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RESEARCH PAPER

Phylogeographic Resolution of the Barnacle (*Chelonibia testudinaria*) from the North-Eastern Mediterranean Loggerhead Sea Turtles Epibiont Community

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Abstract

Barnacles are common epibionts on a wide range of marine organisms, including turtles. *Chelonibia testudinaria* is a successful epibiotic barnacle species, and mainly turtles are responsible for their wide range dispersal. In the present study, the mitochondrial Cytochrome Oxidase I (COI) gene haplotypes of *C. testudinaria* from *Caretta caretta* hosts were evaluated. The samples were collected from three dead *C. caretta* turtle carapaces in 2014 from the Middle East Technical University, Institute of Marine Sciences coastline. Results were also compared with those samples submitted to databases (NCBI and BOLD-system, 139 in total). By comparison, three clades were recorded like previous studies: the Atlantic-Mediterranean clade (Clade-α), the Indian-Pacific Ocean clade (Clade-β), and Magdalena Bay (Eastern Pacific- Clade-γ) clade; all samples collected from Turkish shores clustered in the Atlantic-Mediterranean group (Clade-α). The gene flow between the three clades was deficient and highly significant (0.02, 0.03, and 0.03, respectively). According to network age estimation, present study samples' clade (Clade-α) diverged from the Clade-β approximately 200 kya (SDs=0.22, SDy=4402.90) and Clade-γ 130 kya (SDs=0.17, SDy=3494.55). In the present study, eight haplotypes were observed in total, two of which were specific to the region.

Introduction

Barnacles are a type of arthropod belonging to the subphylum Crustacea, under the superfamily of Coronunloidea. Besides a wide range of other marine organisms, they are common turtles' epibionts (Cheang, Tsang, Chu, Cheng & Chan, 2013). Other known hosts of barnacles include crabs, some marine animals, and inanimate objects such as sea vessels and floating objects (Relini, 1980). Barnacles are filter feeder organisms being continuously carried to new feeding grounds and fresh streams by such hosts, with most of the superfamily specific species to one or a few hosts (Ross & Newman, 1967; Newman & Ross, 1976 ref. in Cheang, Tsang, Chu, Cheng & Chan, 2013). *Chelonibia testudinaria* (Linnaeus, 1758) is an "obligate commensal" species known as the turtle barnacle and attaches onto the carapace, plastron, flippers, head or neck of marine turtles (Hayashi & Tsuji, 2008). It is frequently encountered on the two sea turtles *C. caretta* and *Chelonia mydas* since the Miocene epoch (Blick, Zardus & Dvoracek, 2010).

Although sea turtles are ocean creatures, they are dependent on the land to breed. It is well known that sea turtles return to the shores where they emerged as hatchlings to dig a nest and lay their eggs. Adult turtles travel hundreds, perhaps thousands of kilometers between feeding grounds and nesting beaches (news.nationalgeographic.com/news/2015/01/150115-loggerheads-sea-turtles-navigation-magnetic-field-science/). During these migrations, they commonly engage in symbiotic unions with different species that

encounter along their path. And in this way are thought to be paramount in the distribution and expansion of their common epibiont barnacle *C. testudinaria* (Domènech, Badillo, Tomás, Raga & Aznar, 2015). Among all sea turtles species, the loggerhead turtle, *C. caretta*, is colonized by the largest and the most diverse communities of epibionts (Frick, Williams, & Robinson 1998; Frick, Williams, Markesteyn, Pfaller & Frick, 2004). Third types of epibiont communities were identified for the western Mediterranean loggerhead turtles; 1) obligate epibionts (exclusive to marine turtles worldwide), 2) *Balenophilus manatorum* and *Chelonibia testudinaria* species (facultative for marine turtles); 3) the last group are members of the facultative chelonophilic epibiont taxa which has both commensals or free-living forms (The Epibiont Research Cooperative, 2007; Aznar, Badillo, Mateu & Raga, 2010; McGowin *et al.*, 2011; Hayashi, 2013; Zardus, Lake, Frick & Rawson, 2014; Domènech *et al.*, 2015). Epibionts help to monitor both ecological and evolutionary factors that rule the biotic associations and support glean information about the distribution and ecology of marine turtles (Frick & Pfaller, 2013; Domènech *et al.*, 2015).

Chelonibia testudinaria is most frequently and abundantly observed on the *C. caretta* (Matsuura & Nakamura, 1993; Frick & Ross, 2002), but it can also be found on the external surfaces of other turtles (Kemp's ridley, green, flatback and hawksbill) and animals (Monroe & Garrett, 1979 ref. in Cheang, Tsang, Chu, Cheng & Chan, 2013; Seigel, 1983; Frick & Ross, 2002; Zardus *et al.*, 2014). It is suggested that the migration pattern of *C. caretta* turtles played a vital role in the expansion range of *C. testudinaria* into the Mediterranean Sea (Rawson, Macnamee, Frick, & Williams, 2003). Western and central Mediterranean waters are being mostly used as foraging ground by juvenile–subadult turtles. It was reported that regardless of their geographic origin, most of the turtles change their position into and out of the continental shelf area and take an advantaged from both pelagic and benthic habitats, because of these they are being exposed to a similar pool of epibiont propagules (Paolo Casale *et al.*, 2008; Paolo Casale & Margaritoulis, 2010; Carreras *et al.*, 2011; Clusa *et al.*, 2014).

Whereas, the morphology was accepted as a key factor in identifying species for a long-time. Nowadays, it is accepted that classical taxonomy helps to discriminate only a small fraction of world biodiversity. Besides, those species that have already extinct may not be possible examine via morphology (Carew, Pettigrove & Hoffmann, 2006). Also, the taxonomic classification of some barnacle species had been reported as confusing because of the high degree of morphological variation (Chan, Tsang & Chu, 2007a) These difficulties have been solved by DNA barcoding since its first initiation in 2003 (Hebert, Cywinska, Ball & DeWaard, 2003; Hebert & Gregory, 2005). On the other hand, barcode databases (BOLD system, NCBI, iBOL, etc.) with accumulation of large sequences, offers an effective way for cataloguing biodiversity and novel conservation approaches. Whereas mitochondrial markers have been described as useful tools to investigate 'population genetic' features (Chan, Tsang & Chu, 2007a,b; Tsang *et al.*, 2008), they also help identify geographic resolution the species and also provide information about their host species.

This study aimed to understand the origin, interaction, and phylogeographic resolution of *C. testudinaria*, hosted by *C. caretta* marine turtles on a global scale, and contribute to genetic and ecological research of the Turkish coastline populations.

Materials and Methods

Sampling Location and Host Species

In total, 15 *C. testudinaria* samples were collected from 3 dead *C. caretta* turtle carapaces (6, 5, and 4 samples) in 2014 from the Middle East Technical University, Institute of Marine Sciences coastline (Figure 1). The samples were stored in 70% alcohol until used for DNA isolation. The present study's COI sequences were compared with the selected *C. testudinaria* sequences that mined from the NCBI and BOLD system (139 samples), sampled from the Mediterranean and other worldwide locations (Table S1). The DNA of the present study samples were vouchered.

DNA Isolation, PCR and Sequencing

The tissue of each C. *testudinaria* specimen was placed in a 1.5 ml vial separately, frozen until use. Genomic DNA was extracted using the CTAB protocol (Stewart & Via, 1993). After the dilution (1:100) with molecular grade water, the samples' DNA were kept at 4°C. Forward (LCO1490-F, GGTCAACAAATCATAAAGATATTGG) and revers (HCO2198-R, TAAACTTCAGGGTGACCAAAAAATCA) primers of Folmer *et al.* (1994) were used to amplify the cytochrome oxidase I (COI) gene region during the Polymerase chain reaction (PCR, annealing temperature=48ºC). The PCR products were screened on 1.3% agarose gel and the purification and sequencing processes were performed by Macrogen Inc. (Amsterdam) for both reverse and forward directions. Specimens data were submitted to the Barcode of Life Data System (BOLD, http://www.boldsystems.org, see (Ratnasingham & Hebert, 2007), it is accessible within the project file 'IMS-METU-Animalia' (Table S1). Specimen and sequence pages of BOLD system consist; specimen details, taxonomy, collection data, specimen images, chromatogram, trace files, and primer details.

Statistical Analysis of COI Gene

Sequence alignment of present study samples (15) and those mined from databases (NCBI and BOLD-system, 139 in total) was performed using BioEdit v.7.0.9.0 (Hall, 1999) software. ARLEQUIN 3.11

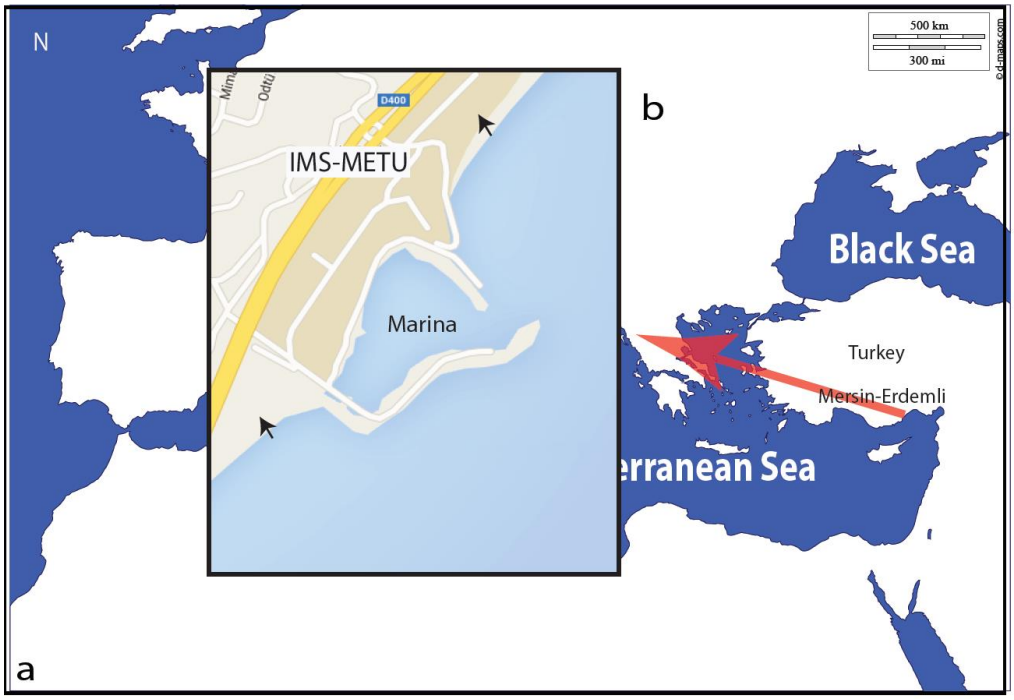


Figure 1. The sampling location of *C. testudinaria* samples from the North-Eastern Mediterranean a) Large scale map b) Local scale map. The black arrows indicate the place where the three dead *C. caretta* individuals were found.

(Excoffier *et al.*, 2005) was used by 10.000 permutations to calculate pairwise genetic distances (h, Weir & Cockerham, 1984) between samples and clades and their significance test.

The DNAsp version 5.0 software (Rozas & Rozas, 1999; Rozas, Sanchez-DelBarrio, Messeguer & Rozas, 2003; Tajima, 1989) was used to perform demographic history and neutrality tests analyses. The number of haplotypes (Nh) and the gene flow parameter (Nm) between the turtle epibionts and clades were estimated (Nei, 1973). The mismatch distribution (pairwise nucleotide differences) analysis was done using the Raggedness Index (r; Harpending, 1994) to calculate the demographic expansion of the samples.

The median-joining algorithm and the default settings of the NETWORK program version 4.6.1.2. (Bandelt, Forster & Rohl, 1999) was used for constructing the network (weight=10 e=0) to estimate the phylogeographic relationships between haplotypes and calculate the standard deviation sigma (SDs) and the standard deviation in a year (SDy). The haplotype clusters have been dated using the rho (q) estimator (Forster, Harding, Torroni & Bandelt, 1996; Saillard, Forster, Lynnerup, Bandelt & Nørby, 2000). To estimates the divergence time, the average number of mutations separating ancestral and descendent haplotypes was used. Mismatch distribution and Raggedness index based on the COI gene for the clusters of *C. testudinaria* was calculated.

Results

We obtained 15 both directions (forward and reverse) COI sequences from 3 *C. testudinaria* individuals. After alignment and trimming, the final length of the COI gene fragment was approximately 550 bp. In total, 73 haplotypes were recorded from the present and previous studies samples. Eight haplotypes were observed in total from the present study samples, two of which were specific to the Mediterranean coast of Turkey. IMS013-15 sample clustered in Haplotype-1 with Greece samples; IMS005-15, IMS007-15, IMS009-15, IMS010-15, IMS011-15, IMS012-15, IMS013-15 samples clustered in Haplotype-19 with Greece, Atlantic, Georgia, Florida, and Puerto Rico samples; IMS008-15 and IMS014-15 samples clustered in Haplotype-33 with Florida samples; IMS017-15, IMS015-15 and IMS006-15 samples clustered in Haplotype-52, 53 and 54 respectively with Greece samples. IMS004-15 and IMS016-15 are observed for the first time in this study as private haplotypes. In total, three clades were observed according to present and previous study results; Atlantic-Mediterranean (Clade-α), Indian-Pacific Ocean (Clade-β), and Magdalena Bay-Equator (Clade-γ); all samples collected from the Turkish Eastern Mediterranean coasts clustered in the Atlantic-Mediterranean clade. Visualization of the network analysis and its supplementary table was given in Table S1 and Figure 2.

There were no significant genetic differences (F_{ST}) between the epibionts of three *C. caretta* individuals (0.07, 0.00, and 0.00, Table 1). On the other hand, very high F_{ST} values were observed between the three clades (0.95, 0.96, and 0.95; P<0,001, Table 2). According to gene flow estimations, Nm values between epibionts of the three *C. caretta* individuals were too high (9.26, 52.8 and 360: Nm > 4 insufficient to avert genetic differentiation, Nm > 1 there is enough gene flow to

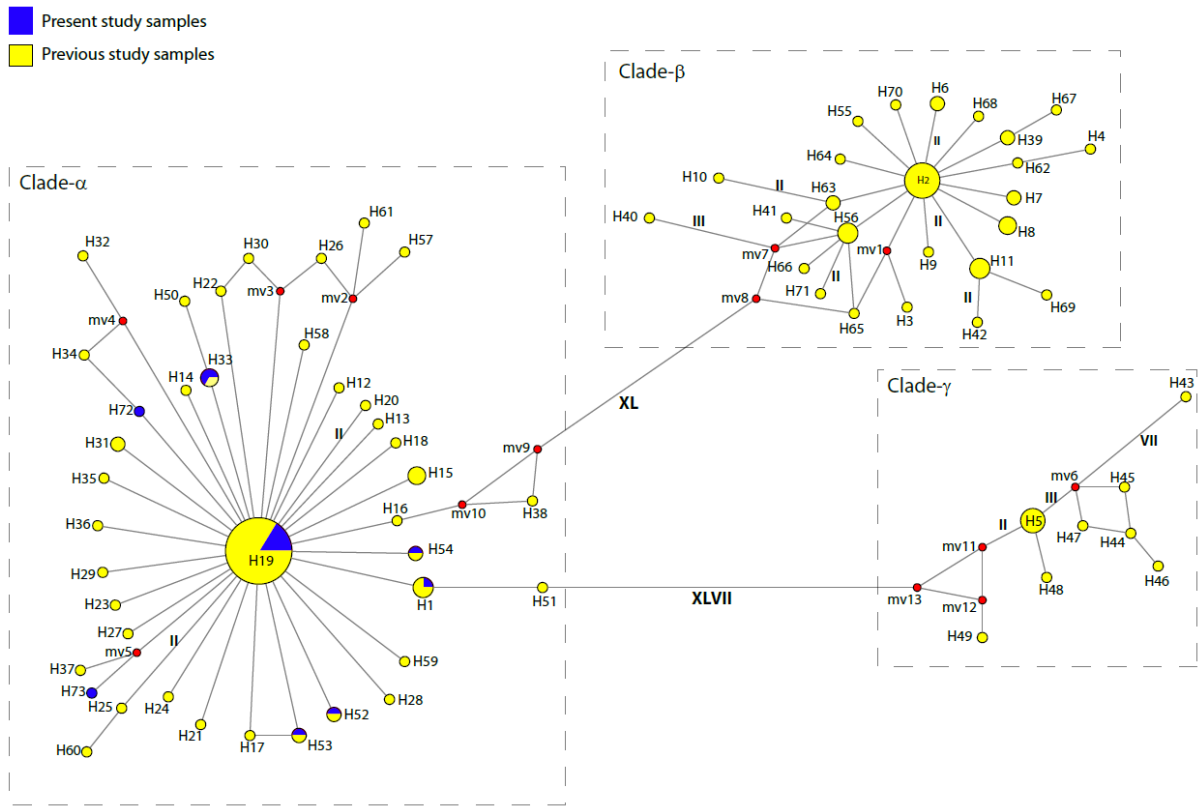


Figure 2. The median-joining network of *C. testudinaria* for the COI haplotypes (H with a corresponding number denotes a specific haplotype). The pie size is proportional to the number of colonies, and colours indicate different samplings/populations. All the present study samples are coloured in blue and previous yellow. Lines without Roman numerals: one mutation step between haplotypes; Roman numerals with vertical black lines: the number of mutations (>1). mv = median vectors; black lines = highlighted mutation positions.

nullify the effects of genetic drift) and between 3 clades were too low (0.02, 0.03 and 0.03, Table 2) (Hudson *et al.*, 1992-Sequence Data Information for clades).

The mismatch distribution plot for the Clade-α and Clade-β was smooth and unimodal, indicating a population expansion. The multimodal pattern of the Clade γ samples mismatch distributions may suggest population subdivision (Figure 3).

According to network age estimation, Clade-α (Atlantic-Mediterranean) diverged from the Clade-β (Indian-Pacific Ocean) approximately 200 kya (SDs=0.22, SDy=4402.90); Clade α and Clade-γ (Magdalena Bay-Equator) diverged ca. 130 kya (SDs=0.17, SDy=3494.55); Clade-β and Clade-γ diverged 665 kya (SDs=0.47, SDy=9512.94).

Discussion

Three major lineages of *C. testudinaria* were described based on the mitochondrial COI gene divergence patterns in the world's oceans; the Eastern Pacific, Western Pacific, and Atlantic (including the Mediterranean) (Cheang *et al.*, 2013; Rawson *et al.*, 2003). Despite the very short free-swimming larval stage (9 days) of *C. testudinaria,* high gene flow was observed between different locations and host populations (Cheang *et al.*, 2013). It can be attributed to their rapid turnover rate, fast growth, a relatively short lifespan, and high juvenile death rates (Casale, Argano, D'Addario & Freggi, 2012). According to the very high gene flow values between the epibionts of the three *C. caretta* individuals of the present study, it is possible to say that those three turtles belonged to the same or connected populations.

Cheang *et al.* (2013) compared 79 *C. patula* individuals collected from diverse benthic crustaceans in Taiwan, Hong Kong, Singapore, and Malaysia and 25 *C. testudinaria* from marine turtles in Taiwan using mitochondrial COI, 12S and 16S rRNA gene regions. According to Cheang *et al.* (2013) *C. testudinaria* samples of their study, together with those from the Pacific coast of Japan (Rawson *et al.*, 2003), clustered with the *C. patula* samples. Based on both morphological and molecular evidence, Rawson *et al.* (2003) proposed that *C. testudinaria* and *C. patula* from South East Asia and Taiwan are conspecific and belong to the western Pacific *C. testudinaria* population. Present study samples were clustered under the Atlantic-Mediterranean cluster. Whereas *C. patula* species has been reported from the Mediterranean coast of Turkey (Bakir, Özcan & Katagan, 2010), the species could not be compared with the present study samples because of the lacking COI sequences. No host-specific phenotypic plasticity has been reported for the *C. testudinaria*'s marine turtles epibiont.

Table 1. Gene flow estimations (Nm) and significance test (F_{ST}) between the 3 *C. caretta*'s epibionts (*C. testudinaria*)

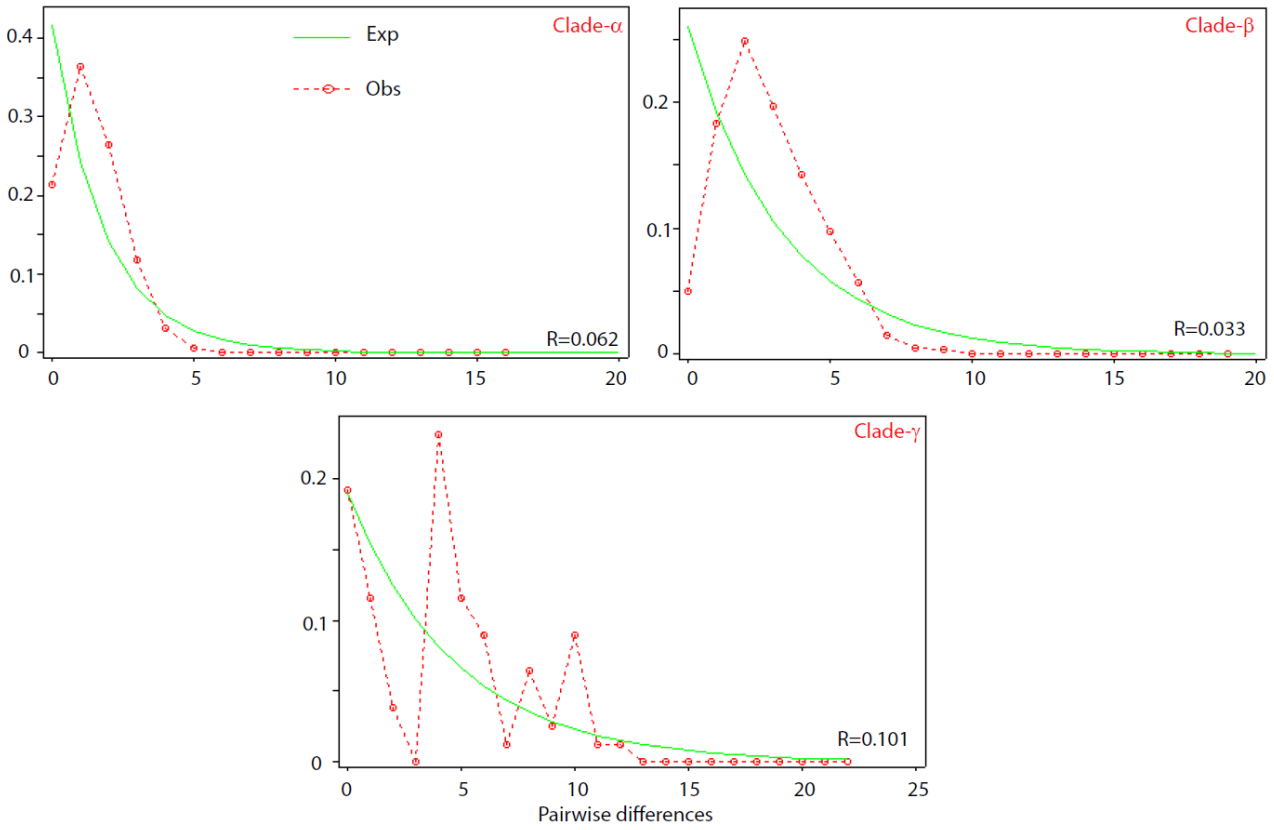
F_{ST}/Nm	*C. caretta-1*	*C. caretta-2*	*C. caretta-3*
C. caretta-1	-	0.07^{ns}	0.00^{ns}
C. caretta-2	9.26	-	0.00^{ns}
C. caretta-3	52.80	360	-

F_{ST} values at above diagonal (^{ns}, P>0.05), Nm values at below diagonal (Nm > 4 insufficient to prevent genetic differentiation, Nm > 1, there is enough gene flow to negate the effects of genetic drift).

Table 2. Gene flow estimations (Nm) and significance test (F_{ST}) between *C. testudinaria* clades (Clade-α: Atlantic-Mediterranean; Clade-β: Indian- Pacific Ocean; Clade-γ: Magdalena Bay-Equator)

F_{ST}/Nm	*Clade-α*	*Clade-β*	*Clade-γ*
Clade-α	-	0.96***	0.95***
Clade-β	0.02	-	0.95***
Clade-γ	0.03	0.03	-

F_{ST} values at above diagonal (***, P<0.001), Nm values at below diagonal (Nm > 4 insufficient to prevent genetic differentiation, Nm > 1, there is enough gene flow to negate the effects of genetic drift).



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A notable population divergence recorded among *C. testudinaria* populations; findings suggested that the Eastern and Western Pacific groups were not only distinct from the Atlantic and Mediterranean populations but also each other (Rawson *et al.*, 2003). On the other hand, the Equator clade was located as solitary and highly divergent from the Australian and Atlantic clades. Present study specimens clustered in the Atlantic group (Clade-∝), Haplotype-1 clustered with those collected from Kyparissia, Greece; Haplotype-19 clustered with Kyparrisia (Greece), Atlantic Ocean (Bulls bay), Atlantic Ocean (Charleston Harbor, Core sound, Hutchinson Island, Virginia Beach, Wellfleet beach), United States (North Carolina, Pamlico sound, Florida,

Manatee River, Keewaydin-Florida), Wassaw Island, Puerto Rico (Mona Island), Haplotype-33 clustered with Keewaydin (Florida), Haplotypes 52, 53 and 54 clustered with Kyparrisia (Greece). Haplotypes 72 and 73 were the private haplotypes for the North-Eastern Mediterranean. Therefore, it is possible to state that those individuals attached to the *C. caretta* hosts in the North-Eastern Mediterranean, and they have the potential to be marker haplotypes to trace the region marine turtles.

It is also possible to say that gene flow differences between the clades may be attributable to the distance between these geographical areas. According to Rawson *et al.* (2003), dispersal of *C. testudinaria* is likely to be partly connected to interactions with its host. It corroborates previous findings that "host migration facilitates long-distance dispersal of *Chelonibia*, while phylogeographic structure only occurs across ocean basins that impact host migration." Migration may be enhanced by the different life stages of the loggerhead turtles. The juveniles spend at least six years in the nursery grounds; Atlantic, Indian and Pacific oceans (Bjorndal, Bolten & Martins, 2000). On the other hand, it is assumed that the adults do vast seasonal migrations between the foraging grounds on coastal edges (Musick & Limpus, 1997). The symbiotic species can attach to the host at any time of this life cycle. According to gene flow and Fst results of the present study, it can be concluded that the migration of the host species is restricted with their clade.

The answers to the questions 'how does this symbiotic relationship affect the host turtles? Do they benefit from it, or are they unaffected, or might they be negatively affected?' still have not been provided precisely. The symbiotic relationship between the loggerhead turtle and its barnacle epibiont may not yet be fully understood. However, it can be concluded that whatever the manner of their union, barnacles are dependent on their hosts to strive. Therefore 'turtle specific barnacles should be considered even more endangered than their hosts (Paolo Casale, Freggi, Basso & Argano, 2004). This states the importance of the conservation efforts for not only the host but also indirectly for its epibiont.

Conclusion

Morphological records of *C. testudinaria* for Turkish shores were provided by Monod (1931) and Geldiay *et al.* (1982). These studies were both based on morphological analysis, since no DNA barcode date of the region's species has been reported. In the present study, for the first time based on mitochondrial DNA evidence, we propose that *C. testudinaria* samples of the Turkish Mediterranean coastline, belong to the Eastern Atlantic population. On the other hand, recording eight haplotypes, from a total of 15 samples, is provide a perspective about the intra-specific distance of the area samples. The present study also provides valuable insight into the turtles' symbiotic fauna's distribution and evolution in the North-Eastern Mediterranean.

Ethical Statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Authors Contributions

- A.K. conceived and planned research.
- E.B. and A.K. carried out the experiments.
- E.B. and A.K. wrote the manuscript.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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