert, 2004). The mean intraspecific divergence was 4.186% for the initial analysis of the samples in this study. The corresponding barcoding gap is 41.86%, which is higher than the mean interspecific distance of 21.07%. In this regard the barcoding gap did not work well. This analysis was repeated, considering each clade within a species as a distinct species. In this second analysis the mean intraspecific distance was 0.61% and the corresponding barcoding gap was 6.1%, which was smaller than the mean interspecific distance of 24%. Hence after this treatment the barcoding gap worked well.

In this study, samples from six different genera, *Calcinus, Clibanarius, Pagurus, Diogenes, Cestopagurus, Diogenes,* were studied. In these six genera, seven species and 46 samples, 42 CO1 sequences were analyzed. Three of seven hermit crab analyzed in this study had unique barcode sequences that were distinct from those found in any other species in BOLD, and represent new entries for BOLD. These species were *Cestopagurus timidus, Pagurus anachoretus and Diogenes avarus*. Actually for *Cestopagurus timidus* the entry represents the first entry for the whole genus.

For two species (*Cestopagurus timidus* and *Diogenes avarus*) sequences from only one sample were available, so for these species it was not possible to undertake more extensive analyses. These species were only analyzed by the ‘identify specimen’ option in BOLD. In the BOLD tree, the *Diogenes avarus* sample fell within the clade for the genus *Diogenes* and is sister to *Diogenes pugilator*. Hence the tree generated under the identify specimen option of BOLD placed *Diogenes avarus* sample in its correct respective genera and worked well. However *Cestopagurus timidus*, unlike *Diogenes avarus*, did not fall in the same clade with any of the congeneric species in the BOLD tree. However the genus *Cestapagarus* is not represented in BOLD, hence this mismatch is normal, by definition.

For another species, *Pagurus cuanensis,* two individuals were sequenced. A neighbor-joining tree could not be prepared for *Pagurus cuanensis* as a minimum of four individuals are needed to prepare it. However we were able to construct a haplotype network and BOLD trees by the 'identify specimen' option for this species. The haplotype network showed that any two samples from this species were differentiated by a maximum of two base pairs from each other. In the BOLD tree, the two samples did not fall in the same clade with any of the congeneric species in the BOLD tree. There exists another entry for *Pagarus cuanensis* in BOLD, from Portugal, and the point that the samples in this study do not group together with that entry suggest that the Turkish and Portuguese populations could comprise separate species.

Three of the other species, *Calcinus tubularis, Pagurus anachoretus*, and *Diogenes pugilator* showed a two-clade pattern both in the neighbor-joining trees, haplotype networks, and BOLD trees. The individual details of these species will be discussed next.

For *Calcinus tubularis,* we had data available from Genbank and BOLD with locality information from East Atlantic and Portugal, which made it possible to make global comparisons. The neighbor-joining tree prepared with our samples from Turkey and BOLD sequences from the East Atlantic showed two different clades. Two samples from the Mediterranean clustered with the samples from East Atlantic formed one clade (1) and the rest of the samples from the Mediterranean and the Aegean formed another clade (2). The haplotypes found in Clade 1 differentiated from the haplotypes found in Clade 2 by six base pairs. The BOLD trees also showed two different clades, one of them consisting of one sample from Portugal and two samples from East Atlantic, and the other consisting of five samples exclusively from Portugal (BOLD Clade 2). In addition, an analysis of the 16S region also showed a two-clade pattern. The samples 99 and 133 clustered in a single clade, whereas the samples from East Atlantic clustered into another. Hence in this study, the haplotype distribution pattern of two clades being found in the East Atlantic and

Portugal geographically expanded into the Turkish coastal waters of the Mediterranean and the Aegean, but to understand these pattern better, more sampling and sequencing should be done in central Mediterranean region as well.

Another species that showed a two-clade pattern is *Diogenes pugilator*. CO1 regions of 18 samples were sequenced for this analysis. From these 18 samples, 16 of them were collected from the Sea of Marmara and the remaining two from the Mediterranean. The neighbor joining tree clustered the sample 171 with the most general haplotype found in the Sea of Marmara despite the 48 base pair difference (Figure 4.30). This grouping is referred to as Clade 1. On the other hand, the other sample collected from the Mediterranean (170) formed another branch which is referred to as Clade 2, with a 60 base pair difference from the most general haplotype found in the Sea of Marmara. We prepared BOLD trees with the 'identify specimen' option with one individual from the samples collected from the Sea of Marmara and the other two samples which were collected from the Mediterranean (170 an 171). The sample 170 did not fall in the same clade with any of the congeneric species in the BOLD tree, however the samples 116 and 171 fell within the *Diogenes* genus and as a sister species to *Diogenes pugilator* in the BOLD tree. These results suggest that sample 170 might represent a species other than *Diogenes pugilator*, whereas the differentiation between sample 170 and the rest of the samples, by 48 bp, represent another two-clade pattern. Additionally, 16S regions of three other samples were sequenced for this analysis (103, 108 and 138). The haplotype network showed two different haplotypes, which were differentiated from each other by only one base pair. This also confirms the shallow differentiation of the samples from the Sea of Marmara.

Another species that showed a two-clade pattern was *Pagurus anachoretus*. For this species CO1 regions of nine samples were sequenced. Here, we present also present the first DNA barcode in BOLD for *Pagurus anachoretus*. Three of the samples were collected from the Aegean (Saros Bay) and six of the samples were collected from the Mediterranean (three from Antakya and three from Kaş). The neighbor-joining tree for *Pagurus anachoretus* showed two clades. The samples from the Aegean, Antakya, and also one sample from Kaş (153) clustered together and formed Clade 1, and the other two samples from Kaş clustered into a different clade, which is referred to as Clade 2. Looking at the haplotype network, again two clusters were observed. In the haplotype network, the samples 154 and 156 clustered together and differentiated from the rest of the samples by 94 base pairs, a high value. In the BOLD tree, a representative samples from clade 1, did not fall in the same clade with any of the congeneric species, although 22 other *Pagurus* species are present in BOLD. This suggests a problem with the barcoding of this species. On the other hand, the representative sample from Clade 2 fell within the *Pagurus* clade as a sister species to *Pagurus pollicarsis*. Additionally 16S region of two samples from the Aegean were sequenced. The two individuals had the same haplotypes for the 16S region, and they also shared the same haplotypes for CO1 region. To understand the haplotype distribution pattern for this species better, more 16S sequencing should be done from Kaş and Antakya locations.

The final species, *Clibanarius erythropus* showed a sigle clade pattern. For this species CO1 region of five samples were sequenced, four from the Aegean and one from the Sea of Marmara. The neighbor-joining tree for this species initially showed two clades; three samples from the Aegean clustered with the sample from the Sea of Marmara, which was initially referred to as Clade 1, and the last sample from the Aegean formed another branch, Clade 2. The haplotype network showed that even though the neighbor-joining tree indicated two different clades, it is more plausible to state that there is one general clade because the genetic differentiation between these two clades is low, by two base pairs. The BOLD trees prepared with representative samples from Clade 1 and Clade 2 placed these two samples in the *Clibanarius erythropus* clade. Two clades were observed in the BOLD tree. BOLD Clade 1 consists of three *Clibanarius erythropus* samples and BOLD Clade 2 consists of four *Clibanarius erythropus* samples. These samples did not have locality information available on BOLD. Although the representative samples fell within different clades in the neighbor-joining tree, they fell in the same clade (BOLD clade 2) in the BOLD tree. This can be explained by the low genetic differentiation that was observed in the haplotype network, discussed above. Additionally, 16S region of two samples were sequenced from the Aegean. Theses samples showed two different haplotypes differentiated from each other by two base pairs. This low differentiation shows that the results of the CO1 and 16S analyses confirm each other, however subsequent genetic analysis from the rest of the species range will be useful to understand the geographic distributions of the two clades retrieved in the BOLD trees better.

In conclusion, in this study we present the first systematic DNA barcoding and genetic cataloging of hermit crabs in Turkey. We provide the first DNA barcodes in BOLD for three species, *Pagurus anachoretus*, *Diogenes avarus* and *Cestopagurus timidus*. The barcode for *Cestopagurus timidus* is also the first for the genus *Cestopagurus*. The barcoding gap worked better when the different clades in each species were treated as separate species, which suggest that the two-clades in these species might represent different taxa. Three of the seven species studied in this study (*Diogenes pugilator, Pagurus anachoretus* and *Calcinus tubularis*), showed a two-clade pattern, both in the neighbor-joining trees and haplotype networks. For *Calcinus tubularis* we were able to make global comparisons, with sequences taken from BOLD and Genbank, from East Atlantic and Portugal, and our results indicate that the two-clade pattern in the East Atlantic and Portugal extends into the Turkish coastal waters of the Mediterranean and Aegean. In *Diogenes pugilator* and *Pagurus anachoretus* a two-clade pattern was observed for the samples from Turkey, however as either no or no geographically explicit information was available for the sequences for these species in the databases, such a global comparison was not possible for these two species. The same was the case for *Clibanarius erythropus* for which a single-clade pattern was observed. In order to understand pyhlogeographic distribution patterns better and for firmer conclusions on intraspecific and interspecific relations of species, we recommend a more detailed generation of CO1 and 16S sequences for studied species both globally and from the Turkish coastal waters.

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