PHYLOGEOGRAPHY AND EVOLUTIONARY HISTORY OF WHITING (*Merlangius merlangus*) AND PICAREL (*Spicara spp*.) ALONG THE TURKISH COASTAL WATERS

by

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ABSTRACT

PHYLOGEOGRAPHY AND EVOLUTIONARY HISTORY OF WHITING (*Merlangius merlangus*) AND PICAREL (*Spicara spp.)* ALONG THE TURKISH COASTAL WATERS

In this study, the effects of the Turkish Straits System (TSS) on the evolutionary history and phylogeography of *Spicara flexuosa* and *Spicara maena*, and *Merlangius merlangus* were investigated. The results of the study indicated that the distinction of the three taxa under *Spicara* is possible with three different mtDNA markers, e.g. (16S, CO1, cyt-b). *S. maena* and *S. flexuosa* were also analyzed using a partial fragment of the nuclear IRBP gene. Molecular results showed that *Spicara flexuosa* was significantly more common in the Turkish coastal waters, when compared to *S. maena*. Morphological measurements revealed that the *S. maena* samples were smaller than the *S. flexuosa* samples. An absence of genetic structure between subpopulations from Turkey indicate the connectivity of the *Spicara flexuosa* populations, suggesting that the TSS is a corridor for gene flow for this species. Considering *Merlangius merlangus*, the mitochondrial DNA (CO1, cyt-b) genes and nuclear DNA (RAG1) gene were used. The results confirmed the existence of two subspecies in the sampling sites used in this study, based on mitochondrial DNA results, one in Turkish coastal waters and Greece (*M. m. euxinus*), and the other (*M. m. merlangus*) in Atlantic. High levels of genetic differentiation was observed along the Turkish coastal waters based on the cyt-b gene, suggesting that TSS potentially represents a barrier to dispersal for this species. As the whiting populations in the Black Sea were found to belong to different stocks when compared to those in the Aegean, separate conservation strategies are necessary for their protection.

ÖZET

TÜRK KIYI SULARINDAKİ MEZGİT (*Merlangius merlangus*) VE İZMARİT'İN (*Spicara spp.)* FİLOCOĞRAFYA VE EVRİMSEL TARİHİ

Bu çalışmada Türk Boğazlar Sistemi'nin (TBS), *Spicara flexuosa* ve *Spicara maena* ile *Merlangius merlangus*'un evrimsel tarihi ve filocoğrafyasına etkisi incelenmiştir. Çalışmanın sonunda *Spicara* cinsine ait üç türün mitokondriyal DNA genlerine (16S, CO1, cyt-b) göre ayırt edilebildiği görülmüştür. *S. maena* ve *S. flexuosa* ise IRBP çekirdek geninin bir parçası kullanılarak da analiz edilmiştir. Moleküler analizler*, S. maena'*yla karşılaştırıldığında, *Spicara flexuosa*'nın Türk kıyı sularında önemli ölçüde yaygın bir tür olduğunu göstermiştir. Morfometrik ölçümler ise *S. maena'* nın *S. flexuosa'*dan küçük olduğunu göstermektedir. *Spicara flexuosa*'nın Türkiye kıyı sularındaki alt populasyonlarında genetik bir ayrımın görülmemesi ise, bu türün bu sularda genetik olarak benzeştiğini, TBS'nin ise bu türün gen akımı için bir koridor görevi üstlendiğini göstermektedir. *Merlangius merlangus* türünde ise mitokondriyal DNA genleri (CO1, cyt-b) ve çekirdek geni (RAG1) kullanılmıştır. Bu çalışmada, mitokondriyal DNA sonuçları baz alındığında, örnekleme yapılan istasyonlarda, biri Türk kıyı suları ve Yunanistan'da (*M. m. euxinus*) ve diğeri de Atlantik'te (*M. m. merlangus*) iki alt türün varlığı doğrulanmıştır. Cyt-b geni baz alındığında ise, Türkiye kıyı sularında yüksek seviyede genetik bir farklılaşmanın görülmesi, TBS'nin bu türün dağılımı için bir bariyer olabileceğini göstermektedir. Ege Denizi ve Karadeniz'deki mezgit popülasyonları farklı stoklara ait oldukları için, korunmaları için farklı koruma stratejileri gerekmektedir.

TABLE OF CONTENTS

ACKNOWLEDGMENTS……...iii
ABSTRACT……iv
ÖZET…….....v
LIST OF FIGURES……....ix
LIST OF TABLES…….....xviii
LIST OF SYMBOLS/ABBREVIATIONS…….....xxii
1. INTRODUCTION……...1
1.1. Phylogeographical Hypotheses and Studies of Marine Fish Species……..2
1.2. General Characteristics of the Study Area…….....4
1.2.1. Hydrological and Biological Characteristics of the Turkish Straits System……....4
1.2.2. Paleoceanography of the Marmara Sea……5
1.2.3. Hydrological Characteristics of the Black Sea…….....6
1.2.4. Geological Evolution of the Black Sea..…….....7
1.2.5. Hydrological Characteristics of the Aegean Sea..……7
1.2.6. Hydrological Characteristics of the Mediterranean Sea……..……8
1.2.7. Geological Evolution and the Origin of the Mediterranean Biota.……..8
1.3. Literature Review For Genetic and Phylogeographic Studies of Marine Fish Species in Turkey …….....9
1.4. The General Biology and Taxonomy of Whiting and Picarel…….....11
1.4.1. Taxonomic Status and Geographical Distribution of *Merlangius merlangus*……....11
1.4.2. Habitat and Biology of *Merlangius merlangus*……..…….....12
1.4.3. Taxonomic Status and Geographical Distribution of *Spicara spp*……13
1.4.4. Habitat and Morphologic Features of *Spicara spp*……..…....15
1.5. Main Aim of the Study…….....16
2. MATERIALS AND METHODS……..18
2.1. Statistical Analyses..……..……..22
2.2. Morphometric Measurements……..……....……25
3. RESULTS AND DISCUSSION……26
3.1. *Spicara flexuosa* (Rafinesque, 1810)*/ Spicara maena* (Linnaeus, 1758).....26
3.1.1. Mitochondrial DNA (16S rRNA gene)……....……26
3.1.2. The CO1 Gene.....…….....…….35
3.1.2.1. Mitochondrial population structure of CO1 gene..……..46

3.1.3. The cyt-b Gene……..……..48
3.1.3.1. Mitochondrial population structure of cyt-b gene……..58
3.1.4. mtDNA Analyses: CO1 and cyt-b Concatenated Data Analyses for *Spicara spp*.....60
3.1.5. Nuclear DNA (IRBP gene)…….……..……63
3.1.5.1. Population structure of the IRBP gene…….....72
3.1.6. Demographic Analyses of *Spicara flexuosa* and *S. maena* mtDNA and Nuclear DNA Results.....73
3.1.7. Morphometric Measurements……..…….....78
3.1.8. Discussion……...…….……81
3.1.8.1. Genetic differentiation of the three *Spicara* species: *Spicara maena, S.flexuosa* and *S. smaris*.....81
3.1.8.2. Connectivity of the Turkish *Spicara flexuosa* populations……..…….....87
3.2. *Merlangius merlangus* (Linnaeus, 1758)……..……92
3.2.1. Mitochondrial DNA (CO1 gene)…….……92
3.2.1.1. Mitochondrial population structure of *Merlangius merlangus* based on the CO1 gene……..…….....99
3.2.2. The cyt-b Gene……...101
3.2.2.1. Mitochondrial population structure of *Merlangius merlangus* based on the cyt-b gene…...……..109
3.2.3. CO1 and cyt-b Concatenated Data for *M. merlangus*……..……111
3.2.4. Nuclear DNA (RAG1 gene)……...116
3.2.4.1. Population structure of *Merlangius merlangus* according to the RAG1 gene……122
3.2.5. Demographic Analyses of *Merlangius merlangus* mtDNA and Nuclear DNA…….123
3.2.6. Discussion on Mitochondrial Phylogeography and Nuclear DNA Structure of *Merlangius merlangus*.…….…….....129
4. CONCLUSIONS AND RECOMMENDATIONS ……137
4.1. *Spicara spp*……..137
4.2. *Merlangius merlangus*……..138
REFERENCES…….140
REFERENCES NOT CITED……...……....174
APPENDIX A: MORPHOMETRIC MEASUREMENTS OF *SPICARA* INDIVIDUALS……175
APPENDIX B: CODON USAGE ANALYSES…….176
APPENDIX C: A LIST OF SEQUENCES WITH AMBIGUITY CODES FOR THE IRBP GENE….……188

APPENDIX D: PHOTOGRAPHS OF *Spicara spp.* SAMPLES COLLECTED FROM ISTANBUL…..…….....204
APPENDIX E: SAMPLING PERIODS AND LOCATIONS OF THE FISH SAMPLES…….216
APPENDIX F: TOTAL NUMBERS OF SAMPLES BASED ON THE SAMPLING SITES AND GENE RESULTS……217

LIST OF FIGURES

Figure 1.1. Turkish Straits Systems (Özsoy et al., 2016)……..……...6

Figure 1.2. Distribution map of *Merlangius merlangus* species (Froese and Pauly, 2017)…….….12

Figure 1.3. Distribution map of *Spicara maena* and *Spicara flexuosa* (Froese and Pauly, 2017)…14

Figure 1.4. Distribution map of *S. smaris* (Froese and Pauly, 2017)……....14

Figure 1.5. Individuals of three species of *Spicara spp.* a) *Spicara maena,* b) *S. flexuosa,* female c)*S. flexuosa*, male adult, d) *Spicara smaris*, male, e) *Spicara smaris* female adult.....16

Figure 2.1. Sampling sites of *Merlangius merlangus* and *Spicara flexuosa/maena* ……....18

Figure 2.2. Gel Image for PCR reactions for 16 SrRNA genes of *Spicara spp*……20

Figure 3.1. The haplotype network based on 16S rRNA sequences of *Spicara spp.*populations. The numbers correspond to the number of mutations between haplotypes. Each of the lines between the three haplogroups represents missing haplotypes……...……..……27

Figure 3.2. Sampling locations of three *Spicara* species, based on the 16S rRNA gene in the Mediterranean region…….28

Figure 3.3. Maximum likelihood tree based on 16S rRNA data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles with different colors. Size of the circles are proportional to the relative frequencies of the haplotypes……..……...…..……...……..…29

Figure 3.4. Neighbour-joining tree based on 16S rRNA data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles with different colors. Size of the circles are proportional to the relative frequencies of haplotypes……30

Figure 3.5. Maximum parsimony tree based on 16S rRNA data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles with different colors. Size of the circles are proportional to the relative frequencies of the haplotypes…….....…….…….……31

Figure 3.6. Bayesian tree based on 16S rRNA data for *Spicara spp.* populations. The node values correspond to posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles with different colors. Size of the circles are proportional to the relative frequencies of the haplotypes……...……..…...…….....32

Figure 3.7. The haplotype network based on CO1 sequences of *Spicara spp.* populations. The numbers correspond to the number of mutations between haplotypes. The line between Hap 11 and 14 represents a missing haplotype. The dashed line represents direct connection between *S. maena* and *S. smaris* haplogroups. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend……...……...…...37

Figure 3.8. Sampling locations of three *Spicara* species, based on the CO1 gene in the Mediterranean region……..…….....38

Figure 3.9. Maximum likelihood tree based on CO1 data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend…….……...39

Figure 3.10. Neighbour-joining tree based on CO1 data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend……....40

Figure 3.11. Maximum parsimony tree based on CO1 data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend…….……...41

Figure 3.12. Bayesian tree based on CO1 data for *Spicara spp.* populations. The node values correspond to posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend…….……42

Figure 3.13. The haplotype network based on the cyt-b data of all *Spicara spp.* populations. The numbers correspond to the number of mutations between haplotypes. The dashed line represents connection between *S. maena* and *S. smaris* haplogroups. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Black circles represent missing haplotypes……...……...49

Figure 3.14. Sampling locations of three *Spicara* species, based on the cyt-b gene, in the Mediterranean……..……...……..……..……50

Figure 3.15. Maximum likelihood tree based on cyt-b data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Size of the circles are proportional to the relative frequencies of the haplotypes……..……...……...…….....51

Figure 3.16. Neighbour-joining tree based on cyt-b data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Size of the circles are proportional to the relative frequencies of the haplotypes…….……..……...……52

Figure 3.17. Maximum parsimony tree based on cyt-b data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Size of the circles are proportional to the relative frequencies of the haplotypes….....53

Figure 3.18. Bayesian tree based on cyt-b data for *Spicara spp.* populations. The node values correspond to posterior probabilities of support for the branches. Locations of the

different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Size of the circles are proportional to the relative frequencies of the haplotypes……...……...……...54

Figure 3.19. Maximum likelihood tree based on cyt-b+CO1 concatenated data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend…….....60

Figure 3.20. Neighbour-joining tree based on cyt-b+CO1 concatenated data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend…….....61

Figure 3.21. Maximum parsimony tree based on cyt-b+CO1 concatenated data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend……..…….....62

Figure 3.22. Bayesian tree based on cyt-b+CO1 concatenated data for *Spicara spp.* populations. The node values correspond to posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend…….……...……63

Figure 3.23. The haplotype network of IRBP gene of *S. flexuosa* and *S. maena*. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Each of the lines between haplotypes represents a single mutational change…….…….64

Figure 3.24. Sampling locations of two *Spicara* species, based on the IRBP gene, in the Mediterranean region……65

Figure 3.25. Maximum likelihood tree based on IRBP data for *S. flexuosa* and *S. maena* populations. The node values correspond to bootstrap support. Locations of the different haplotypes

are given as circles, with each sampling locality indicated by different colors as given in the legend…….....66

Figure 3.26. Neighbour-joining tree based on IRBP data for *S. flexuosa* and *S. maena* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend…….....67

Figure 3.27. Maximum parsimony tree based on IRBP data for *S. flexuosa* and *S. maena* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend…….....68

Figure 3.28. Bayesian tree based on IRBP data for *S. flexuosa* and *S. maena.* The node values correspond to posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend……..…….……..69

Figure 3.29. Mismatch distributions for the CO1 gene for a) *S. flexuosa*, b) *S. maena.* Solid lines represent expected distribution and dotted lines represent observed distribution under the sudden expansion model. Y-axis represents the relative haplotype frequencies…….....75

Figure 3.30. Mismatch distributions for the cyt-b gene for a) *S. flexuosa*, b) *S. maena*. Solid lines represent expected distribution and dotted lines represent observed distribution under the sudden expansion model. Y-axis represents the relative haplotype frequencies…..…76

Figure 3.31. Mismatch distributions for the IRBP gene for a) *S. flexuosa*, b) *S. maena.* Solid lines represent expected distribution and dotted lines represent observed distribution under the sudden expansion model. Y-axis represents the relative haplotype frequencies…..…77

Figure 3.32. Bayesian skyline plot for *S. flexuosa* reflecting changes effective population size against time in thousands of years (Kya) before present……..…….....78

Figure 3.33. Box plots of measurements of each variable investigated, with baseline data for *S. flexuosa* and *S. maena* from Greece, and genetic data results from İstanbul……...…….79

Figure 3.34. Sampling locations of three *Spicara* species, based on the 16S, CO1, cyt-b and the IRBP genes, and the taxonomic evaluations made in previous studies in the Mediterranean ((Imsiridou et al., 2011; Georgiadis et al., 2014; Cannas et al retrieved from GenBank, (2010); and Krey et al retrieved from GenBank, (2007))……..……...……..84

Figure 3.35. Maximum likelihood tree based on CO1 data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend……...93

Figure 3.36. Neighbour-joining tree based on CO1 data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend……...94

Figure 3.37. Maximum parsimony tree based on CO1 data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend……...95

Figure 3.38. Bayesian tree based on CO1 data for *Merlangius merlangus* populations. The node values represent posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend…….……..……...……..…….……..96

Figure 3.39. The haplotype network of the CO1 gene of *Merlangius merlangus*. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Each line represents a single mutation…...……...…….……...……...…….…..97

Figure 3.40. The haplotype network of the cyt-b gene of *Merlangius merlangus*. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Each of the hatches over the lines between haplotypes represents one mutational steps……...…..……...…103

Figure 3.41. Maximum likelihood tree based on cyt-b data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend……..……104

Figure 3.42. Neighbour-joining tree based on cyt-b data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend…….…….105

Figure 3.43. Maximum parsimony tree based on cyt-b data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend…….……..106

Figure 3.44. Bayesian tree based on cyt-b data for *Merlangius merlangus* populations. The node values represent posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles with each sampling locality indicated by different colors as given in the legend……..…..……..……...107

Figure 3.45. Maximum likelihood tree based on cyt-b+CO1 concetanated data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.…….....112

Figure 3.46. Neighbour-joining tree based on cyt-b+CO1 concatenated data for *Merlangius merlangus* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend……113

Figure 3.47. Maximum parsimony tree based on cyt-b+CO1 concatenated data for *Merlangius merlangus* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend……...……..……..114

Figure 3.48. Bayesian tree based on cyt-b+CO1 concatenated data for *Merlangius merlangus* populations. The node values correspond to posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend……...115

Figure 3.49. The haplotype network for the RAG1 gene of *Merlangius merlangus*. Locations of the different haplotypes are given as circles with each sampling locality indicated by different colors as given in the legend. Each line represents a single mutational change…….....……117

Figure 3.50. Maximum likelihood tree based on RAG1 data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles with each sampling locality indicated by different colors as given in the legend……..……..118

Figure 3.51. Neighbour-joining tree based on RAG1 data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles with each sampling locality indicated by different colors as given in the legend……..……...…….119

Figure 3.52. Maximum parsimony tree based on RAG1 data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles with each sampling locality indicated by different colors as given in the legend……..……..120

Figure 3.53. Bayesian tree based on RAG1 data for *Merlangius merlangus* populations. The node values represent posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles with each sampling locality indicated by different colors as given in the legend……...……...121

Figure 3.54. Mismatch distributions based on CO1 gene for a) Total, b) *M. m. euxinus,* c) *M. m. merlangus,* d) TSS, e) Black Sea, f) Aegean populations. The solid lines represent the expected distribution and the dotted lines represent the observed distribution under the sudden expansion model. Y-axis represents the relative haplotype frequencies…….125

Figure 3.55. Mismatch distributions based on cyt-b gene for a) Total b) *M. m. euxinus* c) *M .m. merlangus* d) TSS e) Black Sea f) Aegean populations. The solid lines represent the expected distribution and the dotted lines represent the observed distribution under the sudden expansion model. Y-axis represents the relative haplotype frequencies…….126

Figure 3.56. Mismatch distributions based on RAG1 gene for a) Total b) *M. m. euxinus* c) *M. m. merlangus* d) TSS e) Black Sea f) Aegean populations. The solid lines represent the expected distribution and the dotted lines represent the observed distribution under the sudden expansion model. Y-axis represents the relative haplotype frequencies.....127

Figure 3.57. Bayesian skyline plot for *M. m. euxinus* reflecting changes effective population size against time in thousands of years (Kya) before present……128

LIST OF TABLES

Table 2.1. Primer name and sequences of mtDNA genes for *Spicara flexuosa/maena*……..20

Table 2.2. Primer name and sequences of mtDNA genes for *Merlangius merlangus*……21

Table 2.3. Primer name and sequences of nuDNA genes for *Merlangius merlangus* and *Spicara flexuosa/maena*.....22

Table 3.1. The accession numbers and location information of individuals for 16S rRNA sequences of *Spicara spp*. retrieved from GenBank……..……27

Table 3.2. Total number of mutations (below diagonal) and pairwise sequence divergences and divergence times (above diagonal) based on the 16S rRNA gene among the three species……..…...……..33

Table 3.3. Molecular diversity of *Spicara spp*. based on 16S rRNA region sequences. N: Number of samples; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations, when available are given in parantheses……..…33

Table 3.4. Parsimony informative sites of total *Spicara spp.* based on 155 bp long 16S rRNA region sequences……...……..……34

Table 3.5. The accession numbers and locations of the individuals for CO1 sequences of *Spicara spp*. retrieved from GenBank and BOLD…….....36

Table 3.6. Molecular diversity of *Spicara spp*. based on CO1 region sequences. N: Number of samples; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations are given in parantheses…….....……...43

Table 3.7. Parsimony informative sites of total *Spicara spp*. based on 454 bp long CO1 region sequences……..……..……..……..…….44

Table 3.8. Total number of mutations (below diagonal), pairwise sequence divergences and divergence times (above diagonal) based on the CO1 gene among the three *Spicara* species……..…...……....……..46

Table 3.9. Pairwise Φ_{ST} values of the CO1 gene between three species of *Spicara spp*. Significant *P* (<0.05) values are given in shaded cells……...47

Table 3.10. The accession numbers and locations of individuals for cyt-b sequences of *Spicara spp.* retrieved from GenBank……...……..…….....49

Table 3.11. Molecular diversity of *Spicara spp.* based on cyt-b region sequences. N: Number of samples; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations are given in parantheses…….....55

Table 3.12. Parsimony informative sites of total *Spicara spp.* based on 355 bp long cyt-b region sequences……..…….56

Table 3.13. Total number of mutations (below diagonal), pairwise sequence divergences and divergence times (above diagonal) based on the cyt-b gene among the three *Spicara* species……...……..58

Table 3.14. Pairwise Φ_{ST} values of the cyt-b gene between three species of *Spicara spp*. Significant *P* (< 0.05) values are given in shaded cells……..……...59

Table 3.15. Molecular diversity of *Spicara flexuosa* and *S. maena* based on IRBP gene sequences. N: Number of sequences; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations are given in parantheses…….....……70

Table 3.16. Parsimony informative sites of total *S. maena* and *S. flexuosa* based on 380 bp long IRBP region sequnces…….71

Table 3.17. Pairwise F_{ST} values of IRBP genes between *S. maena* and *S. flexuosa*. Significant *P* (< 0.05) values are indicated in shaded cells…….....72

Table 3.18. Neutrality test results for *S. flexuosa /maena* N: Number of samples; D: Tajima's D; FS: Fu's FS; and rg: raggedness statistic Significant values (*P*< 0.05) are indicated in bold……....74

Table 3.19. Morphometric measurements (HL, BD) of baseline *S. flexuosa* and *S. maena* collected from Greece and *Spicara maena* and *S. flexuosa* samples collected from İstanbul (cm)……...80

Table 3.20. The accession numbers and locations of individuals for the CO1 sequences of *Merlangius merlangus* that were retrieved from GenBank……...…….……..92

Table 3.21. Molecular diversity of *Merlangius merlangus* based on CO1 gene sequences. N: Number of samples; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations are given in parantheses. *Includes *M. m. merlangus* samples detected in these water bodies……...…….……...98

Table 3.22. Pairwise Φ_{ST} values for the CO1 gene of *M. m. merlangus* and *M. m. euxinus*. Significant *P* values are given in shaded cells (< 0.05)……..…...……100

Table 3.23. The accession numbers and locations of individuals for the cyt-b sequences of *Merlangius merlangus* that were retrieved from GenBank…….……...102

Table 3.24. Molecular diversity of *Merlangius merlangus* based on cyt-b gene sequences. N: Number of samples; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations are given in parantheses…….....108

Table 3.25. Pairwise Φ_{ST} values for the cyt-b gene of *M .m. merlangus* and *M. m. euxinus*. Significant *P* values are given in shaded cells (<0.05)……..……...…….....110

Table 3.26. Molecular diversity of *Merlangius merlangus* based on RAG1 gene sequences. N: Number of samples; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations are given in parantheses…….....122

Table 3.27. Pairwise F_{ST} values of the RAG1 gene for *M. m. euxinus*. Significant *P* values are given in shaded cells (< 0.05)……...…….……...123

Table 3.28. Neutrality test results for *M. m. euxinus* and *M. m. merlangus*. N: Number of samples; D: Tajima's D; FS: Fu's FS; and rg: raggedness statistic. Significant values (*P*< 0.05) are indicated in bold…….....124

LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Units used
dH_2O	Distilled water	
KCl	Potassium chloride	
$MgCl_2$	Magnesium chloride	
µl	Microliter	
π	Nucleotide diversity	

Abbreviation	Explanation
AGA	Stop codon
AGG	Stop codon
AIC	Akaike information criterion
AMOVA	Analysis of molecular variance
AUU	Isoleucine
bp	Base pair
BD	Body depth
BOLD	Barcode of Life Database
BSP	Bayesian Skyline Plot
CO1	Cytochrome c oxidase subunit 1
CR	Control region
CUU	Leucine
cyt-b	Cytochrome b
cyt-c	Cytochrome c
D loop	Displacement loop
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
Dxy	Nucleotide substitution values
ESS	Effective sample size
EOL	Encyclopedia of Life
FAO	Food and Agricultural Organisation
Fs	Fu's Fs
H	Haplotype diversity

H^+-ATP	Hydrogen Adenosine Triphosphate	
Hap	Haplotype	
HELCOM	Baltic Marine Environment Protection Commission	
HL	Head length	
IRBP	Interphotoreceptor retinoid binding protein	
ITIS	Integrated Taxonomic Information System	
IUCN	International Union for Conservation of Nature	
Ky BP	Thousands year before present	
MCMC	Markov chain Monte Carlo Calculation Strategy	
Mm	Milimolar	$10^0 mol/m^3$
MSC	Messinian Salinity Crisis	
mtDNA	Mitochondrial DNA	
Mya	Million year ago	
N	Number	
NADH	Nicotinamide adenine dinucleotide	
nuDNA	Nuclear DNA	
numtDNA	Nuclear mitochondrial pseudogenes	
Nh	Number of haplotypes	
Np	Number of polymorphic sites	
PCR	Polymerase chain reaction	
PIS	Parsimony informative sites	
pM	Pico mole	$10^{-6} mol/m^3$
RAD-seq	Restriction site associated DNA sequencing	
RAG1	Recombination activating one	
RFLP	Restriction fragment length polymorphism	
rg	Raggedness statistic	
RNA	Ribonucleic acid	
scnDNA	Single copy nuclear DNA	
SL	Standard length	
SS	Singleton variable sites	
Tris–HCl	Tris hydrochloride	
TBS	Türk Boğazlar Sistemi	
TSS	Turkish Straits System	
UAG	Stop codon	
UAA	Stop codon	

UUU	Phenylalanine
UUG	Leucine
UPGMA	Unweighted pair-group method with arithmetic mean
WGBS	Working Group of Black Sea
WoRMS	World Register of Marine Species
12S rRNA	12S Ribosomal RNA
16S rDNA	16S Ribosomal DNA
16S rRNA	16S Ribosomal RNA

1. INTRODUCTION

Phylogeography is a field of study concerned with investigating the principles of the geographic distributions of species and their genes. As stated by Avise et al. (1987), historical events ultimately shape the geographical distribution of genes, populations and species. In the marine environment, phylogeographic studies help to estimate local levels of biodiversity of the species in restricted geographical areas and dispersal patterns of organisms, and helps with the conservation of species and identification of marine protected areas. In this respect, to understand evolutionary and genetic relationships between species, levels of gene flow, the effects of geographical barriers, and details of life-histories of species can be investigated. In essence, presence of gene flow influences genetic similarity between populations due to dispersal of the individuals between geographical regions (Rocha et al., 2002; Klanten et al., 2007). On the other hand, restriction of gene flow due to barriers, ecological factors and biology of the organisms result from limited dispersal of the species (Rocha et al., 2005). In addition, these approaches can be used to assess genetic identity, nonidentity and parentage (Avise, 1994).

In the aquatic environment, fish are one of the most important natural resources and reduction of the genetic diversity of natural fish populations has become an important fisheries management problem. In this respect, conservation, management and phylogeographic studies of natural fish populations require the use of molecular markers to identify the representatives of individual stocks (Garte, 1994).

Earlier genetic studies on fishes were based on proteins, and allozyme electrophoresis was used to make quantitative assessments of natural fish population structure and stock identification (Clayton et al., 1974; Philip et al., 1983; Allendorf et al., 1987; Ihseen et al., 1988; Billington and Hebert, 1991). Allozyme methods depend on variability of particular enzymes and provide data on single locus genetic variation. However, the main disadvantage of allozyme methods is that they usually show low levels of detectable protein variability to discriminate taxa (Billington and Hebert, 1991).

On the other hand, DNA sequence analyses help to understand genetic relationships of fish species at different taxonomic levels. Most of the initial DNA applications were based on the mitochondrial DNA (mtDNA) (Lansman et al., 1981; Avise, 1987). mtDNA is a double stranded, circular molecule, approximately 15-17000 base pairs long in animals (Ladoukakis and Zouros,

2017). It includes 13 protein coding genes (seven sub-units of NADH-ubiquinone oxireductase, three sub-units of cytochrome c oxidase, two sub-units of the H^+-ATP synthase and cytochrome b), 22 transfer RNA, two ribosomal RNAs and also non-coding control region displacement (D) loop, which evolves rapidly and contains sites for replication and transcription (Graur and Li, 2000).

mtDNA is a maternally inherited molecule, and has been widely used in phylogeographic studies of fish species (Bernatchez, 2001; Rocha et al., 2007; Ketmaier et al., 2008). The rapid evolution rate of mtDNA makes it useful to discriminate closely related species, more so than other molecular markers (Graur and Li, 2000). Moreover, mtDNA has some other advantages in animal population genetics. The mtDNA of different species of animals are amplified by universal primers, and can easily be isolated from the rest of the cells' DNA. Mutation rate of the mtDNA is high, and these high mutation rates provide more information on genetic divergence over evolutionary time scales. Also haploid mtDNA has a lower effective population size than diploid nuclear DNA, and therefore is more likely to show differences in intraspecific population structure, and help with detection of reduction of genetic variability due to population bottlenecks (Nei and Tajima, 1981; Birky et al., 1983). Unlike nuclear DNA, haplotype frequencies can drift rapidly in mtDNA, creating genetic differences among populations in relatively short timescales (Friesen et al., 1996).

Apart from mtDNA, nuclear DNA (nuDNA) is also useful for phylogeographic analyses of fishes (Li et al., 2009; Lautredu et al., 2013). However, single-copy nuclear DNA (scnDNA) or non-repetitive DNA loci that consist of exons (coding) and introns (noncoding) regions are not sufficient for an effective understanding phylogeography of fish species. Informative scnDNA genotypes combined with mtDNA provide more discriminatory resolution for identification of fish population genetic structure (Bagley and Gall, 1998). In the scnDNA, non-coding intron sequences are more variable than coding regions (exons). Consequently, researchers more traditionally examine introns, exons and/or specific mtDNA genes to obtain a more complete picture of fish population genetics (Dominques et al., 2007; Li and Orti, 2007; Varon and Orti, 2009; Ilves and Taylor, 2009; Carvajal-Vallejos et al., 2010).

1.1. Phylogeographical Hypotheses and Studies of Marine Fish Species

As mentioned in the previous section, historical biogeographical factors, as well as ecology and behavior of organisms have important roles in shaping genetic structure of species (Avise et al.,

1987). These concepts help to invoke some intraspecific phylogeographical hypotheses, as outlined below:

i) Gene flow, absence of barriers and life histories of species lead to shallow phylogeographic population structure.

ii) Presence of barriers to gene flow ultimately shapes phylogenetic gaps between intraspecific monophyletic groups. This phenomenon leads to deep phylogeographic population structure within species.

In this perspective, mtDNA phylogeographic studies of marine fish species have shown that in many cases, long-term biogeographical barriers and different oceanic regions limiting genetic exchange provide explanation for major deep phylogeographic structure, such as seen in coastal killifish (*Fundulus heteroclitus*) (Powers et al., 1991), deep sea fish (*Cyclothone alba*) (Miya and Nishida, 1997), European hake (*Merluccius merluccius*) (Quenteiro and Rey-Mendez, 2000; Lo Brutto et al., 2004), red mullet (*Mullus barbatus*) (Maggio et al., 2009), and mackerel (*Trachurus sp*.) (Cardenas et al., 2005).

In other respects, shallow intraspecific phylogeographic structure and strong lineage connections were observed in several pelagic marine fish such as tuna (*Thunnus thynnus*) (Graves and Dizon, 1989; Scoles and Graves 1993; Ward et al., 1994; Chow and Ushiama, 1995), anchovy (*Engraulis encrasicolus*) (Magoulas et al., 1996), horse mackerel (*Trachurus trachurus*) (Cimmaruta et al., 2008; Comesana et al., 2008), Adriatic and Mediterranean sardines (*Sardina pilchardus*) (Carvalho et al., 1994), and North Atlantic sardines (*Sardina pilchardus*) (Baibai et al., 2012). In addition, mtDNA lineage separation within regional populations of most marine fish species appear to be shallow and show star-like lineage connections; these species include the Atlantic herring (*Clupea harengus*) (Kornfield and Bogdanopwicz, 1987), the hake (*Merluccius sp*.) (Becker et al., 1988; Lundy et al., 2000), Atlantic cod (*Gadus morhua*) (Carr and Marshall, 1991; Arnason et al., 1992; Arnason and Palsson, 1996), and sardine (*Sardina pilchardus*) (Atarhouch et al., 2006). Other studies have demonstrated that some of the bottom-dwelling marine fish species display only shallow intraspecific phylogeographic structure, such as the hammerhead shark (*Sphyra lewini*) (Duncan et al., 2006; Quattro et al., 2006, and some coral reef fish (*e.g. Halichoeres radiatus*) (Rocha et al., 2007; Horne et al., 2008).

However, shallow phylogeographic structure may not always result in genetic homogenity. Significant genotypic frequency differences were observed within several fish species among different geographical regions, such as seen in swordfish (*Xiphias gladius*) (Kotoulas et al., 1995), sardine (*Sardina pilchardus*) (Grant et al., 1998), and horse mackerel (*Trachurus trachurus*) (Karaiskou et al., 2003), and these results had relevant and important implications for stock identification and population management (Spanakis et al., 1989; Roldan et al., 1998; Castillo et al., 2004; Kristoffersen and Magoulas, 2008; Baibai et al., 2012).

1.2. General Characteristics of the Study Area

1.2.1. Hydrological and Biological Characteristics of the Turkish Straits System

Turkish Straits System (TSS), consisting of the Bosphorus, the Sea of Marmara and the Dardanelles, plays a significant role for the protection of biodiversity in the Black Sea (Öztürk, 1998). The TSS is a biological corridor for migratory species of fish and mammals from both the Black Sea and the Mediterranean Sea, and also feeding and spawning ground for the pelagic fish of Atlantic origin during their migrations from the Black Sea to the Sea of Marmara or vice versa. However, due to presence of anthropogenic pollution, Bosphorus lost some of its past significance as a biological corridor (Öztürk and Öztürk, 1996). The TSS also serves as an acclimatization zone for marine species (such as introduced marine invertebrates and fish species) in the Sea of Marmara and the Black Sea, due to its distinguishing hydrographical characteristics (Öztürk and Öztürk, 1996). Apart from being an acclimatization zone, TSS serves as a physical barrier against dispersion of fish larvae, planktonic species, and fish. This physical barrier (Nara passage zone of the Dardanelles Strait) prevents the fish larvae and planktonic species entering into the Black Sea from the Aegean, due to strong vertical current structure in the Dardanelles, the suboxic anoxic basin in the Sea of Marmara, and sudden temperature and salinity changes between the Aegean and the Black Sea (Öztürk and Öztürk, 1996).

Considering hydrology, temperature, salinity, and current systems are some of the important physical parameters in the TSS. The temperature and the salinity of the surface layer of the Bosphorus are under the influence of the Black Sea, and range from 4 to 24 °C and 18 ‰ to 23 ‰, respectively. On the other hand, the temperature and the salinity of the surface layer of the Dardanelles, which are under the influence of the Mediterranean Sea, range from 6 to 26 °C and 24 ‰ to 37 ‰ respectively (Kocataş et al., 1993).

TSS consists of two layers of water, Black Sea and Mediterranean origin, separated by a transitional layer. The Black Sea water enters the Bosphorus as an upper layer and exits through the Dardanelles Strait. On the other hand, the Aegean water enters the system as a lower layer, and exits into the Black Sea through the Bosphorus underflow. The upper layer contains around 230 km^3 of water, and average residence time of this water is four to five months (Beşiktepe et al., 2000). Life in the upper layer is nourished by the brackish waters of the Black Sea. The lower layer that contains about 3400 km^3 of water, is influenced by the Aegean Sea and the Mediterranean, with a renewal time of six to seven years (Tuğrul and Salihoğlu, 2000). Finally, the Sea of the Marmara (Figure 1.1) is a relatively small basin (size: ~70 km x 250 km, surface area: 11500 km^2, maximum depth 1350 m), located between Europe and Asia, and connecting with the Mediterranean and the Black Sea, respectively through the Dardanelles (length:-75 km, width: -1.3-7.0 km) and the Bosphorus (length: 35 km, width: -0.7-3.5 km) (Özsoy et al., 2016).

1.2.2. Paleoceanography of the Marmara Sea

During the early and Middle Miocene, the current location of the Sea of the Marmara was covered by the lakes, and fluviolacustrine conditions prevailed, as indicated by the deposition of the fresh water ostracod and mollusks deposits in its sediments (Yaltırak, 1995). In the late Miocene, the first brackish marine water (Paratethys) was inundated and this incursion occupied the fluviolacustrine area mentioned above. This intrusion might have been the result of desiccation of the Mediterranean during the Messinian (Hsü, 1978). In the late Pliocene, the faunal distribution of the sea indicates that, the Sea of the Marmara was first connected to Paratethys and then to the Mediterrenean. At that time, the Strait of the Dardanelles was first formed (Erol, 1992). During the glacial periods in the Pleistocene, the brackish-marine conditions influenced by the Black Sea were observed, however, during the interglacial period, the basin was covered by water from both the Mediterranean and the Black Sea. Approximately 20 Kya, when Karangat Sea (the name referring to the Black Sea during the Riss-Würm Interglacial) was replaced by the Neoeuxian lake (the name of Black Sea during Upper Würm Glacial), its salinity was reduced after losing its contact with the surrounding seas. At this period, the supply of the meltwater from glaciers to the Pontic basin was direct, and the water level in the Black Sea increased quickly, surpassing the Bosphorus sill level by around 12 ky BP. During the Neoeuxinian phase, there was a freshwater outflow from the Neoeuxian lake in to the Aegean Sea through the Bosphorus and Dardanelles (Kvasov, 1975). About 7500 ya, around the beginning of the Holocene, however, contact with the Mediterranean was re-established when the Mediterranean and the Black Sea reached similar water levels (Ryan et al., 1997; Panin and

Popescu, 2007). At that time, Mediterranean marine fauna and flora were established in the Sea of the Marmara (Zaitsev and Mamaev, 1997), and subsequently, suboxic conditions in the Marmara Sea was replaced by fully marine conditions. These suboxic conditions are currently observed below depths of 100 m in the Sea of Marmara (Aksu et al., 2002).



Figure 1.1. Turkish Straits Systems (Özsoy et al., 2016).

1.2.3. Hydrological Characteristics of the Black Sea

The Black Sea, which is connected with the Sea of Azov through the Kerch Strait in the north, and with the Mediterranean in the south through the TSS, is one of the largest and deepest inland seas in the world (Oğuz et al., 1990). The conservation mass and salinity of the Black Sea are maintained by the double layer flow of the TSS. The less saline water of the Black Sea flows into the Mediterranean Sea through the Bosphorus, on the other hand more saline water enters the Black Sea via the Dardanelles, as mentioned above. The exchange of the water maintains the salt balance of the Black Sea. The salinity of the deep water is 22.2 -22.4 ‰, and the salinity of the surface water is 13-18.5 ‰, and is influenced by evaporation, precipitation and river run-off (Özsoy et al., 2002). As a result of this, the Black Sea is characterized by a stable pycnocline between 100 to 200 meters in water depth that separates brackish surface water (~18 ‰) from more saline deep water (~22 ‰) of Mediterranean origin (Özsoy et al., 2002). Stratification leads to anoxic conditions below depths of ~150 m. The Black Sea waters are exposed to mixing and convection throughout the year especially during the winter months, however in the summer, temperature stratification is observed.

1.2.4. Geological Evolution of the Black Sea

During early or Middle Miocene, Black Sea basin was filled by salt water of Mediterranean origin, and the fauna was that of the Meditteranean. Connection between Black Sea and other seas was lost at the end of the Middle-Miocene. The formation of the Black Sea began at the end of the Miocene (7-5 Mya), as a result of the formation of several brackish lakes in these basins. One of them was the Sarmatic Sea that consisted of the modern Black Sea, Aral Sea, Azov Sea and Caspian Sea (Kosswig, 1954). The salinity of the Sarmatic Sea was very low since it was separated from the oceans, and due to inflow from rivers. Due to these extremely freshwater conditions, the fauna of the Sarmatic Sea changed, and marine species, such as Echinoderms and Cephalopods dissapperad (Kosswig, 1954). At the end of the Miocene, the Sarmatic Sea connected to the world ocean, and at the beginning of the Pliocene, the Pontian Sea lake was formed. The connection to the ocean was severed and marine fauna disappeared in the Pontian Sea-lake (3-1.5 My BP), and were replaced by the brackish-water fauna (Caspers, 1957). In the Quaternary Period and the ice ages, the salinity and composition of the Black Sea were changed, the Pontian Sea-lake shrunk in the size, and Chaudian Sea-lake was formed. In the late Mindel Glaciation (500.000 to 400.000 ya) Chaudian Sea lake turned into the Paleoeuxian basin and connected to the Sea of the Marmara through the Bosphorus. At that time, the Sea of the Marmara was isolated from the Mediterranean and was influenced by the low saline conditions. In the Riss-Wurm Interglacial Period, (150.000-100.000 ya), the Black Sea got connected to the Mediterranean, and following the opening of the Dardanelles, Karangat Sea was formed with its salinity higher than that of the present day the Black Sea. Approximately, 20 000 to 18 000 ya, the Karangat Sea was replaced by the Neoeuxian lake, its salinity was reduced due to its lost communication with the sea, however, Pontian species survived. About, 10.000 ya, modern Black Sea was established and finally, 7000 (ya), Black Sea was connected to the Mediterranean Sea through the Dardanelles and Bosporus (Zaitsev and Mamaev, 1997).

1.2.5. Hydrological Characteristics of the Aegean Sea

The Aegean Sea, located between Greece to its west and Anatolia to its east, is connected to the Marmara and the Black Sea by the Bosphorus and Dardanelles, and has a total area of 214.000 square kilometres. The general circulation of the Aegean Sea is determined by the different current systems. The northern Aegean Sea is influenced by the presence of the low saline and low temperature Black Sea waters that enter the Aegean through the straits of the Dardanelles, and by the fresh water discharge of the rivers into the Thermaikos Gulf. As opposed to this, a large part of the northeast

Aegean is affected by the warm and saline waters from the Mediterrenean Sea. The presence of such different water-masses create strong temperature and salinity differences between cold and fresh areas to the north and northwestern, and warm and saline areas to the south and southeastern of the northern Aegean basin (Nittis and Perivoliotis, 2002). Moreover, southern and central portions of the Aegean Sea are also influenced by the Mediterranean waters.

1.2.6. Hydrological Characteristics of the Mediterranean Sea

The Mediterranean Sea is considered to be one of the most important semi-enclosed water bodies in the world, and it contains 4 % of the marine fish species biodiversity of the world (Keskin et al., 2011). The Levantine Sea, which is adjacent to the southern coast of Turkey, is located to the eastern part of the Mediterranean Sea, and is connected to the Aegean Sea through the islands of Crete, Karpathos and Rhodes. It is characterized by Levantine surface and deep water which are warmer (16-29 ^{0}C) and more saline (37-39 ‰) than the Aegean and the Black Sea waters. The water is characterized by some circulation and gyres, and surface water temperatures that range between 19 ^{0}C to 22 ^{0}C in the winter months. On the other hand, temperature ranges from 25 to 27 ^{0}C in the summer months. Salinity values are generally about 38.8 ‰ in the summer, but in the winter salinity values can reach values higher than 39.1 ‰ on the surface water. On the other hand, deep water temperature and salinity are generally at 14 ^{0}C and 38.8 ‰, respectively (Özsoy et al., 1989).

1.2.7. Geological Evolution and the Origin of the Mediterranean Biota

The breakup and dispersal of the African and Eurasian plates generated a series of sub-basins cumulatively referred to as Tethys. During the Mesozoic, the Tethys surrounded the continental masses of the Pangea, forming a large gulf and forcing Pangea into the west (Por, 1989; Goffredo and Dubinsky, 2014). This process was important and led to separation of the eastern Mediterranean, Paratethys, which is currently represented by its residuals, the Black Sea and the Caspian Sea. In the Miocene and Pliocene (23-3 Mya), the western Mediterranean and the eastern Mediterranean had very similar oceanographic conditions. However, at the the end of the Miocene (ca. 5.6 Mya), Messinian Salinity Crises led to dramatic changes in the sedimentation dynamics and to complete extinction of ancient marine biota in the Mediterranean and Black Sea (Kosswig, 1954). After the opening of the Gibraltar Strait in the post-Messinian period, the normal oceanic conditions were established in the Pliocene and the Pleistocene (Krijgsman et al., 1999), and these conditions led to the continuous exchange of the Mediterranean and Atlantic marine biota through the Gibraltar Strait.

This was the only connection between the Mediterranean Sea and other oceans until the opening of the Suez Canal in 1869. For this reason, the native biota in the Mediterranean is of Atlantic origin. The Mediterranean Sea was transformed to an inland sea after the last glaciation period in the late Pleistocene (100.000 ya), and divided into two basins, the eastern and western Mediterranean (Kosswig, 1954). Formation of endemic species in the Mediterranean was accelerated by the partial isolation of the Mediterranean from the Atlantic (Goren, 2014), with climatic and hydrologic variability being the two main factors causing the high diversity of species in this sea (Bianchi and Mori, 2000).

1.3. Literature Review on Genetic and Phylogeographic Studies of Marine Fish Species in Turkey

Genetic and phylogeographic studies of fish species focus on freshwater and marine resources in Turkey. However, in this review, only marine fish studies are summarized. Focusing on individual species, Turan and Gurlek (2003) investigated genetic and morphologic structure of the European hake (*Merluccius merluccius*) in the Sea of Marmara and northeastern Mediterranean Sea. Genetic analysis was conducted by protein electrophoresis. Although they found 100 % morphometric differentiation of the samples, no allele frequency differences were detected between the hake stocks in the Sea of Marmara and northeastern Mediterranean.

Phylogenetic relationships of nine mullet species (*Mugil cephalus, Mugil soiuy, Liza ramada, Liza aurata, Liza abu, Liza saliens, Liza carinata, Chelon labrosus, Oedalechilus labeo)* in the Mediterranean and the Black Sea region was studied by Turan et al. (2005), with allozyme electrophoresis. Phylogenetic relationships of these mullet species was not clear based on an UPGMA tree. As opposed to Turan et al. (2005), Ergüden et al. (2010) investigated the same nine species based on 16S rDNA mtDNA sequence data. The clear identification of the genus and species based on 16S rDNA data showed that mtDNA had greater resolution than allozymes in inferring evolutionary relationships in this group of species.

Ergüden and Turan (2005) investigated genetic and morphological relationships of the sea bass (*Dicentrarchus labrax)* in the Turkish seas. Allozymes methods were used to understand the extent of the genetic differentiation. This preliminary study revealed that the morphological differentiation of the sea bass populations in four different seas was not reflected in genetics. Mitochondrial and

microsatellite nuclear DNA analyses were suggested to help detection of the corresponding genetic component of the phenotypic differentiation.

Genetic and morphological divergence and phylogenetic relationships of four species -*Mullus barbatus*, *Mullus surmuletus*, *Upeneus moluccensis*, *Upeneus pori*- and one subspecies *M. barbatus ponticus*, were investigated with allozymes and morphological data (Turan, 2006) and mtDNA cyt-b, 12S rRNA, and cty-c oxidase genes (Keskin and Can, 2009). The amount of genetic distance (0.034) between *M. barbatus barbatus* and *M. barbatus ponticus* was found to be large enough to supported the idea that *M. barbatus ponticus* was a subspecies of *M. barbatus.* Fisher's exact test and neighbour-joining analysis also confirmed this result. As opposed to Turan (2006), Keskin and Can (2009) showed that based on mtDNA analyses, genetic differences between *M. barbatus* and *M. barbatus ponticus* were not large, and they concluded that *M. barbatus ponticus* should not be considered as a separate subspecies.

Phylogenetic relationships of the three species of *Trachurus,* horse mackarel (*T. trachurus*), Mediterranean horse mackarel (*T. mediterraneus*), and blue jack mackarel (*T. picturatus*) from the coastal regions of Turkey was investigated by Bektaş and Beldüz (2008) using entire mtDNA control region (CR) and partial cytochrome (cyt-b) sequences. The results indicated no geographical differences in haplotype frequencies, and revealed genetic similarities that suggested gene flow between populations in the Black Sea and the Mediterranean Sea. As opposed to Bektaş and Beldüz (2008), a sharp genetic and geographical break between the Mediterranean horse mackarel (*Trachurus mediterraneus)* and Atlantic horse mackarel (*Trachurus trachurus*) were detected between the Black Sea and northeastern Mediterranean, by restriction fragment length polymorphism (RFLP) analysis of the mtDNA 16S gene, from seven locations in the Black Sea, the Sea of Marmara, the Aegean and eastern Mediterranean (Turan et al., 2009a; 2009b).

In another study, Sarmaşık et al. (2008) investigated genetic and morphological differences among the Turkish sardine (*Sardina pilchardus*) populations in the Aegean, the Sea of Marmara and the Mediterranean Sea by using mtDNA sequences from the cyt-b gene. Phylogenetic trees (neigbour-joining and maximum likelihood) demonstrated small genetic divergence between the populations. This result confirmed that genetic mixture and gene flow were the main concepts that shaped the sardine populations in the Turkish coastal waters.

Genetic and morphological differentiation of European anchovy (*Engraulis encrasicolus*) was investigated through allozyme analyses performed by the Erdoğan et al. (2009) in the Black Sea, the Sea of Marmara and the Aegean Sea. They found that the Aegean and the Sea of Marmara samples were the most isolated when compared to the others. The analyses also indicated high variability, but low levels of differentiation in the eastern Black Sea samples. On the other hand, Keskin and Atar (2012) studied European anchovy (*Engraulis encrasicolus*) from all Turkish coastal waters using the mitochondrial CO1 gene, and assessed the genetic structure among these populations. They indicated that the highest nucleotide divergence values were found between eastern Mediterranean and northern Aegean Sea, and the lowest between eastern Black Sea and the Sea of Marmara.

1.4. The General Biology and Taxonomy of Whiting and Picarel

1.4.1. Taxonomic Status and Geographical Distribution of *Merlangius merlangus*

FishBase, World Register of Marine Species (WORMS), California Academy of the Sciences (Catalog of Fishes) (Eschmeyer et al., 2018), ITIS (Integrated Taxonomic Information System), EOL (Encyclopedia of Life) and Marine Species Identification Portal are online databases that provide information about taxonomy, geographical distribution, ecology, behavior and habitat of fish and other animal species. All these online databases and other sources categorize whiting under the order Gadiformes and Gadidae family, as predominantly marine benthopelagic fish, found in the world oceans especially circumpolar to temperate waters, mainly in the northern hemisphere. *Merlangius merlangus* inhabits coastal regions in the eastern North Atlantic between Iceland to Portugal, and is also distributed in the Black Sea, the Aegean, the Adriatic and neighbouring areas, with a limited distribution in northwestern Mediterranean (Bini, 1969; Svetovidov, 1973; Baily, 2008; Whitehead et al., 1986; Wheeler, 1991; Cohen et al., 1990; Parin et al., 2014). (Figure 1.2). According to California Academy of the Sciences (Catalog of Fishes), the type locality of *Merlangius merlangus* is European Sea (Linnaeus, 1758). *Merlangius merlangus* is divided into two subspecies, namely *M. merlangus merlangus* and *M. merlangus euxinus*, and the type locality of *Merlangius merlangus euxinus* is Crimea, Black Sea (Nordmann, 1840). *M. merlangus merlangus* occurs along the European coasts from Iceland and south-western Barents Sea to northern coasts of Portugal and western Baltic, and with a restricted distribution in the western Mediterranean (Schmidt, 1905; Ancona, 1933). *M. merlangus euxinus*, on the other hand, inhabits the Black Sea, adjoining areas of the Azov Sea, the Sea of Marmara, the Aegean Sea and the Adriatic (Svetovidov, 1935; Vodjanitzki and Kazanova, 1954).

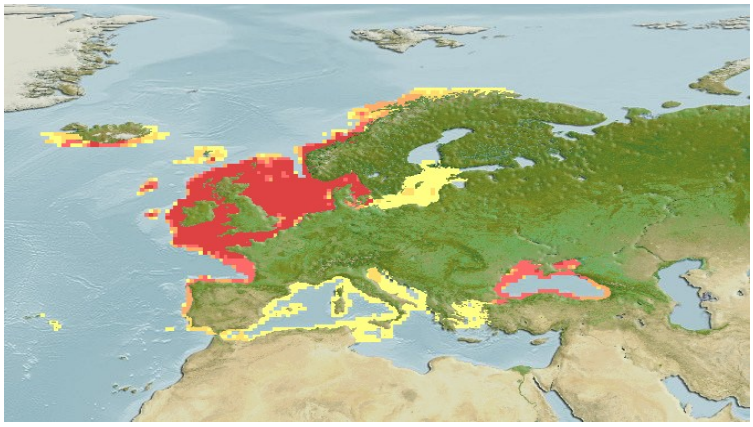


Figure 1.2. Distribution map of *Merlangius merlangus* (Froese and Pauly, 2017).

The most important diagnostic feature of the two *Merlangius merlangus* subspecies is the presence/absence of a small barbel on the chin. In greater detail, the two subspecies are identified by these diagnostic features: *Merlangius merlangus euxinus* (Nordmann, 1840) has a barbel on the chin, with its pectoral fin reaching about 15.4-18.2 % of the body length. On the other hand, *Merlangius merlangus merlangus* does not have a barbel on the chin, and its pectoral fin is around 13.8-15.6 % of the body length (Whitehead et al., 1986). Another striking feature is that its three dorsal fins are isolated by a small distance, whereas, two anal fins are located very close to each other; and the pectoral fins have a slightly elongated ray that reaches beyond to the origin of anal fin. The three dorsal and two anal fins which are unique and diagnostic for the Gadoids, comprise strong evidence supporting the monophyly of Gadinae (Svetovidov, 1948). A small dark spot is another distinguishing feature in the pectoral fin, and the color of the species is variable.

1.4.2. Habitat and Biology of *Merlangius merlangus*

Merlangius merlangus is a benthopelagic, eurohaline, and eurothermal species that is mainly observed on mud, gravel, sand and rock areas and lives in shallow waters, and rarely below 200 m. The juveniles are found in shallower waters than adults, and between 5 to 30 m depth, and can tolerate low salinities. Migration of the species is observed through the open sea after the first year, and occurs between the spawning areas and different feeding areas. Spawning of the species is generally detected at depths spanning 20 to 150 m. throughout the winter and spring season in the Mediterranean, and during the whole year in the Black Sea. Fecundity of the species is very high and depends on the female size. The eggs of the species are pelagic, and its larvae become demersal when they reach 5 to 10 cm in length. During the pelagic period of their life, larvae of the species are dispersed via sea

currents. Adult size of the species is between 23 to 70 cm (Svetovidov, 1948; Nagabhushanam, 1964; Garrod and Gambell, 1965; Bini, 1969; Fischer et al., 1987; Cohen et al., 1990).

1.4.3. Taxonomic Status and Geographical Distribution of *Spicara spp.*

Phylogenetic position of the Sparidae and Centracanthidae family members is uncertain based on the literature (Orell et al., 2002; Orell and Carpenter, 2004; Chiba et al., 2009). The monophyly of the family Sparidae has not yet been supported, primarily because of the uncertainty of the position of its sister family Centracanthidae (*Spicara* and *Centracanthus* spp.). According to Nelson (2006) the two families are distinct and separate. The inclusion of the family Centracanthidae in the family Sparidae has repeatedly been considered, based on both morphological (Jordan and Fesler, 1893; Day, 2002) and molecular studies (Orrell and Carpenter, 2004; Hanel and Tsigenoplous, 2011). Both Orell et al. (2002) and Orell and Carpenter (2004) showed that the currently defined sub-families of Sparidae were not monophyletic and *Spicara* was the member of the Sparidae family group. Later, Chiba et al. (2009) found that Sparidae family members divided into the three major clades, and some sub-clades. However, most of the sub-families in the Sparidae were not monophyletic. The Sparidae or Centracanthidae family consists of two genera (*Spicara* and *Centracanthus*) (Froese and Pauly, 2017) and nine species, namely *Spicara maena*, *Spicara smaris*, *Spicara alta*, *Spicara melanurus*, *Spicara martinicus*, *Spicara nigricauda*, *Spicara axillaris* and *Spicara austrialis* and *Centracanthus cirrus*. The genus *Spicara* was also found to be nonmonophyletic; for instance, *Spicara alta* was placed in Clade A and *S. maena*, *S. flexuosa* and *S. smaris* were found in the Clade B of the Sparidae family (Chiba et al., 2009; Sanciangco et al., 2016). Studies conducted by the Santini et al. (2014) also found nonmonophyly of the genus *Spicara*, specifically *Spicara maena*, *S. smaris*, and *S. axillaris* were found in different subclades. On the other hand, other Sparidae family members such as *Spondylisma cantharus* were observed to be more closely related to *S. maena* and *S. smaris*, when compared to other *Spicara* species such as *S. austrialis*, *S. axillaris*, *S. alta* (Chiba et al., 2009; Santini et al., 2014; Sanciangco et al., 2016). According to California Academy of the Sciences (Catalog of Fishes), World Register of Marine Species (WORMS), ITIS, EOL and Marine Species Identification Portal, *Spicara* species are currently in the Centracantidae family, however, based on the FishBase data, *Spicara* spp. is placed within the Sparidae.

According to California Academy of the Sciences (Catalog of Fishes), the type locality of *Spicara* is Sicily, Mediterranean Sea (Rafinesque, 1810) and *Spicara flexuosa* is considered as a valid species. WORMS (World Register of Marine Species), ITIS, and EOL accept *Spicara flexuosa* as a

valid species (Baily, 2017). Based on FishBase and Marine Species Identification Portal (Froese and Pauly, 2017), on the other hand, *Spicara flexuosa* is considered as a synonym of *Spicara maena*, however recent morphological (Minos et al., 2013) and genetic studies (Imsiridou et al., 2011; Georgidias et al., 2014 and Bektaş et al., 2018) suggest that *Spicara flexuosa* should be classified as a distinct species.

Picarels are small to medium sized fish, and the genus is native to the eastern Atlantic Ocean and the western Indian Ocean. *Spicara maena/ flexuosa* is of Atlanto-Mediterrenean origin and inhabits temperate to warm waters and is largely distributed in the Adriatic, the Ionian and the Mediterranean Sea, and is also found from Portugal to Morocco in the Atlantic, and with a limited distribution in the Black Sea (Dulcic et al., 2000; Froese and Pauly, 2017) (Figure 1.3). *Spicara flexuosa* is observed in the eastern Atlantic, Mediterranean, Portugal and the Black Sea (Salekhova, 1979; Tortonose, 1986; Mytilineou and Papaconstantinou, 1991; Golani et al., 2006). Finally, *Spicara smaris* is recorded in the Mediterranean, Atlantic coasts of Portugal, Morocco and the Black Sea (Tortonose, 1986) (Figure 1.4).

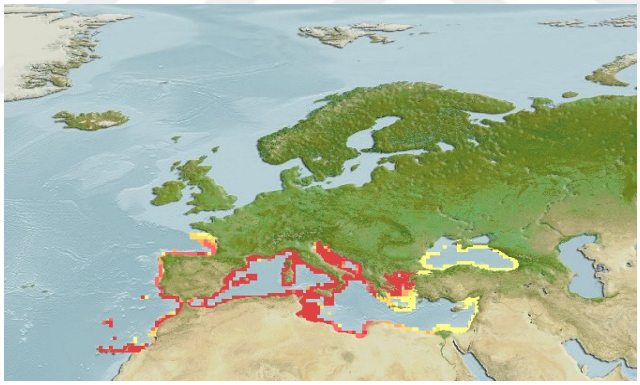


Figure 1.3. Distribution map of *Spicara maena* and *Spicara flexuosa* (Froese and Pauly, 2017).

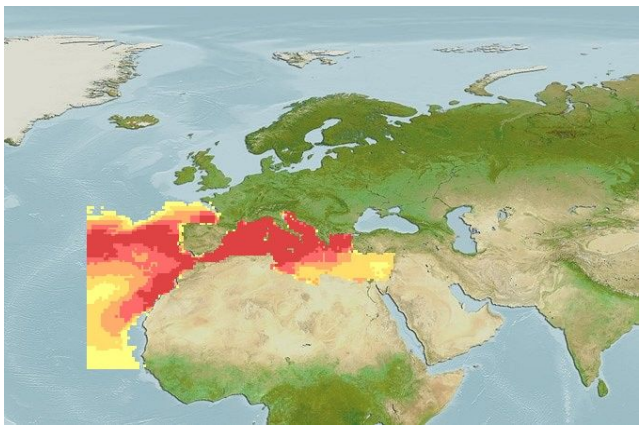


Figure 1.4. Distribution map of *Spicara smaris* (Froese and Pauly, 2017).

1.4.4. Habitat and Morphologic Features of *Spicara spp.*

The three species of *Spicara* are demersal to mid water, and neritic species. Adults are found in the neritic zone, and generally observed in temperate and warm waters over the *Posidonia* beds, and on sand or muddy bottoms (Tortonose, 1986). *Spicara maena* is a gregarious to schooling species, and is usually found in mud, and bottom regions of rocks at depths ranging from 20 to 120 m. Although it is a demersal species, it usually inhabits the coastal waters in warm seasons, and migrates into deeper waters (100 m) in colder months. The individuals are demersal spawners, and spawning occurs near the coastal waters between the months of May and August. Spawning and reproduction are increased with female's age and size (Dulcic et al., 2000). *Spicara flexuosa* is an inshore-pelagic schooling, non-migratory, necto-benthonic species found in sand or muddy bottom, and observed at depths down to 130 m., although it occasionally moves to surface water column (Imsiridou et al., 2011). Spawning is observed between months of March to May. Their eggs are also demersal. *Spicara smaris* prefers deep water, especially in winter and is found over vegetated bottoms and posidionia beds, at depths ranging from 15 to 100 m. Spawning is observed between months of February to May (Tortonose, 1986). Due to its deep water preferences, *Spicara smaris* is rarely observed in the poorly oxygenated and polluted deep waters of the Black Sea (100-150 m) (Banarascu, 1964; FAO, 1973).

Considering morphology of the three species, *S. maena* has a cylindric and elongated body, and its head is shorter than the body depth. The lateral line of the body which is covered with a wavy grey-bluish and yellow stripe is the main diagnostic feature of *Spicara maena*. A black mark which is larger than that found in *S. flexuosa* and *S. smaris* occurs on the side above the pectoral fin, and the scales are covered on the gills. Also, a blue spot appears on the head and the body. The females reach up to 21 cm and males up to 25 cm (Akşiray, 1954; Tortonose, 1986; Lythgoe and Lythgoe, 1992). (Figure 1.5). *Spicara flexuosa* has clear yellowfish or grey brownish color, and is smaller than *S. maena*. Its size reaches to a maximum of 18 cm. in females, and 21 cm. in males. (Akşiray, 1954, Tortonose, 1986; Lythgoe and Lythgoe, 1992). The most distinguishing feature of the *Spicara flexuosa* is that its eye depth is greater than that of *S. maena* (Figure 1.5). Finally, *Spicara smaris* is grey, brown and silvery in color and has a smaller size and body depth than *S. maena* and *S. flexuosa.* Its size reaches to a maximum of 15 cm. in females, and 21 cm. in males in the Mediterranean region (Akşiray, 1954, Tortonose, 1986; Lythgoe and Lythgoe, 1992). (Figure 1.5).

Morphologic misidentification among the three *Spicara* species is possible, due to sexual dimorphism and protogynous hermaphroditism. Previous studies revealed that sex reversals which

can be affected by the protoygnous hermaphroditism (sex change from females to males) (Lepori, 1960; Reinboth, 1962; Salekhova, 1979; Vidalis and Tsimenidis, 1996; Dulcic et al., 2000; Karakulak et al., 2006; Saygılı et al., 2016 a,b) were widely observed in picarels, which contributes to difficulties in their morphological identification. In addition, some species-specific characteristics and secondary sex characteristics (e.g. size, body color and marking) contribute to confused taxonomy. For instance, due to sexual dimorphism and protogynous hermaphroditism, male *S. smaris* and female *S. flexuosa*, and male *S. flexuosa* and female *S. maena* are hard to differentiate (Zei, 1941; Pollard ad Pichot, 1971; İlkyaz et al., 2007) (Figure 1.5).

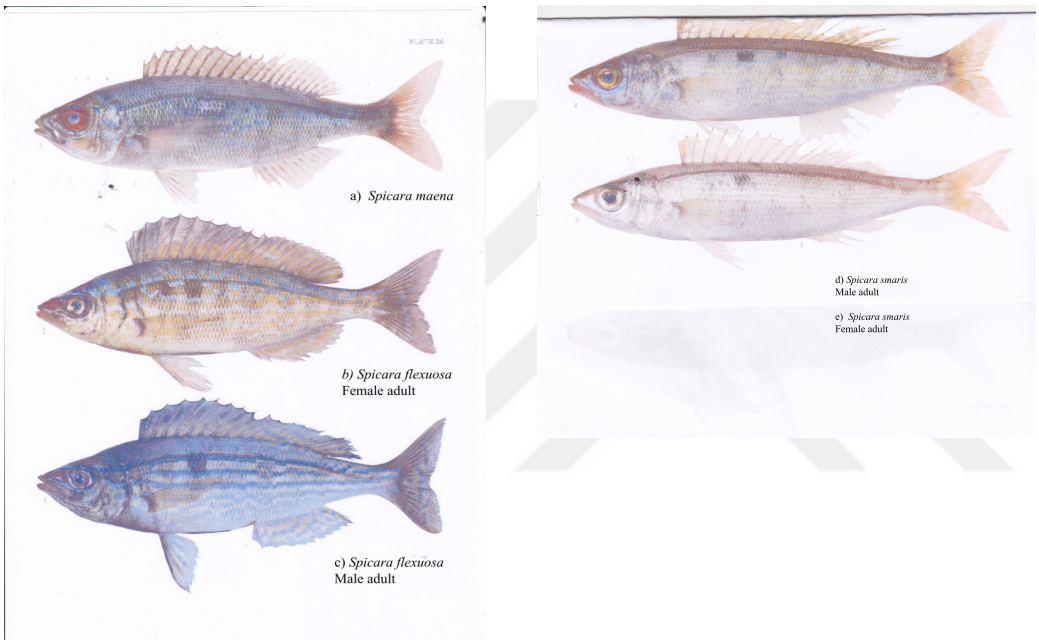


Figure 1.5. Individuals of three species of *Spicara*: a) *Spicara maena,* b) *S. flexuosa,* female *c) S. flexuosa,* male adult, d) *Spicara smaris*, male, e) *Spicara smaris* female adult (Iglesias, 2013).

1.5. Main Aim of the Study

The aim of the study is to help better understand the effect of the Turkish Straits System (TSS) on the evolutionary history of fish species. Historical and hydrographic peculiarities, and also the formation of this transion zone between the Mediterranean and the Black Sea served as a biogeographic boundary that controls fauna and flora characteristics in the TSS system. In this respect, to better understand the influence of the TSS system on intraspecific gene flow, two marine fish species, with wide geographic distributions around the Turkish coasts, namely *Merlangius merlangus* and *Spicara* spp. were investigated.

Focusing on the *Spicara* genus in greater detail, we see that phylogenetic relationships and morphologic discrimination of *Spicara maena* and *Spicara flexuosa* are not clear in the Mediterranean region. Besides, questions on the genetic relationships and the amount of genetic divergence between the three species of *Spicara* have not been addressed in the Turkish coastal waters. Specifically, *S. maena* is considered to be the dominant species found along the Turkish coasts, rather than *S. flexuosa*. In this perspective, another aim of the study is to investigate whether *S. maena*, or *S. flexuosa* is the common species in the Turkish coastal waters using a molecular approach, as their identification based on morphology is not uncontentious.

2. MATERIALS AND METHODS

Individuals of *Merlangius merlangus* (whiting) and *Spicara flexuosa/maena* (picarel) were collected from a total of 13 stations from the Black Sea (4), the TSS (4), the Aegean Sea (3), and the Mediterranean Sea (2) (Figure 2.1).

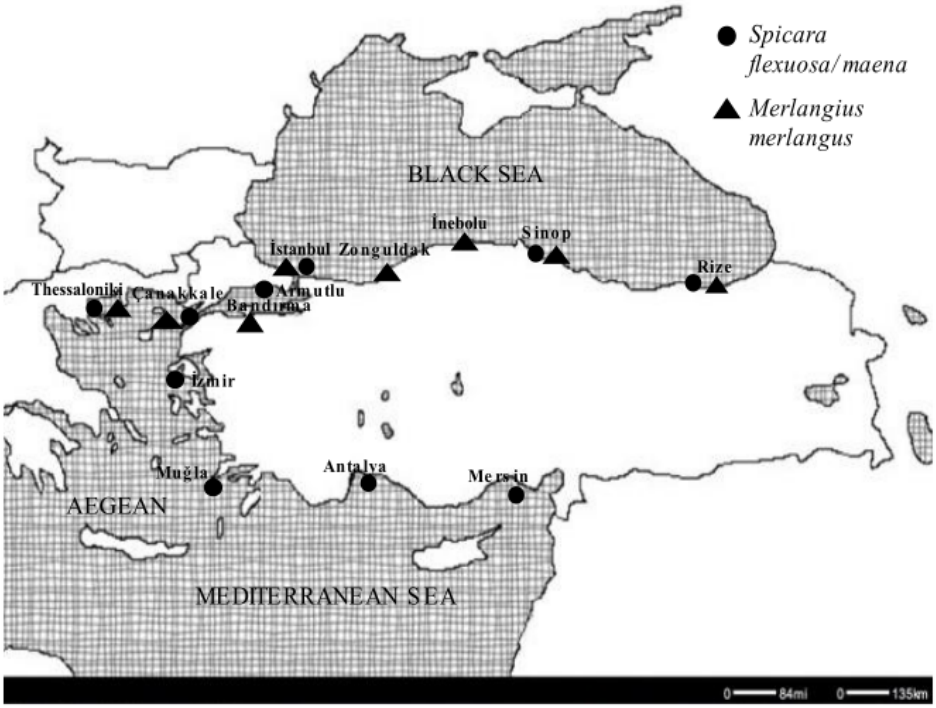


Figure 2.1. Sampling sites of *Merlangius merlangus* and *Spicara flexuosa/maena*.

While individual samples of *M. merlangus* were collected from the Black Sea, the Sea of the Marmara, and Northwestern Aegean coast of Greece, *S. flexuosa/ maena* were sampled from all coastal regions in Turkey and Northwestern Aegean coast of Greece. A total of 200 samples of *M. merlangus* and 273 specimens of *S. flexuosa/ maena* (picarel) were collected between 2013-2017, from fishermen, who were using trawlers and hand-line fishing. Locations and sampling periods are given in Appendix Table E.1. Fresh tissue of 25 individuals of *M. merlangus* and *S. flexuosa/maena* (muscle or caudal) were collected from each sampling location, and preserved in 80 % ethanol at room temperature in the field, and at -20 °C in the laboratory, until further processing.

Total genomic DNA was isolated from the muscle or caudal tissue with a DNA Tissue Extraction Kit (Invitrogen), following the manufacturer's protocols, and subsequently stored at -20 ^{0}C. To infer

evolutionary history and distribution of genetic variability of the two species, both mitochondrial DNA (mtDNA) and nuclear DNA (nuDNA) markers were used.

CO1 and cyt-b genes were used for amplification of the mtDNA region of both species. To amplify CO1 gene in *Spicara flexuosa*/*maena*, a universal forward primer was used as described by the Ward et al. (2005), and a degenerate reverse primer was used as described by Kochzius et al. (2010) (Table 2.1). The PCR reactions were performed in a total reaction volume of 25 µL, containing 2.5 µL of 10X Taq Buffer with KCl (100 mM Tris–HCl, 500 mM KCl, pH 8.8), 2 µL of $MgCl_2$ (25 mM), 0.5 µL of dNTPs (10 mM each), 0.5 µL of each primer (20 pM/µL), 0.25 µL of Taq polymerase (5U/µL), and 5 µL of DNA. PCR thermal profile was: 95 °C for 2 min for the initial denaturation step, followed by 35 cycles at 95 °C for 30 s, 52 °C for 40 s, 72 °C for 1 min, and with a final extension step of 72 °C for 10 min (Keskin and Atar, 2012).

To amplify the cyt-b gene in *Spicara flexuosa*/*maena* the Cytb I 1F and Thr Fish R primer pair was used (Sevilla et al., 2007) (Table 2.1). The PCR reactions were performed in a total reaction volume of 25 µl, containing 2.5 µL of 10X Taq Buffer with KCl (100 mM Tris–HCl, 500 mM KCl, pH 8.8), 2 µL of $MgCl_2$ (25 mM), 0.5 µL of dNTPs (10 mM each), 0.5 µL of each primer (20 pM/µL), 0.25 µL of Taq polymerase (5U/µL), and 5 µL of DNA. Thermal cycle conditions for PCR amplification were as follows: 95 °C for 2 min for the initial denaturation step, followed by 35 cycles at 95 °C for 30 s, annealing at 56 °C for 40 s, extension at 72 °C for 1 min, and with a final extension step of 72 °C for 7 min (Sevilla et al., 2007).

In addition to the COI and cyt-b genes, 16S rRNA gene was also amplified for 16 selected individuals from Turkish coastal waters and Greece for additional confirmation of taxonomic status of *Spicara flexuosa/maena.* New primers were designed using Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on a published sequence of *S. maena* from GenBank (JF795031) (Turan, 2011) (Table 2.1). The gel image of the PCR reactions was taken and is represented below (Figure 2.2). The PCR reactions were performed in a total of 25 µl reaction volume, containing 2.5 µL of 10 X Taq Buffer with KCl (100 mM Tris–HCl, 500 mM KCl, pH 8.8), 2 µL of $MgCl_2$ (25 mM), 0.5 µL of dNTPs (10 mM each), 0.5 µL of each primer (20 pM/µL), 0.25 µL of Taq polymerase (5U/µL), and 5 µL of DNA. Thermal cycle conditions for PCR amplification were as follows: 95 °C for 2 min for the initial denaturation step, followed by 35 cycles at denaturation 95 °C for 30 s, annealing at 56 °C for 40 s, extension 72°C for 1 min, and with a final extension step of 72 °C for 7 min (Turan, 2011).

Table 2.1. Primer name and sequences of mtDNA genes for *Spicara flexuosa / maena*.

Gene	Name	Primer sequences (5'-3')	References
Cyt-b	Cyt-bI1F	CGATTCTTCGCATTCCACTTCCT	Sevilla et al. (2007)
Cyt-b	ThrFish R	ACCTCCGATCTTCGGATTACAAGACC	Sevilla et al. (2007)
COI	COI-Fish-F2	TCGACTAATCATAAAGATATCGGGAC	Ward et al. (2005)
COI	COI-Fish-R1	TAGACTTCTGGGTGGCCRAARAAYCA	Kochzius et al. (2010)
16 S	16 S F1	GTGTGCTGCATGGGAAAGAC	This study
16 S	16 S R1	CTGGTCCACATGGGGGTTTT	This study

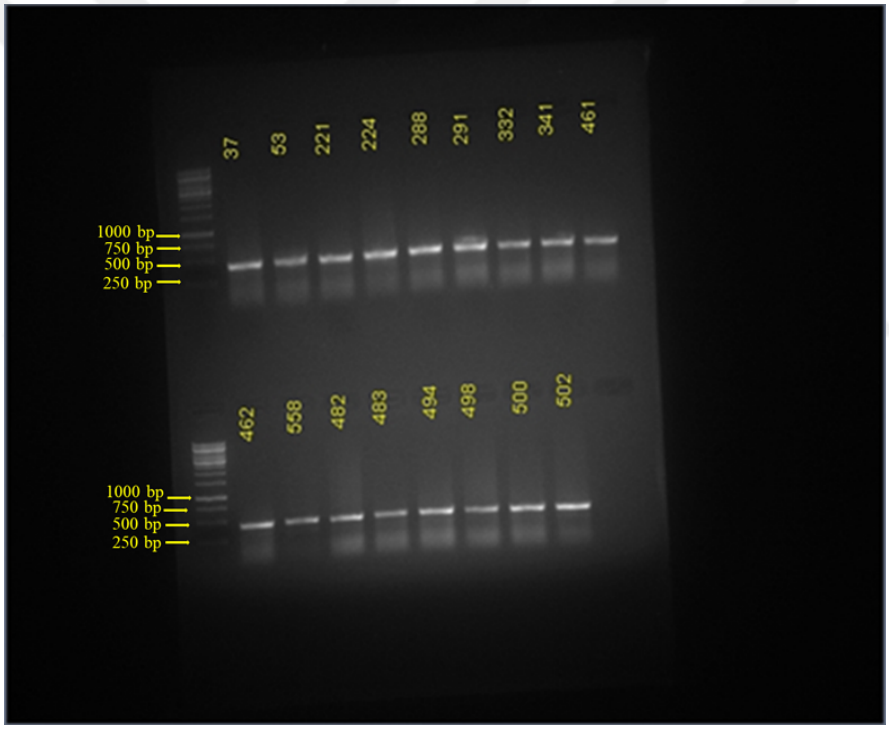


Figure 2.2. Gel image for PCR reactions for the 16 SrRNA gene of *Spicara spp.*

In order to amplify the CO1 gene in *Merlangius merlangus*, primer pairs were used as described by the Eiriksson and Arnason (2014) and Kochzius et al. (2010) (Table 2.2). The PCR reactions were performed in a total reaction volume of 25 µl, containing 2.5 µL of 10 X Taq Buffer with KCl (100 mM Tris–HCl, 500 mM KCl, pH 8.8), 2 µL of $MgCl_2$ (25 mM), 0.5 µL of dNTPs (10 mM each), 0.5 µL of each primer (20 pM/µL), 0.25 µL of Taq polymerase (5U/µL), and 5 µL of DNA. PCR thermal profile was: 94 °C for 5 min for the initial denaturation step, followed by 35 cycles of denaturation at

94 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 1 min, and with a final extension step of 72 °C for 7 min.

To amplify the cyt-b gene in *Merlangius merlangus*, GluDg and Cb3h primers were used as described by Roques et al. (2006) (Table 2.2). The PCR consisted of 5 µL of each template DNA, 0.5 µl of dNTPs (10 mM each), 3 µl of 10x buffer, 2.5 µl of $MgCl_2$ (25mM), 0.5 µl of each primer (20µM), 0.2 µl of Taq (5 U/µl), and dH_2O to a final volume of 25 µl. PCR thermal profile was: 95 °C for 3 min for the initial denaturation step, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 45 °C for 45 s, extension at 72 °C for 1 min, and with a final extension step at 72 °C for 5 min.

Table 2.2. Primer name and sequences of mtDNA genes for *Merlangius merlangus.*

Gene	Name	Primer sequences (5'-3')	References
Cyt-b	GluDg	TGACTTGAARAACCAYCGTTG	Roques et al. (2006)
Cyt-b	CB3H	GGCAAATAGGAARTATCATTC	Roques et al. (2006)
CO1	COI-Fish-F1	TCAACCAACCACAAAGACATT GGCAC	Eiriksson and Arnason, (2014)
CO1	COI-Fish-R1	TAG ACT TCT GGG TGG CCR AAR AAY CA	Kochzius et al. (2010)

The PCR strategy for the nuclear DNA region of *Spicara flexuosa*/*maena* included the amplification of the interphotoreceptor retinoid binding protein (IRBP) gene. New primers were designed in Primer Blast for amplifying a partial IRBP gene fragment, based on alignments of published sequences for *Spicara maena* (Lautredeu et al., 2013) (JX627893) from GenBank (Table 2.3). The PCR consisted of 5 µL of each template DNA, 0.7 µl of dNTPs (10 mM each), 3 µl of 10x buffer, 2.0 µl of $MgCl_2$ (25mM), 0.6 µl of each primer (20µM), 0.2 µl of Taq (5 U/µl), and dH_2O to a final volume of 25 µl. PCR thermal profile was: 94 °C for 2 min for the initial denaturation step, followed by 50 cycles at 94 °C for 30 s, annealing at 55 °C for 40 s, extension at 72 °C for 1 min, and with a final extension step at 72 °C for 5 min.

To amplify a nuclear DNA region for *Merlangius merlangus*, the recombination activating gene (RAG1) was used (Hansen and Kaattari, 1996; Willett et al., 1997). New primers were designed in Primer Blast for a partial RAG1 gene fragment, based on alignments of published sequences for *Merlangius merlangus* (Owens, 2015) (KP644393) in GenBank (Table 2.3). The PCR reactions were performed in a total reaction volume of 25 µl, containing, 3 µL of 10 X Taq Buffer with KCl (100 mM Tris–HCl, 500 mM KCl, pH 8.8), 2 µL of $MgCl_2$ (25 mM), 0.5 µL of dNTPs (10 mM each), 1

µL of each primer (20 pM/µL), and 0.25 µL of Taq polymerase (5U/µL), and 5 µL of DNA. PCR thermal profile was: 94 °C for 5 min for the initial denaturation step, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 40 s, extension at 72 °C for 1 min, and with a final extension step at 72 °C for 7 min.

Table 2.3. Primer name and sequences of nuDNA genes for *Merlangius merlangus* and *Spicara flexuosa*/*maena.*

Gene	Name	Primer sequences (5'-3')	References
RAG1	F2	TGCGTTACTCCTTCACCGTC	This study
RAG1	R2	CCTAAGCTCTTCGGCGGATT	This study
IRBP	IRBP F1	ACGCCTGATGGTCTCCTTTG	This study
IRBP	IRBP R1	TTCGAGTTTCACAGAGCCCC	This study

All amplified products were sequenced commercially (Macrogen, Holland) in the forward direction, with the respective forward primer for each gene. In the nuclear DNA sequence analysis of IRBP gene (for *Spicara flexuosa/ maena*), all ambiguous sites were re sequenced in the reverse direction for confirmation.

2.1. Statistical Analyses

All chromotgrams were edited and aligned manually with Sequencher v. 5.4.1 (GeneCodes Corp.). In the nuclear DNA data analysis, base positions that were ambigious were split into nonambigious sets (a haplotype with an ambiguity was defined as two distinct haplotypes) prior to subsequent phylogenetic analyses.

Phylogenetic relationships between haplotypes were determined by neighbour-joining, maximum likelihood, and maximum parsimony methods using the sofware MEGA 7.0 (Kumar et al., 2016). The robustness of the trees was tested with 1000 replicates. Nucleotide substitution models to be used were determined using MODELTEST v.3.7 (Posada and Crandall, 1998), with the AIC (Akaike, 1974) and the AICc (Hurvich and Tsai, 1989) statistics. The general time reversable model that fit the data best was used for building the maximum likelihood trees for the different species and gene combinations. Considering neighbour-joining trees, maximum composite likelihood distances

were used based on previous studies in the literature (Tamura et al., 2004; Kumaraswamy-Naidu et al., 2010). The phylogenetic relationships between haplotypes were also determined with Bayesian analysis using the program BEAST v.1.8.3 (Drummond et al., 2012) under the Hasegawa-Kishino-Yano substitution model, with the length of chains determined in order to obtain effective sample size (ESS) values higher than 200, and these results were visualized in Tracer (Rambaut et al., 2014). The final MCMC calculation strategy resulted in two runs with chain lengths of 10000000, sampling every 1000 generations. The phylogenetic relationships between haplotypes were also determined for *Spicara spp*. and *Merlangius merlangus*, using concetanated cyt-b and CO1 data.

To describe genetic diversity of all samples, number of haplotypes, haplotype diversity (h) (Nei and Tajima, 1981), nucleotide diversity (**π**) (Nei, 1987), and number of polymorphic, singleton and parsimony informative sites were calculated with DnaSP v.5.10.1 (Rozas, 2009), for all populations. Codon usage analyses were conducted for the mtDNA genes to determine if the amplified fragments indeed were from mtDNA, or if they represented nuclear mitochondrial pseudogenes (numtDNA). Codon frequencies and relative synonomous codon usage were calculated with the program DAMBE ver 6.4.1 (Xia, 2017).

Haplotype networks of the sequences of individuals from different geographical regions were constructed by using the median-joining method as implemented in the software PopART v.1.7. (Bandelt et al., 1999). Genetic structure between populations and subpopulations was estimated by AMOVA (Analysis of molecular variance), using the program Arlequin v.3.5.2.2 (Excoffier and Lischer, 2010). Arlequin was also used to calculate pairwise Φ_{ST} and F_{ST} values between populations, and test their significance (with 10000 permutations). As a separate note, some populations where sample size were smaller than seven were excluded for Φ_{ST} analysis. Considering the CO1 gene, for *S. flexuosa*, seven individuals each from Portugal and Italy, and one individual from Sardinia, and for *S. maena* four individuals from Sardinia and Malta were excluded for this analysis due to low sample sizes. For cyt-b gene, for *S. flexuosa,* five individuals from Sinop, four individuals each from Italy and Greece, and for *S. maena* one individual from Spain were excluded for this analysis due to low sample sizes. Regarding *Merlangius merlangus* species for CO1 gene, six *Merlangius merlangus euxinus* (İnebolu) samples were also excluded from this analysis.

Mismatch distributions of the number of pairwise differences (Slatkin and Hudson, 1991; Rogers and Harpending, 1992) between haplotypes, which can result from demographic or spatial population expansion, were estimated with DNAsp. v.5.10.11 (Rozas, 2009). The neutrality tests of Tajima's D

(Tajima, 1989) and Fu's Fs (Fu, 1997) were also performed to detect signatures of population growth, if any. The negative values of Tajima's D and Fu's Fs generally indicate population growth or selection, and positive values show population decline. The raggedness statistic "rg" (Harpending et al., 1993; Harpending, 1994), which quantifies the smoothness of the fit of the observed mismatch distribution to one expected under a population expansion model was calculated using DNasp v.5.10.11. Significance of neutrality tests and rg values were also tested with 1000 coalescence simulations done in DnaSP v.5.10.11. Past population demography of *Spicara flexuosa* and *M. m. euxinus* was also reconstructed using Bayesian skyline plots (BSP), as implemented in BEAST v1.8.4 (Drummond et al., 2012). Skyline-plot methods are based on coalescent theory, which quantifies the relationship between the genealogy of the sequences and the demographic history of the population (Kingman, 1982a, b), and they are widely used in phylogeographic studies of marine fishes (Grant et al., 2012), among other taxa. In terms of the analyses parameters, a strict molecular clock was set at 1.2 % substitution/site/Myr, using the molecular clock rate of 1.2 % *per* million years for the CO1 gene for marine teleosts (Bermingham et al., 1997). Two billion iterations were used as the chain length for both *Spicara flexuosa* and *M. merlangus euxinus*, and the first two hundred million iterations were discarded as burn-in. Tree prior was set as 'Coalescent', Bayesian Skyline with group size m = 10, and auto-optimize options were used (Drummond et al., 2005). The BEAST input file was produced using BEAUTi v.1.8.4 (Drummond et al., 2012), and the median and corresponding credibility intervals of the BSP were determined using TRACER v.1.6. (Rambaut et al., 2014).

To estimate evolutionary divergence and times of phylogenetic separation of three *Spicara* species, a molecular clock hypothesis was tested, using Tajima's relative rate test as implemented in MEGA 7.0 (Kumar et al., 2016). *S. smaris* was used as an outgroup of the examined genes (16S, CO1, cyt-b), and p-distances were used. Divergence times between three species were calculated by using the molecular clock rate of 1 % *per* million years for the 16S rRNA gene in Perciformes fish (Tringali et al., 1999), 1.2 % *per* million years for the CO1 gene for marine teleosts (Bermingham et al., 1997); and 0.77 % *per* million years for the cyt-b gene of vertebrates (Caccone et al., 1997). Molecular clock hypothesis was also tested, to estimate evolutionary divergence and times of phylogenetic separation of the two *Merlangius merlangius* subspecies. *Melanogrammus aeglefinus* was used as an outgroup in the CO1 gene. Divergence times between two subspecies were again calculated by using the molecular clock rate of 1.2 % *per* million years for the marine teleosts (Bermingham et al., 1997).

2.2. Morphometric Measurements

In the section above, morphological identification of the *Spicara* species was not carried out, as samples were provided by multiple contacts from Turkey and Greece, and it was not possible to standardize the morphological methods for their application by these different sample providers. To understand the concordance between genetic and morphological characteristics, and their use in species identification (as *maena*/ *flexuosa*), an additional 23 *Spicara* individuals were collected from İstanbul. Morphologic and genetic assignment of collected 23 individuals were made based on data from five *S. maena,* five *S. flexuosa* collected by Krey et al in 2003, which were morphologically identified following Fish Trace protocols and procedures by Kullander and Noren (2003). Regarding the molecular analyses, five PCRs were not successful, (for samples with the codes İstanbul 12, 16, 17, 18, 21, see Appendix D), and therefore these samples were not included in the morphological measurements. Two characters were used for morphological measurements: head length (HL) and body depth (BD). Head related characteristics were widely used for the morphological identification of Sparids (Palma and Andrede, 2002; Delavira and Agostinho, 2001) and *Spicara* species (Golani et al., 2006; Minos et al., 2013). All morphological measurements were done to the nearest 0.1 cm with a vernier caliper (Appendix Table A.1). Each specimen was photographed after taking their measurements (Appendix Figure D. 1-23).

3. RESULTS AND DISCUSSION

3.1. *Spicara flexuosa* (Rafinesque, 1810) / *Spicara maena* (Linnaeus, 1758)

3.1.1. Mitochondrial DNA (16S rRNA gene)

The mitochondrial 16S rRNA gene is considered to be a useful evolutionary marker for fish, and it has produced robust phylogenies at various taxonomic levels (Karaiskou et al., 2003; Perez et al., 2005; Turan et al., 2008; Turan, 2011). A partial fragment of the 16S rRNA gene was amplified for a total 16 selected individuals from Turkish coastal waters and Greece (Appendix Table F.1) for confirmation of the taxonomic status of *Spicara flexuosa*/ *maena.* To determine the species identity of the *Spicara* samples collected from Turkish and Greece coasts in this study, six *S. maena*, four *S. flexuosa,* five *S. smaris* sequences from Greece, and one most closely related outgroup species' sequence from *Spondylisma cantharus* were added from GenBank (Table 3.1). Considering the source studies for some of these sequences, and their resolution for diagnosing the three *Spicara* species, Imsiridou et al. (2011) found interspecific sequence polymorphism between *S. maena* and *S. flexuosa.* The sequences submitted by Georgiadis et al. (2014) were previously used to diagnose three *Spicara* species (including *S. smaris*), by using real-time (melt-curve haplotype specific) PCR. In the final data set, for the 16S rRNA gene, a 155 bp fragment was analyzed, and a total of five haplotypes were found within 32 individuals, whose relationships are represented as a haplotype network in Figure 3.1.

Table 3.1. The accession numbers and location information of individuals for 16S rRNA sequences of *Spicara spp*. retrieved from GenBank.

Species	Accession numbers	Location
S. maena	AJ247298	France
S. maena	KF796618	Greece
S. maena	FJ625835	Greece
S. maena	JF795031	Turkey
S. maena	JF795030	Turkey
S. maena	AF247434	Italy
S. flexuosa	JF795029	Turkey
S. flexuosa	KF796616	Greece
S. flexuosa	KF796617	Greece
S. flexuosa	FJ625836	Greece
S. smaris	JF795032	Turkey
S. smaris	JF795033	Turkey
S. smaris	JF795034	Turkey
S. smaris	KF796614	Greece
S. smaris	KF796615	Greece

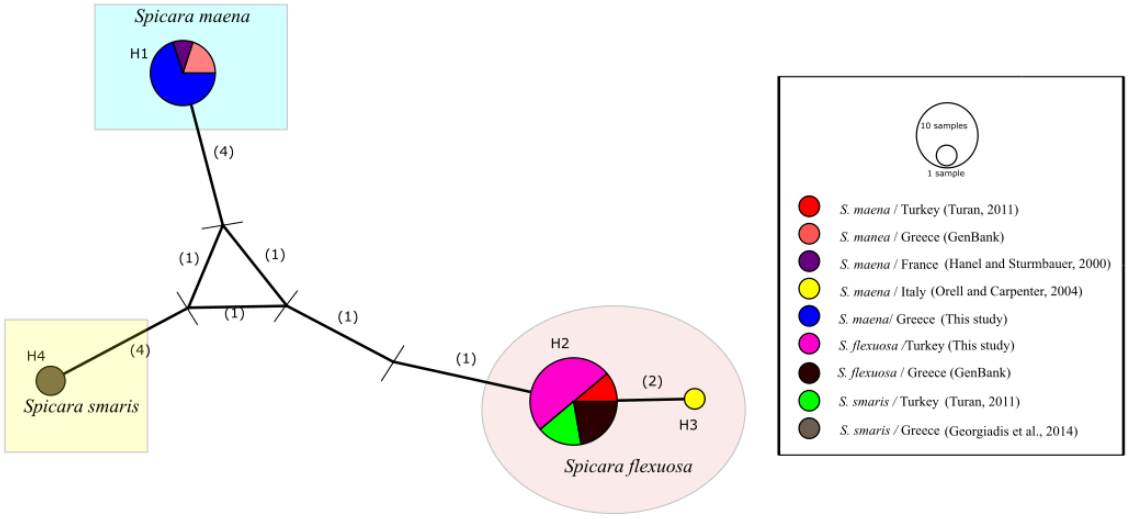


Figure 3.1. The haplotype network based on 16S rRNA sequences of *Spicara spp.* populations. The numbers correspond to the number of mutations between haplotypes. Each of the lines between the three haplogroups represents missing haplotypes.

The results of the haplotype network show three distinct *Spicara* haplotypes/haplogroups, corresponding to three different species. According to classification of Imsiridou et al. (2011) and Georgiadis et al. (2014), those samples collected from Turkish coastal waters, which all have Hap 2, were clustered with *S. flexuosa* from Greece. On the other hand, samples collected from Greece coasts for this study, which all have Hap 1, were clustered with *S. maena*. Thus, the samples collected from Turkey and Greece corresponded to *S. flexuosa* and *S. maena*, respectively, based on results for the 16S gene.

Focusing on the other GenBank entries, based on the 16S classification that is followed here, samples from Italy (Orell and Carpenter, 2004) previously identified as *S. maena*, should be classified as *S. flexuosa*. Again, based on this classification, *S. maena* and *S. smaris* collected by Turan (2011) should be classified as *S. flexuosa*. The classification of samples, whose sequences were retrieved from GenBank, based on studies of Imsiridou et al. (2011), Georgiadis et al. (2014) and Hanel and Sturmbauer (2000) as *S. maena* was confirmed as correct. The distribution of the three *Spicara* species based on the 16S gene data from the literature and this study is given in Figure 3.2. This shows an overlapping distribution of three *Spicara* species only along the Greece coast. *Spicara flexuosa* was recorded in all sampling locations in Turkish coasts from our study, as well as in Italy, Greece and Hatay (Turkey) from GenBank. *S. maena* was recorded in Greece/northern Aegean (Thessaloniki), (collected in this study) and Greece (Thessaloniki), France (Corsica) from GenBank. Finally, *S. smaris* was only seen in Greece (Thessaloniki) (GenBank).

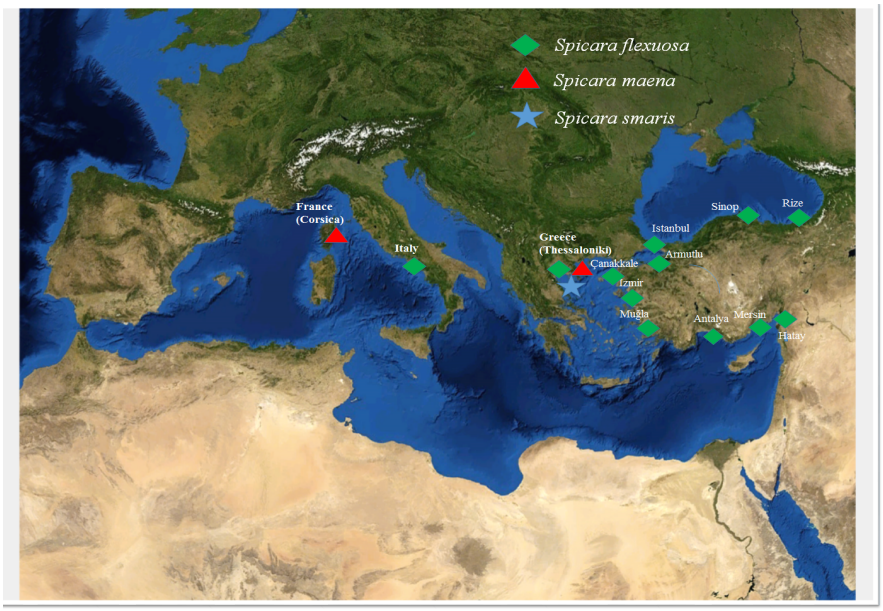


Figure 3.2. Sampling locations of three *Spicara* species, based on the 16S rRNA gene, in the Mediterranean region.

Phylogenetic relationships between haplotypes were determined by maximum likelihood (Figure 3.3), neighbour-joining (Figure 3.4), maximum parsimony (Figure 3.5), and Bayesian trees (Figure 3.6).

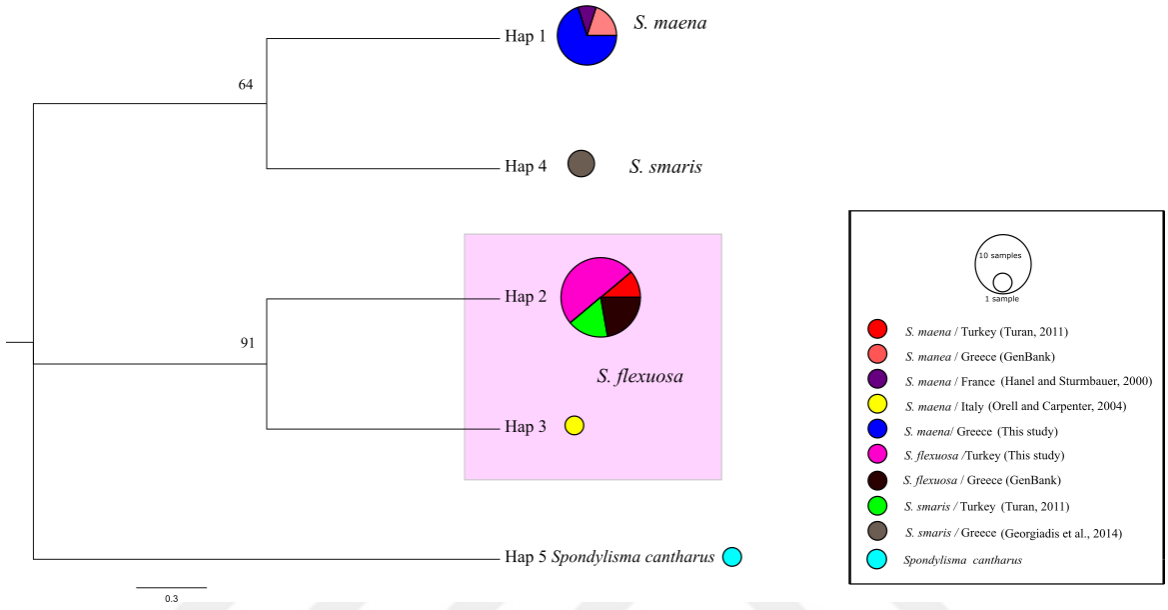


Figure 3.3. Maximum likelihood tree based on 16S rRNA data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles with different colors. Size of the circles are proportional to the relative frequencies of the haplotypes.

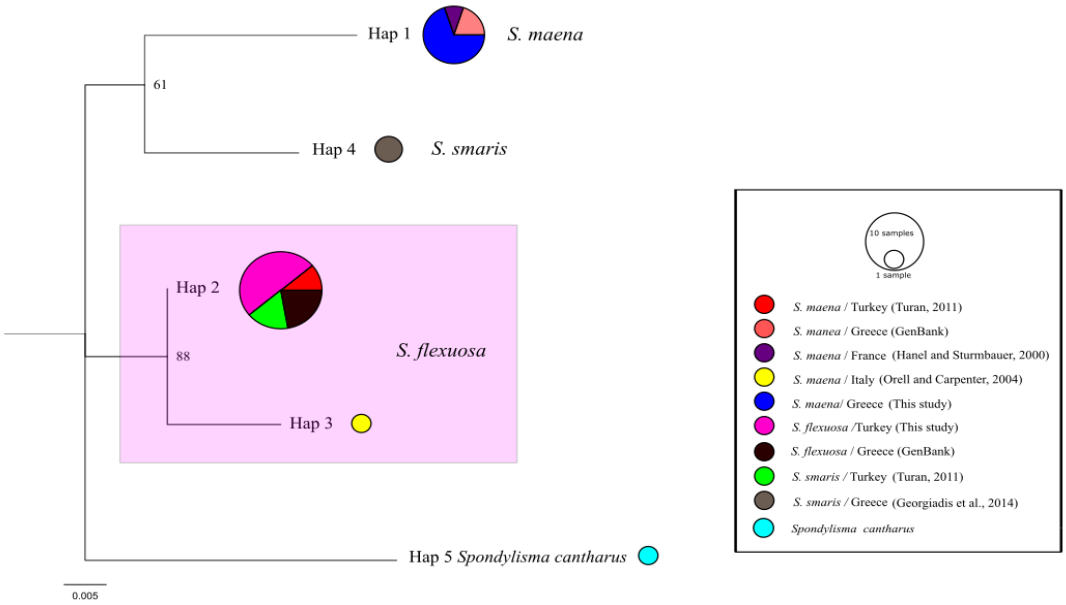


Figure 3.4. Neighbour-joining tree based on 16S rRNA data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles with different colors. Size of the circles are proportional to the relative frequencies of the haplotypes.

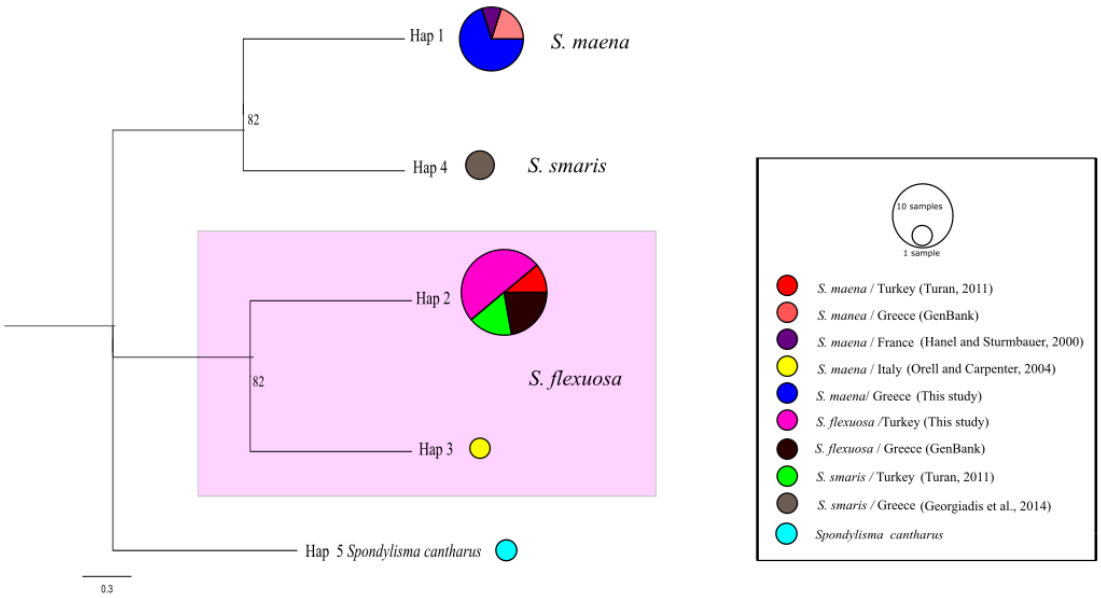


Figure 3.5. Maximum parsimony tree based on 16S rRNA data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles with different colors. Size of the circles are proportional to the relative frequencies of the haplotypes.

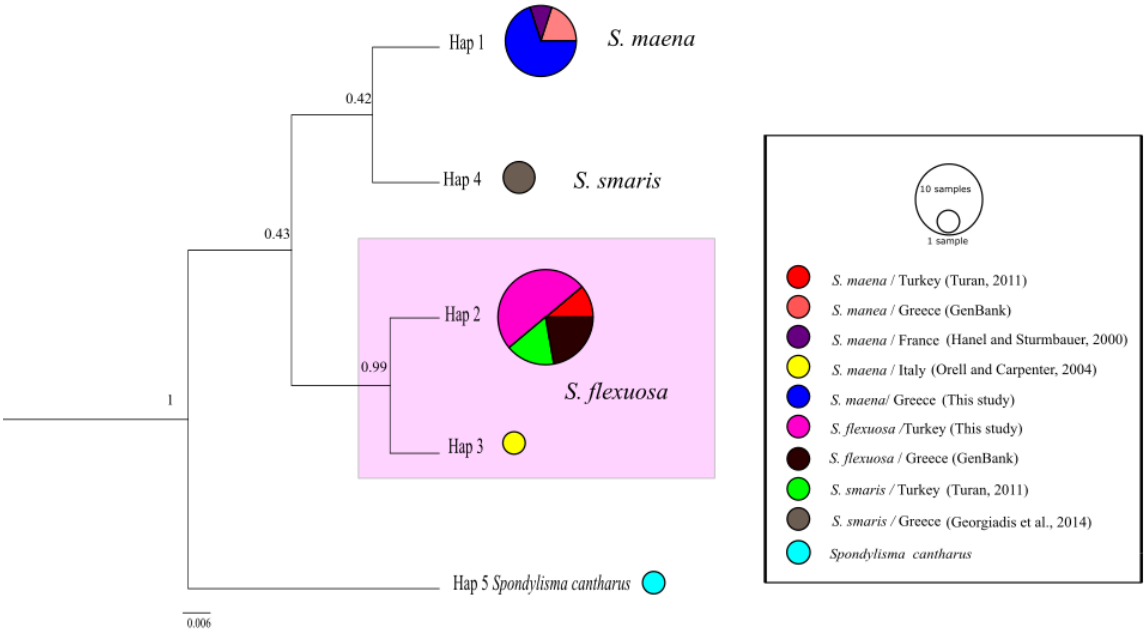


Figure 3.6. Bayesian tree based on 16S rRNA data for *Spicara spp.* populations. The node values correspond to posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles with different colors. Size of the circles are proportional to the relative frequencies of the haplotypes.

The phylogenetic trees obtained by maximum likelihood, neighbour-joining, maximum parsimony, and Bayesian methods revealed the same topology with high bootstrap values. All analyses revealed three different haplogroups that corresponded to *Spicara flexuosa*, *S. maena* and *S. smaris*.

The total number of mutations and pairwise sequence divergences, and corresponding divergence times among the three species are given in Table 3.2, which shows that the total number of mutations, pairwise sequence divergence were approximately equal between the three species. Considering evolutionary divergence times and phylogenetic separation of the three species, molecular clock hypothesis was not rejected among the three *Spicara* species. Divergence times indicated that the three species were differentiated during the late Pliocene. Codon usage analyses was not conducted for this gene, since 16S rRNA is not a protein encoding gene.

Table 3.2. Total number of mutations (below diagonal) and pairwise sequence divergences and divergence times (above diagonal), based on the 16S rRNA gene among the three species.

Species name	*S. maena*	*S. flexuosa*	*S. smaris*
S. maena		4.5 %-2.6 Mya	5.8 %-2.9 Mya
S. flexuosa	9		4.5 %-2.6 Mya
S. smaris	9	9	

Molecular diversity indices and base compositions of parsimony informative sites for the 16S rRNA gene of the three *Spicara* species are shown in Table 3.3 and Table 3.4, respectively. As shown in Table 3.3., the highest haplotype and nucleotide diversities were found in *Spicara flexuosa* populations. Among the three species, a total of 18 nucleotide sites were variable of which 11 positions were parsimony informative (Table 3.4).

Table 3.3. Molecular diversity of *Spicara spp*. based on 16S rRNA region sequences. N: Number of samples; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations, when available, are given in parantheses.

Species name	**N**	**Nh**	**Np**	**h**	**π**	**SS**	**PIS**
S. flexuosa	19	2	2	0.105 (0.092)	0.00136 (0.00119)	2	0
S. maena	10	1	0	0	0	0	0
S. smaris	2	1	0	0	0	0	0

Table 3.4. Parsimony informative sites of total *Spicara spp.* based on 155 bp long 16S rRNA region sequences.

		Parsimony Informative Sites of 16S rRNA										
Species name	Codes of samples	15	16	17	50	58	66	80	82	92	97	104
S.cantharus	GQ485282	A	A	A	G	G	C	G	A	G	A	C
S.flexuosa	JF795033	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	JF795034	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	10 462 16S F1	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	4 224 16S F1	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	AF247434	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	11 558 16S F1	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	3 221 16S F1	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	JF795029	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	8 341 16S F1	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	KF796616	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	9 461 16S F1	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	KF796617	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	FJ625836	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	JF795032	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	JF795030	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	7 332 16S F1	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	1 37 16S F1	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	5 288 16S F1	.	T	.	.	.	.	A	T	.	.	.
S.flexuosa	JF795031	.	T	.	.	.	.	A	T	.	.	.
S.maena	AJ247298	C	T	T	.	A	T	A	.	.	T	T
S.maena	14 494 16S F1	C	T	T	.	A	T	A	.	.	T	T
S.maena	KF796618	C	T	T	.	A	T	A	.	.	T	T
S.maena	18 500 16S F1	C	T	T	.	A	T	A	.	.	T	T
S.maena	13 483 16S F1	C	T	T	.	A	T	A	.	.	T	T
S.maena	19 502 16S F1	C	T	T	.	A	T	A	.	.	T	T
S.maena	12 482 16S F1	C	T	T	.	A	T	A	.	.	T	T
S.maena	FJ625835	C	T	T	.	A	T	A	.	.	T	T
S.maena	15 495 16S F1	C	T	T	.	A	T	A	.	.	T	T
S.maena	20 507 16S F1	C	T	T	.	A	T	A	.	.	T	T
S.smaris	KF796614	.	G	.	A	A	-	T	.	A	G	.
S.smaris	KF796615	.	G	.	A	A	.	T	.	A	G	.

Of all the parsimony informative sites, two positions were parsimony informative for *S. smaris* and *S. maena*, four for *S. smaris* and *S. flexuosa*, and four for *S. maena* and *S. flexuosa*. These results indicate three species seem to be more or less equally distinct from each other, as observed in the pairwise sequence divergences (Table 3.2).

3.1.2. The CO1 Gene

The CO1 gene was amplified in 199 *Spicara flexuosa*/*maena* individuals (176 from Turkey and 23 from Greece), from 10 sampling locations (Appendix Table F.1). In order to identify the *Spicara* specimens collected in this study based on the CO1 gene, six sequences ((HM038500 (*Spicara flexuosa*), HM038501, HM038502, HM038503 (*Spicara maena*), and HM038505, HM038506 (*Spicara smaris*)) from Sardinia (Cannas et al retrieved from GenBank, 2010) were added as previously submitted to GenBank in 2010 (The title of this submission was 'Molecular evidence of the erroneous synonimization of *Spicara flexuosa* and *S. maena* as a single species and of the polyphyly of the Centracanthidae family'). Based on these submissions, Cannas et al. (2010) were able to diagnose *S. flexuosa* and *S. maena* using the CO1 gene. In addition to these, 30 *Spicara smaris*, 21 *Spicara maena*, and one *Spondylisma cantharus* (outgroup) sequences were added from GenBank and the Barcode of Life Database (BOLD) to the data set. GenBank and BOLD accession numbers, and locality information for these individuals are given in Table 3.5. A fragment of 454 bp region was used for all analyses, and a total of 19 haplotypes were found within 257 specimens (Figure 3.7).

Table 3.5. The accession numbers and locations of the individuals for CO1 sequences of *Spicara spp*. retrieved from GenBank and BOLD.

Species	Accession numbers	Location	Species	Accession numbers	Location	Accession numbers	Location
S. flexuosa	HM038500	Sardinia	*S. smaris*	KM538579	Israel	KC501594	Turkey
S. maena	HM038501	Sardinia	*S. smaris*	KM538577	Israel	JQ624001	Turkey
S. maena	HM038502	Sardinia	*S. smaris*	KM538576	Israel	KC501598	Turkey
S. maena	HM038503	Sardinia	*S. smaris*	BIM303-13	Israel	KC594593	Turkey
S. maena	KM538575	Israel	*S. smaris*	KT074093	Italy	KM538578	Israel
S. maena	KM538574	Israel	*S. smaris*	HM038506	Sardinia		
S. maena	KM538573	Israel	*S. smaris*	HM038505	Sardinia		
S.maena	KM538572	Israel	*S. smaris*	KT883623	Italy		
S. maena	KM538571	Israel	*S. smaris*	KJ709920	Portugal		
S. maena	KM538570	Israel	*S. smaris*	KJ709921	Portugal		
S. maena	KM538569	Israel	*S. smaris*	KC501612	Turkey		
S. maena	KM538568	Israel	*S. smaris*	KC501611	Turkey		
S. maena	KM538567	Israel	*S. smaris*	KC501610	Turkey		
S. maena	KM538566	Israel	*S. smaris*	KC501609	Turkey		
S. maena	KM538565	Israel	*S. smaris*	KC501608	Turkey		
S. maena	KM538564	Israel	*S. smaris*	KC501607	Turkey		
S. maena	BIM012-13	Israel	*S. smaris*	KC501606	Turkey		
S. maena	KJ709919	Malta	*S. smaris*	KC501605	Turkey		
S. maena	KJ768312	Portugal	*S. smaris*	KC501604	Turkey		
S. maena	JQ774742	Portugal	*S. smaris*	KC501603	Turkey		
S. maena	JQ774741	Portugal	*S. smaris*	KC501602	Turkey		
S. maena	JQ774740	Portugal	*S. smaris*	KC501601	Turkey		
S. maena	JQ774739	Portugal	*S. smaris*	KC501600	Turkey		
S. maena	JQ774738	Portugal	*S. smaris*	KC501599	Turkey		
S. maena	KT883622	Italy	*S. smaris*	KC501596	Turkey		
S. smaris	KC501597	Turkey	*S. smaris*	KC501595	Turkey		

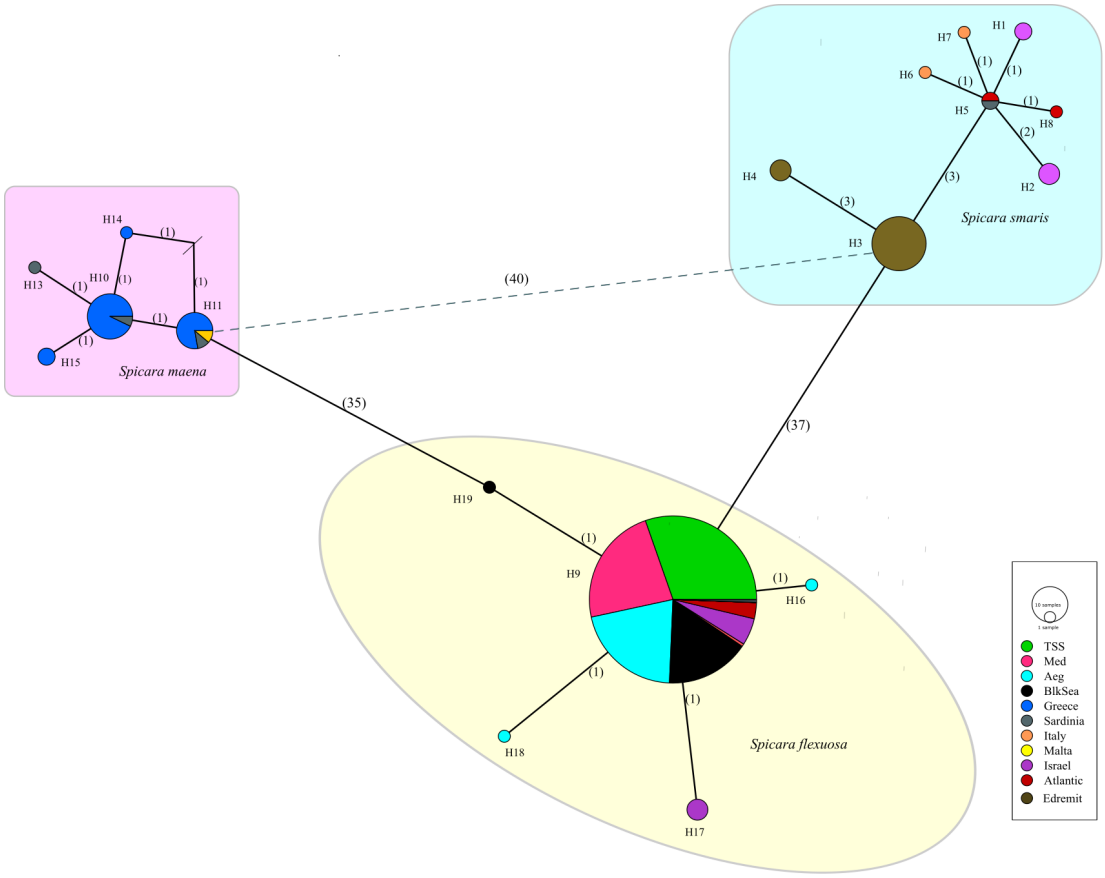


Figure 3.7. The haplotype network based on CO1 sequences of *Spicara spp.* populations. The numbers correspond to the number of mutations between haplotypes. The line between Hap. 11 and 14 represents a missing haplotype. The dashed line represents a direct connection between *S. maena* and *S. smaris* haplogroups. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

Based on the classification of Cannas et al. (2010) three haplogroups were determined as to correspond to *Spicara flexuosa, S. maena* and *S. smaris* (Figure 3.7). As can be seen in the haplotype network, samples collected from Turkey, which have Hap 9, cluster with *S. flexuosa* from Sardinia, and those collected from Greece, which have Hap 10 and Hap 11, cluster with *S. maena* samples from Sardinia and Malta, respectively. Thus, based on the CO1 gene, in a parallel manner to the 16S rRNA, the samples we collected from Turkish and Greek coastal waters were identified as *S. flexuosa;* and *S. maena*, respectively.

The results also indicate that samples from Italy, Israel, Portugal (Atlantic), (previously identified as *S. maena* in GenBank) (Hap 9, Hap 17), should also be classified as *S. flexuosa*. On the other hand, the identification of the samples from Greece, Sardinia and Malta as *Spicara maena* is

consistent with the classification of Cannas et al. (2010). Finally, based on these results and including data from GenBank and BOLD, *Spicara smaris* was recorded from Edremit-Turkey, Sardinia, Israel, Portugal (Atlantic) and Italy (Figure 3.8).

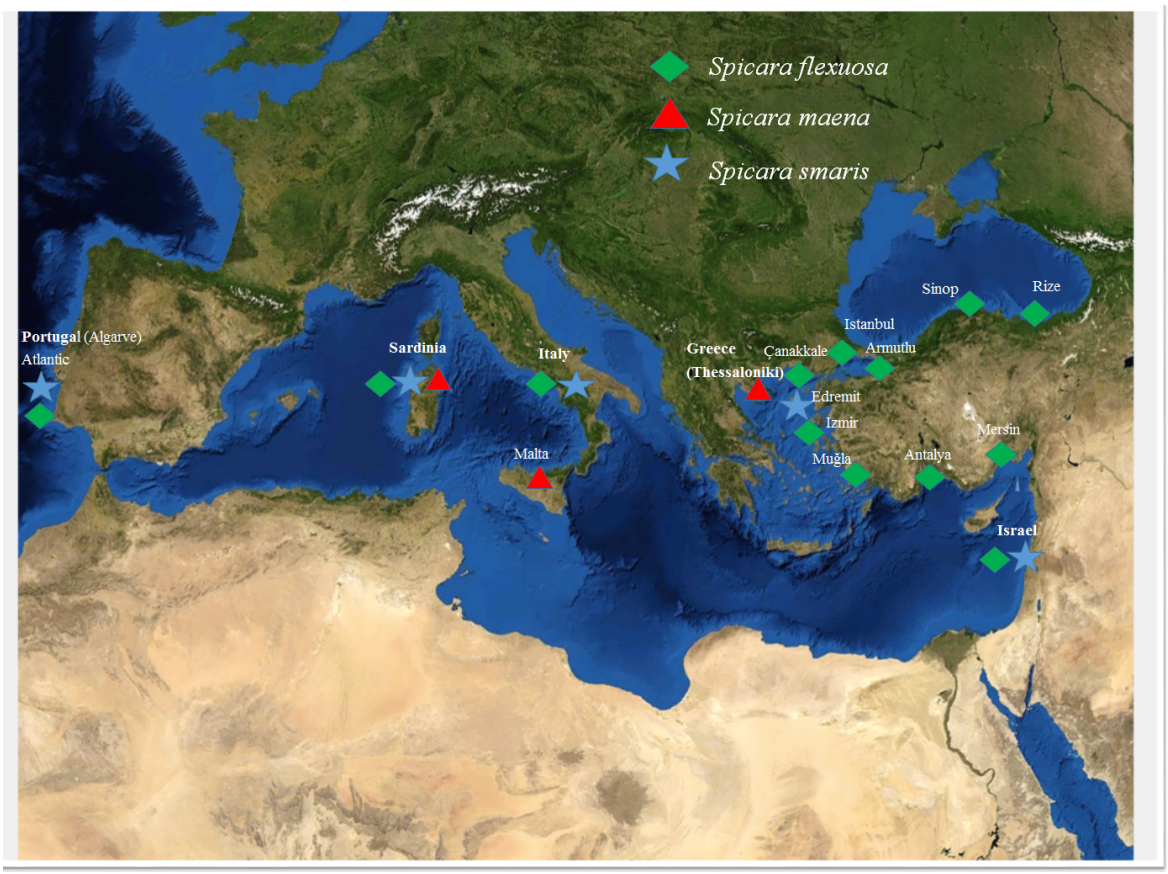


Figure 3.8. Sampling locations of three *Spicara* species, based on the CO1 gene, in the Mediterranean region.

Phylogenetic relationships between haplotypes were determined by the maximum likelihood (Figure 3.9), neighbour-joining (Figure 3.10), maximum parsimony (Figure 3.11) and Bayesian trees (Figure 3.12). In these trees, the species classification of Cannas et al. (2010) was followed.

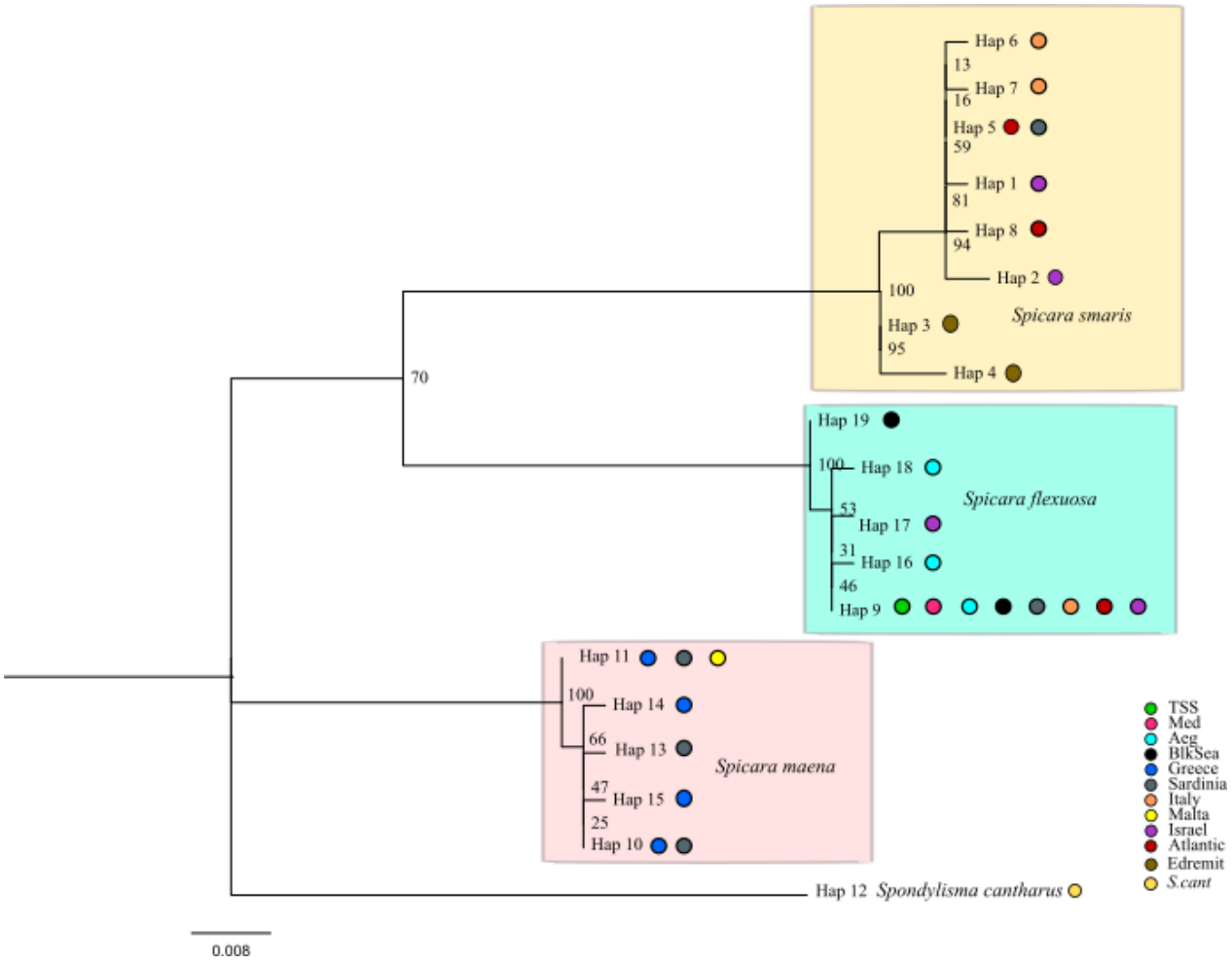


Figure 3.9. Maximum likelihood tree based on CO1 data for *Spicara spp*. populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

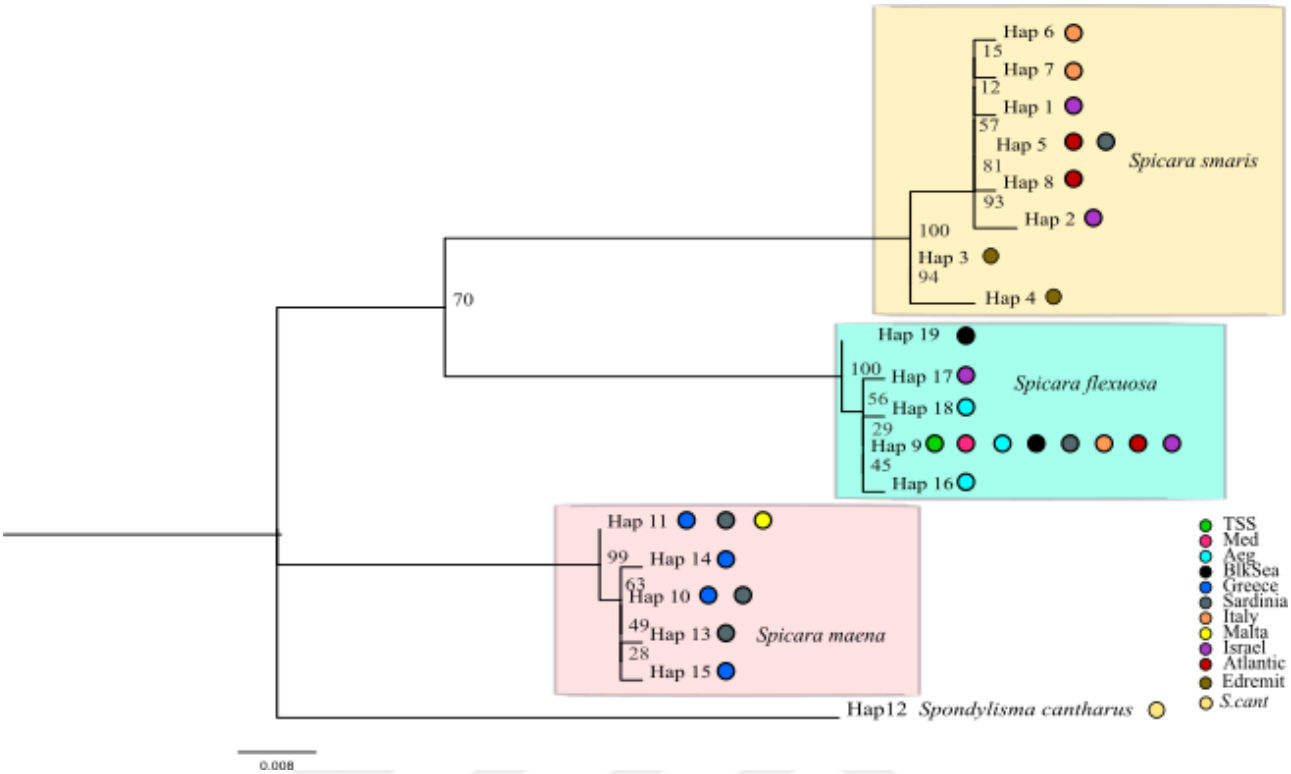


Figure 3.10. Neighbour-joining tree based on CO1 data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

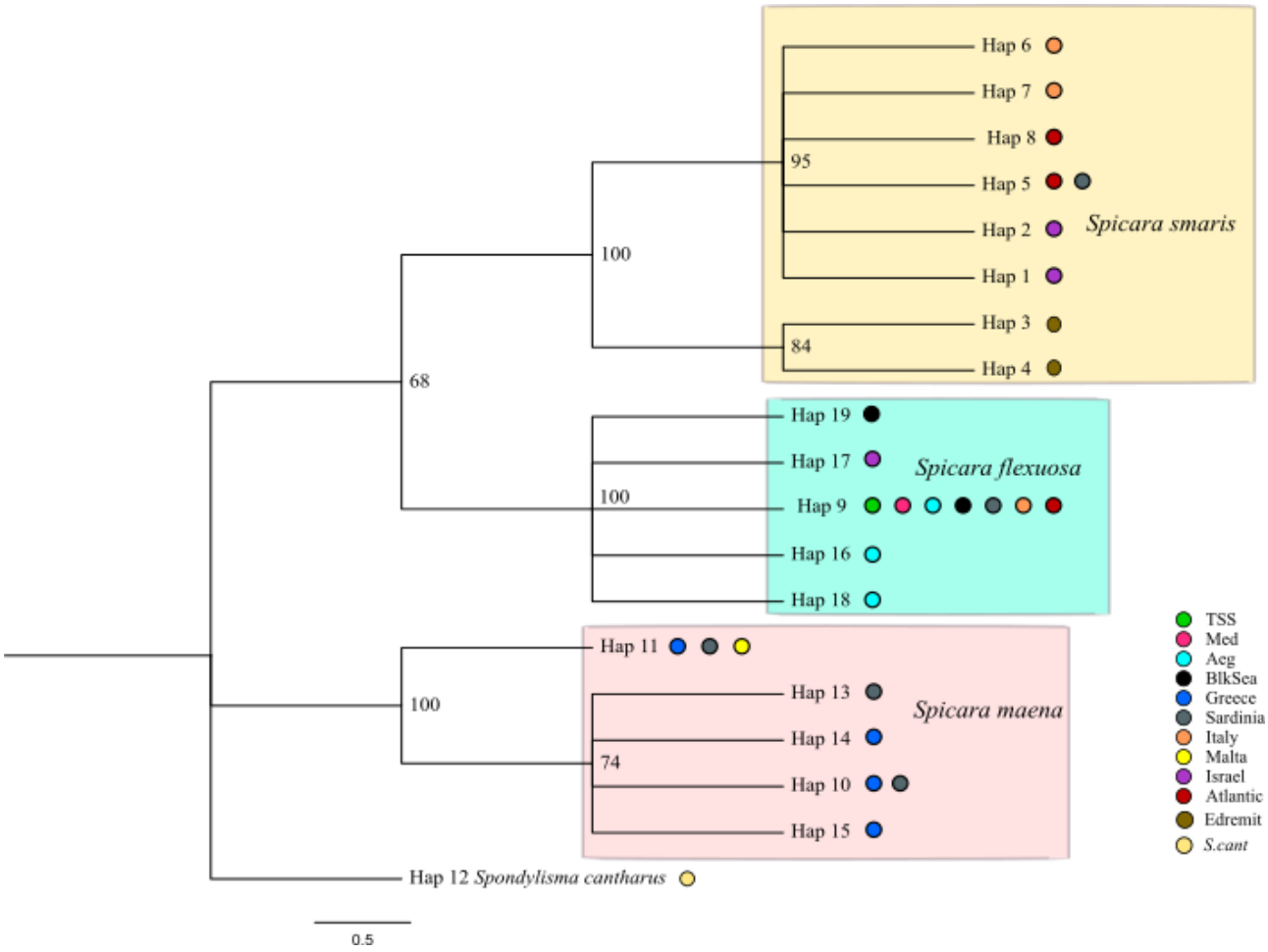


Figure 3.11. Maximum parsimony tree based on CO1 data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

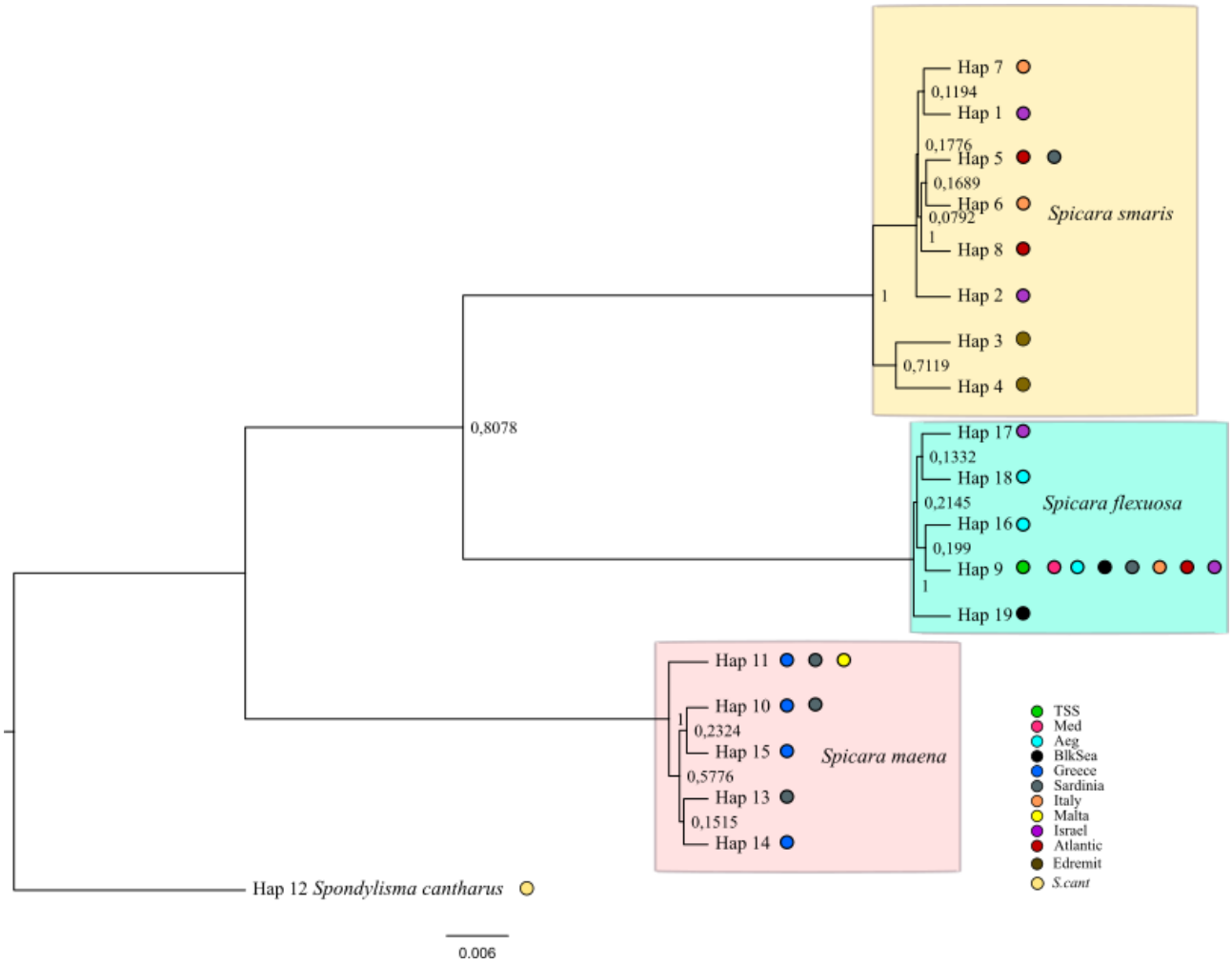


Figure 3.12. Bayesian tree based on CO1 data for *Spicara spp.* populations. The node values correspond to posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

All of the phylogenetic trees, built using different methods, revealed the three different haplogroups with high bootstrap values. Molecular diversity indices, and information on parsimony informative sites for the CO1 gene of the three *Spicara* species are shown in Table 3.6 and Table 3.7, respectively. Seven individuals from Portugal and Italy, one individual from Sardinia (*S. flexuosa*), and four individuals from Sardinia and Malta (*S. maena*) were eliminated from molecular diversity analyses due to their low sample sizes.

Considering codon analyses, average number of codon analyzed for this gene was 151. As can be seen in Appendix Table B.1, AUU (Isoleucine) was the most detected aminoacid, however stop codons were not observed in the CO1 gene.

Table 3.6. Molecular diversity of *Spicara spp*. based on CO1 region sequences. N: Number of samples; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations are given in parantheses.

	Sampling sites	**N**	**Nh**	**Np**	**h**	**π**	**SS**	**PIS**
Spicara flexuosa	İstanbul	17	1	0	0	0	0	0
	Çanakkale	19	1	0	0	0	0	0
	Armutlu	22	1	0	0	0	0	0
	Rize	19	2	1	0.105(0.092)	0.00023(0.00020)	1	0
	Sinop	13	1	0	0	0	0	0
	İzmir	24	2	1	0.083(0.075)	0.00018 (0.00017)	1	0
	Muğla	17	1	0	0	0	0	0
	Antalya	23	2	1	0.087(0.078)	0.00019(0.00017)	1	0
	Mersin	22	1	0	0	0	0	0
	Israel	13	2	1	0.385 (0.132)	0.00085 (0.00029)	0	1
	Total *S. flexuosa*	**189**	**5**	**4**	**0.062 (0.024)**	**0.00014(0.00005)**	**3**	**1**
	TSS	**58**	**1**	**0**	**0**	**0**	**0**	**0**
	Black Sea	**32**	**2**	**1**	**0.063 (0.058)**	**0.00014(0.00013)**	**1**	**0**
	Mediterranean	**45**	**2**	**1**	**0.044 (0.042)**	**0.00010 (0.00009)**	**1**	**0**
	Aegean	**41**	**2**	**1**	**0.049 (0.046)**	**0.00011 (0.00010)**	**1**	**0**
S. maena	Greece	23	4	3	0.605 (0.079)	0.00153 (0.00028)	1	2
	Total *S. maena*	**23**	**4**	**3**	**0.605(0.079)**	**0.00153 (0.00028)**	**1**	**2**
S. smaris	***Spicara smaris***	**32**	**8**	**12**	**0.639 (0.092)**	**0.0554 (0.00088)**	**3**	**9**

Among the three species, *Spicara smaris* had the highest (h=0.639; π=0.0554) and *S. flexuosa* had the lowest (h=0.062; π=0.00014) mean haplotype and nucleotide diversities, respectively. Considering *S. flexuosa* populations, Black Sea had the highest haplotype (h=0.063) and nucleotide (π=0.00014) diversities (Table 3.6).

Table 3.7. Parsimony informative sites of total *Spicara spp*. based on 454 bp long CO1 region sequences.

		Parsimony Informative Sites of COI region																														
Species name	Hap Numbers	4	13	16	20	22	28	37	49	55	67	73	76	79	85	97	112	115	118	124	130	136	139	151	154	163	169	172	175	178	181	187
S.cantharus	Hap 12	C	A	A	A	T	T	C	A	A	C	C	C	A	T	C	A	A	T	G	C	C	C	G	G	T	C	C	T	G	A	A
S.flexuosa	Hap 9	.	.	G	.	C	C	.	G	.	T	.	.	.	.	T	G	T	C	T	.	.	.	.	.	.	A	A	C	A	.	.
S.flexuosa	Hap 16	.	.	G	.	C	C	.	G	.	T	.	.	.	.	T	G	T	C	T	.	.	.	.	.	.	A	A	C	A	.	.
S.flexuosa	Hap 17	.	.	G	.	C	C	.	G	.	T	.	.	.	.	T	G	T	C	T	.	.	.	.	.	.	A	A	C	A	.	.
S.flexuosa	Hap 18	.	.	G	.	C	C	.	G	.	T	.	.	.	.	T	G	T	C	T	.	.	.	.	.	.	A	A	C	A	.	.
S.flexuosa	Hap 19	.	.	G	.	C	C	.	G	.	.	.	.	.	.	T	G	T	C	T	.	.	.	.	.	.	A	A	C	A	.	.
S.maena	Hap 10	.	.	.	.	C	.	.	.	G	.	T	.	.	C	.	G	.	C	T	.	T	.	A	A	.	.	T	C	A	.	.
S.maena	Hap 11	.	.	.	.	C	.	.	.	G	.	T	.	.	C	.	G	.	C	T	.	T	.	A	A	.	.	T	C	A	.	.
S.maena	Hap 13	.	.	.	.	C	.	.	.	G	.	T	.	.	C	.	G	.	C	T	.	T	.	A	A	.	.	T	C	A	.	.
S.maena	Hap 14	.	.	.	.	C	.	.	.	G	.	T	.	.	C	.	G	.	C	T	.	T	.	A	A	.	.	T	C	A	.	.
S.maena	Hap 15	.	.	.	.	C	.	.	.	G	.	T	.	.	C	.	G	.	C	T	.	T	.	A	A	.	.	T	C	A	.	.
S.smaris	Hap 1	T	T	.	C	.	.	T	.	.	T	.	T	G	.	T	.	T	.	A	T	.	T	A	A	C	.	T	.	.	G	G
S.smaris	Hap 2	T	T	.	C	.	.	T	.	.	T	.	T	G	.	T	.	T	.	A	T	.	T	A	A	C	.	T	.	.	G	G
S.smaris	Hap 3	T	T	.	C	C	.	T	.	.	T	.	T	.	.	T	.	T	.	A	T	.	T	A	A	C	.	T	.	.	G	G
S.smaris	Hap 4	T	T	.	C	C	.	T	.	.	T	.	T	.	.	T	.	T	.	A	T	.	T	A	A	C	.	T	.	.	G	G
S.smaris	Hap 5	T	T	.	C	.	.	T	.	.	T	.	T	G	.	T	.	T	.	A	T	.	T	A	A	C	.	T	.	.	G	G
S.smaris	Hap 6	T	T	.	C	.	.	T	.	.	T	.	T	G	.	T	.	T	.	A	T	.	T	A	A	C	.	T	.	.	G	G
S.smaris	Hap 7	T	T	.	C	.	.	T	.	.	T	.	T	G	.	T	.	T	.	A	T	.	T	A	A	C	.	T	.	.	G	G
S.smaris	Hap 8	T	T	.	C	.	.	T	.	.	T	.	T	G	.	T	.	T	.	A	T	.	T	A	A	C	.	T	.	.	G	G

Table 3.7. Parsimony informative sites of total *Spicara spp*. based on 454 bp long CO1 region sequences (cont.).

		Parsimony Informative Sites of COI region																																
Species name	Hap Numbers	190	193	196	199	200	211	223	232	236	247	253	283	301	304	328	331	337	346	358	361	362	383	385	388	391	397	409	418	427	436	448	454
S.cantharus	Hap 12	T	C	A	A	A	T	C	T	T	C	C	C	C	C	C	C	A	G	A	G	G	T	A	C	C	C	C	A	G	A	A	C
S.flexuosa	Hap 9	C	.	.	.	.	C	T	.	.	T	.	T	T	T	T	.	G	A	.	A	T	.	.	.	.	T	T	.	.	.	C	T
S.flexuosa	Hap 16	C	.	.	.	.	C	T	.	.	T	.	T	T	T	T	.	G	A	.	A	T	.	.	.	.	T	T	.	.	.	C	T
S.flexuosa	Hap 17	C	.	.	.	.	C	T	.	.	T	.	.	T	T	T	.	G	A	.	A	T	.	.	.	.	T	T	.	.	.	C	T
S.flexuosa	Hap 18	C	.	.	.	.	C	T	.	.	T	.	T	T	T	T	.	G	A	G	A	T	.	.	.	.	T	T	.	.	.	C	T
S.flexuosa	Hap 19	C	.	.	.	.	C	T	.	.	T	.	T	T	T	T	.	G	A	.	A	T	.	.	.	.	T	T	.	.	.	C	T
S.maena	Hap 10	C	G	G	.	.	.	.	C	C	.	T	T	.	.	.	.	G	.	.	.	.	C	G	.	.	T	A	G	.	.	T	.
S.maena	Hap 11	C	G	G	.	.	.	.	.	C	.	T	T	.	.	.	.	G	.	.	.	.	C	G	.	.	T	A	G	.	.	T	.
S.maena	Hap 13	C	G	G	.	.	.	.	C	C	.	T	T	.	.	.	.	G	.	.	.	.	C	G	.	.	T	A	G	.	.	T	.
S.maena	Hap 14	C	G	G	.	.	.	.	C	C	.	T	T	.	.	.	.	.	.	.	.	.	C	G	.	.	T	A	G	.	.	T	.
S.maena	Hap 15	C	G	G	.	.	.	.	C	C	.	T	T	.	.	.	.	G	.	.	.	.	C	G	.	.	T	A	G	.	.	T	.
S.smaris	Hap 1	.	.	.	G	G	C	T	.	.	.	T	T	.	.	.	T	G	A	.	A	.	-	.	T	T	.	.	.	A	C	T	.
S.smaris	Hap 2	.	T	.	G	G	C	T	.	.	T	T	T	.	.	.	T	G	A	.	A	.	.	.	T	T	.	.	.	A	C	T	.
S.smaris	Hap 3	.	.	.	G	G	C	T	.	.	.	T	T	.	.	.	.	G	A	.	A	.	.	.	T	T	.	.	.	A	C	T	.
S.smaris	Hap 4	.	.	.	G	G	C	T	.	.	.	T	T	.	.	.	.	G	A	.	A	.	.	.	T	T	.	.	.	A	C	T	.
S.smaris	Hap 5	.	.	.	G	G	C	T	.	.	.	T	T	.	.	.	T	G	A	.	A	.	.	.	T	T	.	.	.	A	C	T	.
S.smaris	Hap 6	.	.	.	G	G	C	T	.	.	.	T	T	.	.	.	T	G	A	.	A	.	.	.	T	T	.	.	.	A	C	T	.
S.smaris	Hap 7	.	.	.	G	G	C	T	.	.	.	T	T	.	.	.	T	G	A	.	A	.	.	.	T	T	.	.	.	A	C	T	.
S.smaris	Hap 8	.	.	.	G	G	C	T	.	.	.	T	T	.	.	.	T	G	A	G	A	.	.	.	T	T	.	.	.	A	C	T	.

Total number of haplotypes, singleton variable and parsimony informative sites were also high within the *Spicara smaris* samples (Table 3.6). Among the three species, out of 454 bp, a total of 85 (18 %) nucleotide sites were variable, of which 63 (13.8 %) positions were parsimony informative (Table 3.7). As seen in Table 3.7., a total of 26 positions were parsimony informative for grouping *S. maena* and *S. flexuosa,* 18 for *S. flexuosa* and *S. smaris*, and 15 for *S. maena* and *S. smaris*.

Considering evolutionary divergence times and phylogenetic separation of the three species, molecular clock hypothesis was not rejected among the three species. Total number of mutations and pairwise sequence divergences, and divergence times among the three species are also given in Table 3.8. Total number of mutations, sequence divergences and divergence times indicate similar levels of differentiation among the three *Spicara* species. Estimated divergence time revealed that three species began to diversify during the late Pliocene.

Table 3.8. Total number of mutations (below diagonal), pairwise sequence divergences and divergence times (above diagonal) based on the CO1 gene, among the three *Spicara* species.

Species name	*S. maena*	*S. flexuosa*	*S. smaris*
S. maena		8.113 % -3.33 Mya	9.314 % -3.83 Mya
S. flexuosa	43		8.459 % - 3.5 Mya
S. smaris	56	51	

3.1.2.1. Mitochondrial population structure of CO1 gene. Analysis of molecular variance (AMOVA) and pairwise Φ_{ST} values are given in Table 3.9. Since haplotype network and phylogenetic trees revealed three species based on the CO1 gene, pairwise Φ_{ST} values were calculated for each of these groups. For *Spicara flexuosa* individuals, Φ_{ST} values were also computed taking data from all sampling locations (subpopulations) in Turkish coastal waters, as well as Israel subpopulations from GenBank. AMOVA analysis, however, was only undertaken for the Black Sea, TSS, Aegean and Mediterranean groups in *Spicara flexuosa*.

Table 3.9. Pairwise Φ_{ST} values of the CO1 gene between three species of *Spicara spp*. Significant *P* (< 0.05) values are given in shaded cells.

			İstanbul	Çanakkale	Armutlu	Izmir	Muğla	Rize	Sinop	Antalya	Mersin	Israel	Greece	*S. smaris*
Spicara flexuosa	TSS	**İstanbul**	0.00											
		Çanakkale	0.00	0.00										
		Armutlu	0.00	0.00	0.00									
	Aeg.	**İzmir**	-0.01	-0.01	-0.00	0.00								
		Muğla	0.00	0.00	0.00	-0.01	0.00							
	BlkSea	**Rize**	-0.00	0.00	0.00	0.00	-0.00	0.00						
		Sinop	0.00	0.00	0.00	-0.02	0.00	-0.02	0.00					
	Med.	**Antalya**	-0.01	-0.00	-0.00	0.00	-0.01	0.00	-0.02	0.00				
		Mersin	0.00	0.00	0.00	-0.00	0.00	0.00	0.00	-0.00	0.00			
		Israel	0.20	0.21	**0.24**	**0.19**	0.20	**0.16**	0.16	**0.18**	**0.24**	0.00		
S. maena		**Greece**	**0.98**	**0.98**	**0.99**	**0.98**	**0.98**	**0.98**	**0.98**	**0.98**	**0.99**	**0.98**	0.00	
S. smaris		***S. smaris***	**0.95**	**0.95**	**0.96**	**0.96**	**0.95**	**0.95**	**0.95**	**0.96**	**0.96**	**0.95**	**0.95**	0.00

Pairwise Φ_{ST} values were the highest (Φ_{ST}=0.99), and significant between the pairwise comparisons of *S. flexuosa, S. maena* and *S. smaris*. However, considering subpopulations for *S. flexuosa,* low level of genetic structure (Φ_{ST}= 0.00) was observed among individuals in the Turkish coastal waters. Taking data from GenBank into account as well, Israel populations were significantly differentiated from some Turkish populations, specifically Armutlu (Φ_{ST}=0.24), İzmir (Φ_{ST}=0.19), Rize (Φ_{ST}=0.16), Antalya (Φ_{ST}=0.18) and Mersin (Φ_{ST}=0.24) (Table 3.9).

AMOVA within *S. flexuosa* individuals showed that group variation was very low among the four regions (namely Black Sea, TSS, Aegean, and the Mediterranean) around the Turkish coasts. Only 1.07 % variation was found between these four seas, none (-1.48 %) of the variation was among populations within groups, and practically all (100.40 %) of the variation was within populations. AMOVA analyses did not show any hierarchical regional structuring among populations within groups or among groups.

3.1.3. The cyt-b Gene

The cyt-b gene was amplified in 157 individuals of *Spicara flexuosa*/*maena* (139 from Turkey, 18 from Greece), from 10 sampling locations (Appendix Table F.1). A 355 bp fragment was used for all analyses. For this gene, to determine the species identity of the *Spicara* samples collected in this study, two *S. maena* (EU036504, EU036505), two *S. flexuosa* (EU03650, EU036503), and two *S. smaris* (EU036506, EU036507) sequences from Greece (Krey et al., 2007) were added, as previously submitted to GenBank in 2007. Krey et al. (2007) were able to differentiate the three *Spicara* species by using cyt-b gene in a submission with an associated unpublished study entitled 'The sequence of the cytochrome b gene from 50 marine fish species of the North Aegean Sea (Eastern Mediterranean)'. In addition, two individuals of *Spicara smaris*, three *Spicara maena* (Italy and Spain) and one outgroup species *Spondylisma cantharus* were added from GenBank. Accession numbers and locality information for these individuals are given in Table 3.10.

Table 3.10. The accession numbers and locations of individuals for cyt-b sequences of *Spicara spp*. retrieved from GenBank.

Species	Accession numbers	Location
S. maena	EF439598	Spain
S. maena	EU036505	Greece
S. maena	EU036504	Greece
S. maena	AF240737	Italy
S. maena	KT883671	Italy
S. flexuosa	EU036503	Greece
S. flexuosa	EU036502	Greece
S. smaris	EU036507	Greece
S. smaris	EU036506	Greece
S. smaris	EF439600	Spain
S. smaris	KT883672	Italy

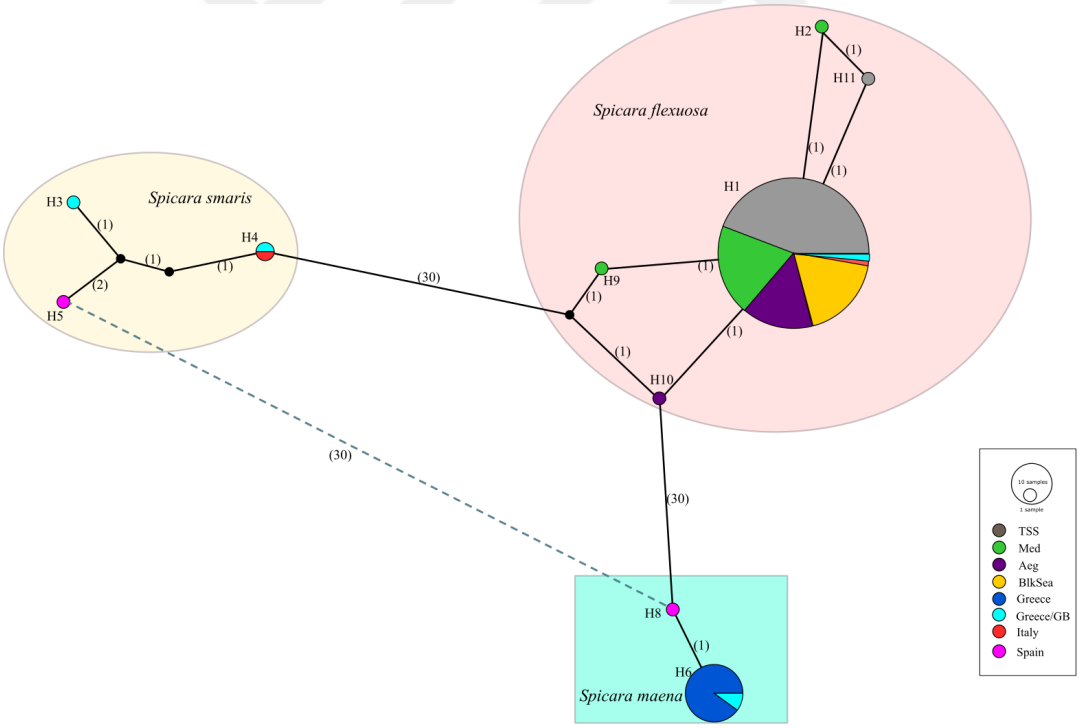


Figure 3.13. The haplotype network based on the cyt-b data of all *Spicara spp.* populations. The numbers correspond to the number of mutations between haplotypes. The dashed line represents connection between *S. maena* and *S. smaris* haplogroups. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Black circles represent missing haplotypes.

A total of 11 haplotypes were found in 169 individuals (Figure 3.13). Based on the classification of Krey et al. (2007), samples collected from Turkish coastal waters, all of which had Hap 1, were clustered within *S. flexuosa* from Greece (GenBank), whereas samples collected from Greece, all of which had Hap 6, were clustered with *S. maena* from Greece (GenBank).

As can be seen in haplotype network (Figure 3.13), *Spicara flexuosa* samples from Italy (previously identified as *S. maena* in GenBank), Greece (GenBank) (Hap 1), and samples collected from Turkish coastal waters were in the *S. flexuosa* haplogroup. One sample from Spain, which was classified previously as *S. maena* (GenBank) (Hap 8) was detected in the *S. maena* haplogroup. Finally, samples from Greece, Spain, Italy, (Hap 3, Hap 4, and Hap 5) retrieved from GenBank were in the *S. smaris* haplogroup. Based on the cyt-b gene results, overlapping distribution for the three species was only seen in Greece, *S. maena* was found in Greece and Spain, and *S. smaris* was recorded in the Mediterranean locations, except Turkish coastal waters.

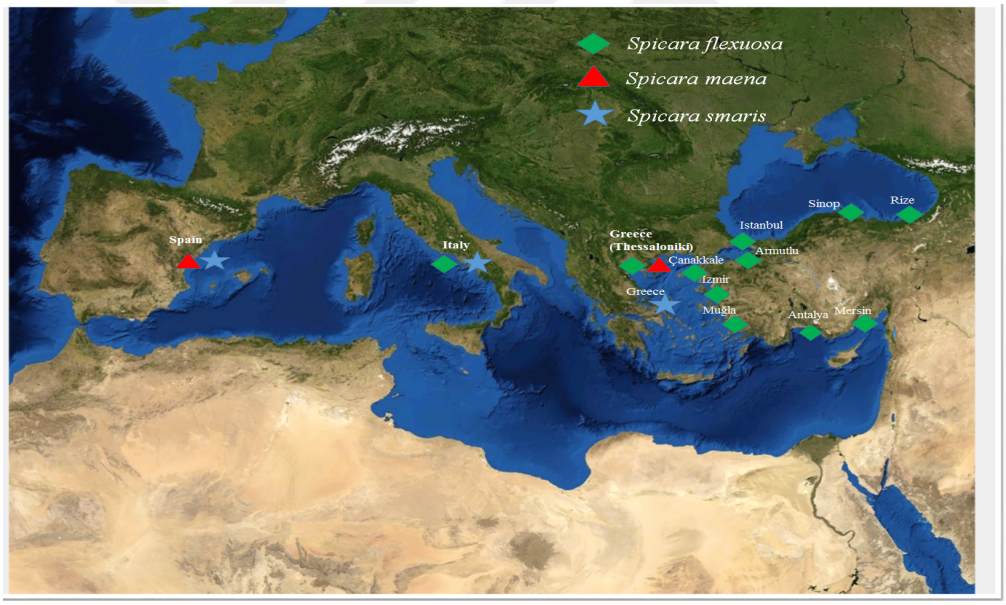


Figure 3.14. Sampling locations of three *Spicara* species, based on the cyt-b gene, in the Mediterranean.

Phylogenetic relationships between haplotypes were determined by maximum likelihood (Figure 3.15), neighbour-joining (Figure 3.16), maximum parsimony (Figure 3.17), and Bayesian trees (Figure 3.18).

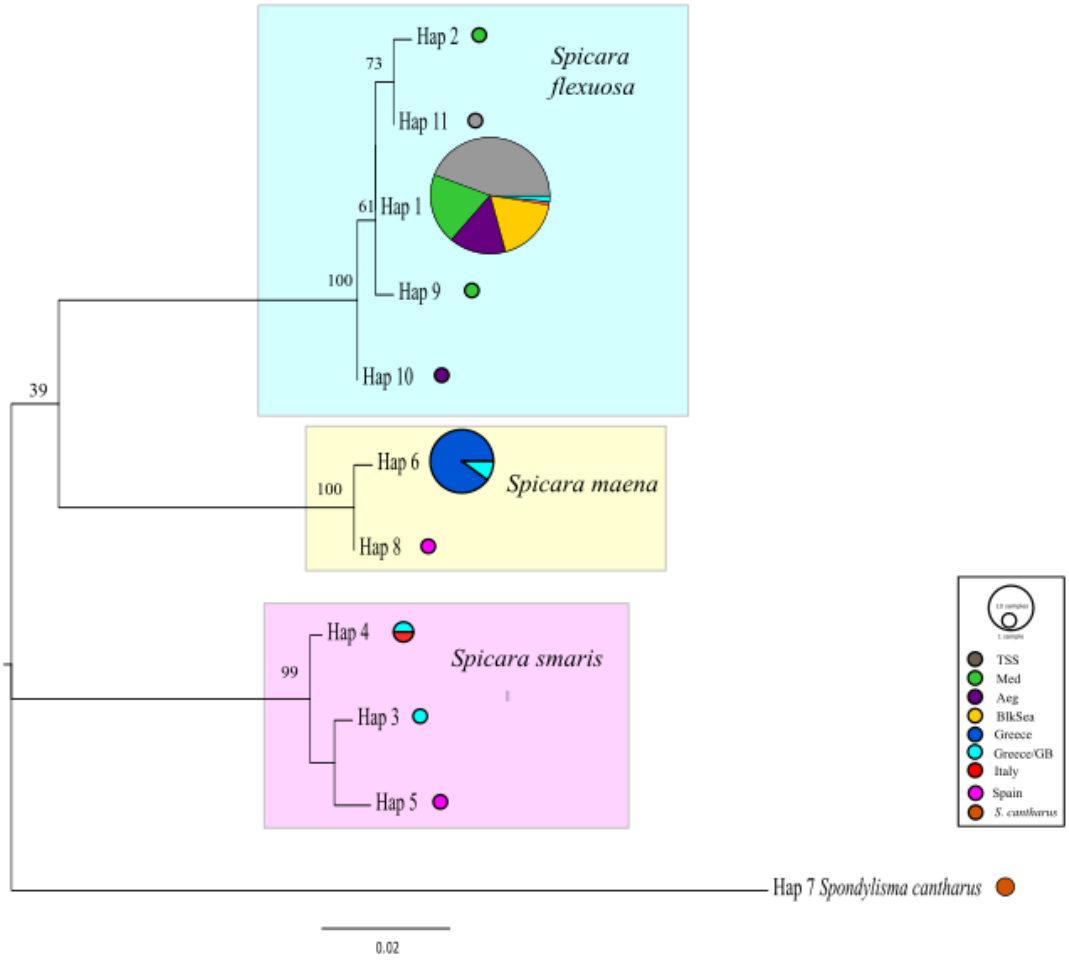


Figure 3.15. Maximum likelihood tree based on cyt-b data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Size of the circles are proportional to the relative frequencies of the haplotypes.

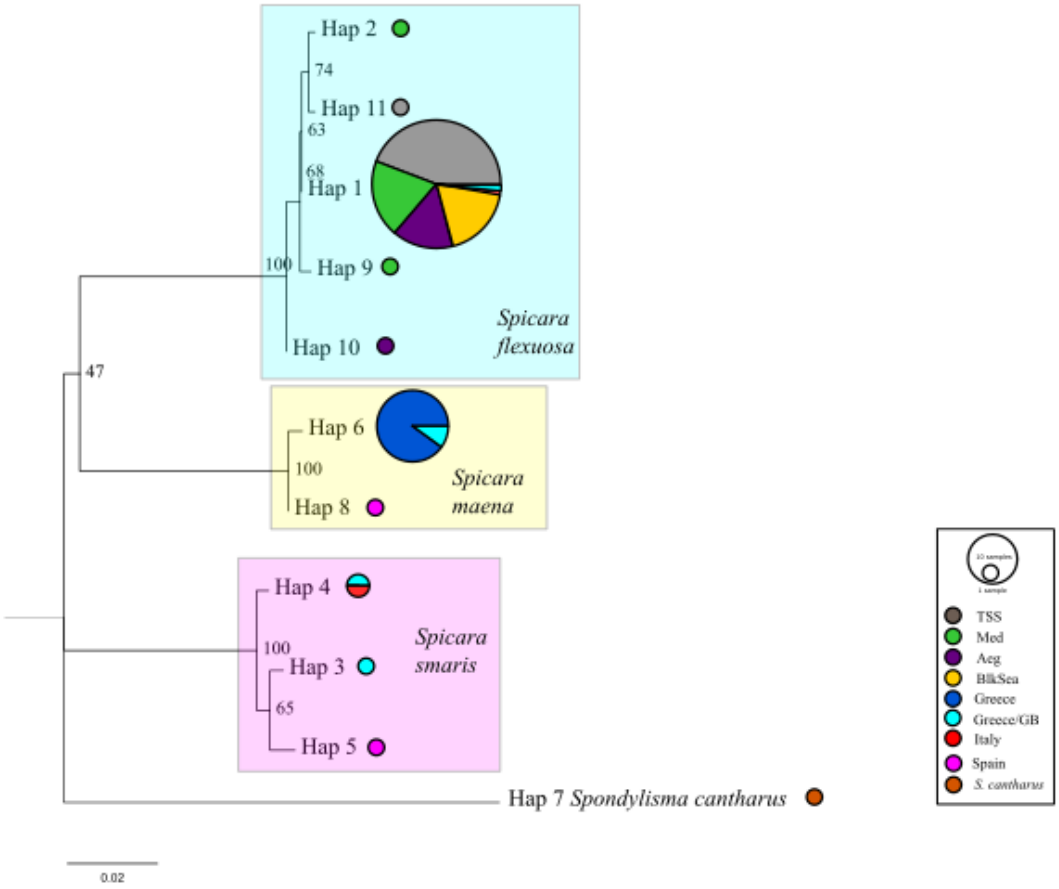


Figure 3.16. Neighbour-joining tree based on cyt-b data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Size of the circles are proportional to the relative frequencies of the haplotypes.

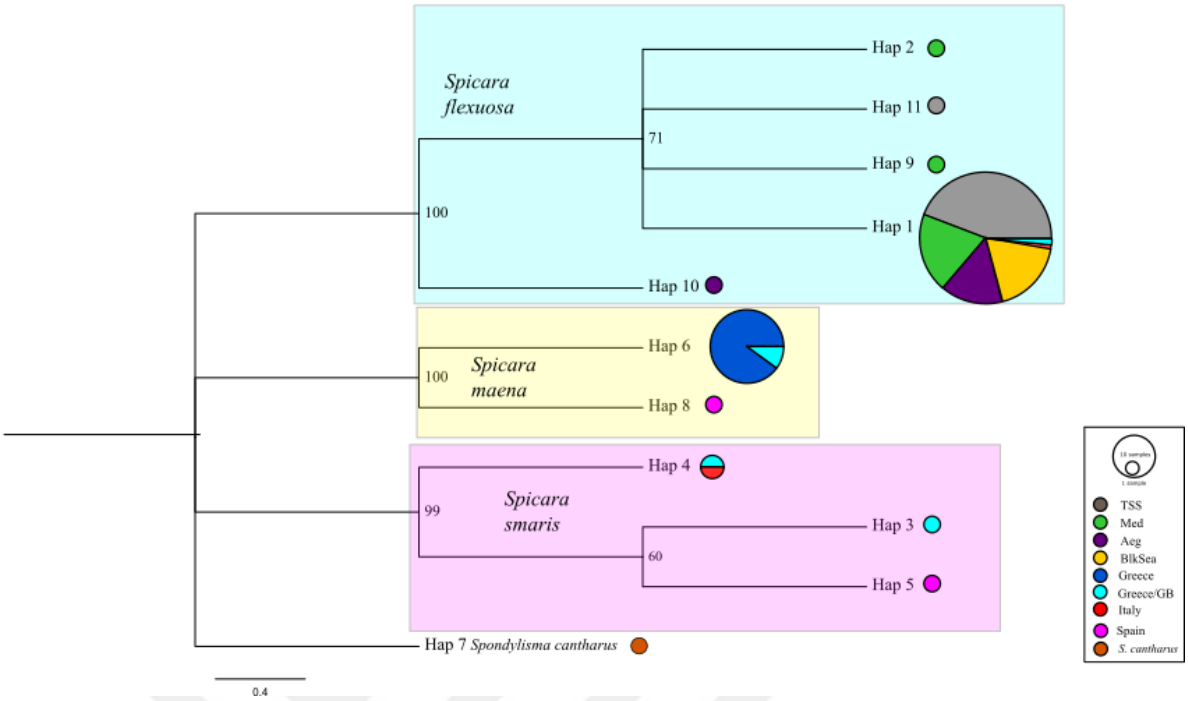


Figure 3.17. Maximum parsimony tree based on cyt-b data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Size of the circles are proportional to the relative frequencies of the haplotypes.

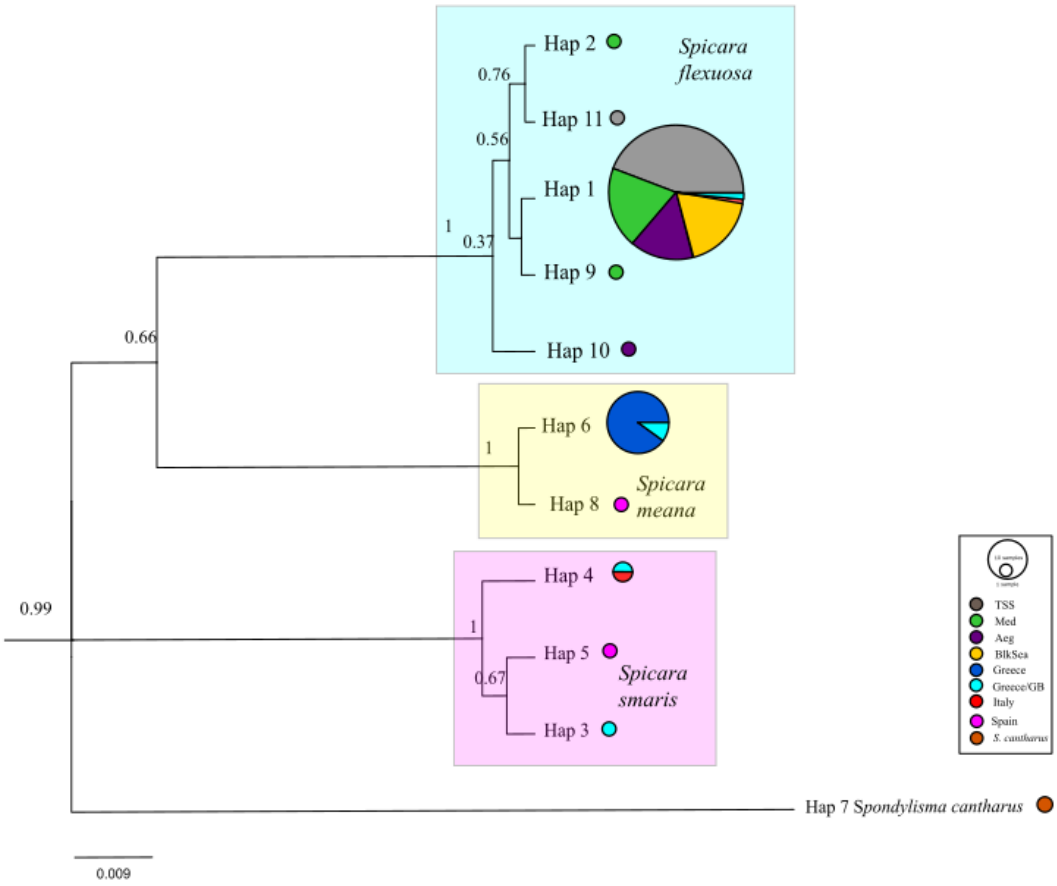


Figure 3.18. Bayesian tree based on cyt-b data for *Spicara spp.* populations. The node values correspond to posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Size of the circles are proportional to the relative frequencies of the haplotypes.

Maximum likelihood (Figure 3.15), neighbour-joining (Fig 3.16), maximum parsimony (Fig 3.17) and Bayesian trees (Fig 3.18) revealed the same tree topology with high bootstrap values. In the cyt-b trees, *S. flexuosa* and *S. maena* were closely related to each other when compared to *S. smaris.* As shown in all of the phylogenetic trees, *Spicara smaris* is the basal species as compared to the two other *Spicara* species.

Molecular diversity indices of cyt-b gene of all *Spicara spp.* populations and parsimony informative sites are shown in Table 3.11 and Table 3.12, respectively. As done with the phylogenetic trees and haplotype network, *Spicara spp.* populations were analyzed as three different species in terms of their parsimony informative sites. On the other hand, molecular diversity indices analyses were performed for *S. flexuosa* and *S. maena*, only. *S. smaris* (n=4), Sinop samples (n=5), four *S.*

flexuosa individuals from Italy and Greece, and one individual from Spain (*S. maena*) were excluded from this analysis due to their low sample sizes.

Table 3.11. Molecular diversity of *Spicara spp*. based on cyt-b region sequences. N: Number of samples; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations are given in parantheses.

	Sampling sites	**N**	**Nh**	**Np**	**h**	**π**	**SS**	**PIS**
Spicara flexuosa	İstanbul	20	1	0	0	0	0	0
	Çanakkale	17	2	1	0.118 (0.101)	0.00033 (0.00028)	1	0
	Armutlu	25	1	0	0	0	0	0
	Rize	20	1	0	0	0	0	0
	İzmir	7	2	1	0.286 (0.196)	0.00080 (0.00055)	1	0
	Muğla	15	1	0	0	0	0	0
	Antalya	21	2	1	0.095 (0.084)	0.00027(0.00024)	1	0
	Mersin	9	2	1	0.389 (0.164)	0.00110(0.00046)	0	1
	Total *S. flexuosa*	**134**	**5**	**3**	**0.073 (0.031)**	**0.00021 (0.00009)**	**2**	**1**
	TSS	**62**	**2**	**1**	**0.032 (0.031)**	**0.00009 (0.00009)**	**1**	**0**
	Black Sea	**20**	**1**	**0**	**0**	**0**	**0**	**0**
	Mediterranean	**30**	**3**	**2**	**0.191(0.093)**	**0.00055 (0.00028)**	**1**	**1**
	Aegean	**22**	**2**	**1**	**0.091 (0.081)**	**0.00026 (0.00023)**	**1**	**0**
S. maena	Greece	20	1	0	**0**	**0**	0	0
	Total *S. maena*	**20**	**1**	**0**	**0**	**0**	**0**	**0**

As shown in Table 3.11, the mean highest haplotype and nucleotide diversities (h= 0.073; π=0.00021) were found in *Spicara flexuosa* populations. Specifically, Mersin (h=0.389; π=0.00110) and İzmir (h= 0.286; π=0.00080) had the highest haplotype and nucleotide diversities, respectively. Considering regions, Mediterranean had the highest (h=0.191; π=0.00055) haplotype and nucleotide diversities. On the other hand, *S. maena* had the lowest (h=0, π=0) haplotype and nucleotide diversities.

Table 3.12. Parsimony informative sites of total *Spicara spp*. based on 355 bp long cyt-b region sequences.

		Parsimony Informative Sites of cyt-b. region																							
Species name	Hap Numbers	3	14	20	38	39	44	53	68	80	86	101	104	107	110	113	116	122	131	134	137	149	155	162	167
S.cantharus	Hap 7	C	T	C	C	T	C	C	G	G	G	C	A	T	T	C	A	A	A	C	G	C	C	G	A
S.flexuosa	Hap 1	.	C	T	T	.	T	.	A	.	A	A	.	C	G	T	.	.	G	.	.	.	.	.	G
S.flexuosa	Hap 2	.	C	T	T	.	T	.	A	.	A	A	.	C	G	T	.	.	G	.	.	.	.	.	G
S.flexuosa	Hap 9	T	C	T	T	.	T	.	A	.	A	A	.	C	G	T	.	.	G	.	.	.	.	.	G
S.flexuosa	Hap 10	.	C	T	T	.	.	.	A	.	A	A	.	C	G	T	.	.	G	.	.	.	.	.	G
S.flexuosa	Hap 11	.	C	T	T	.	T	.	A	.	A	A	.	C	G	T	.	.	G	.	.	.	.	.	G
S.maena	Hap 6	.	C	.	T	.	.	.	A	A	.	A	G	.	.	.	G	G	.	T	C	.	T	A	.
S.maena	Hap 8	.	C	.	T	.	.	.	A	A	.	A	G	.	.	.	G	G	.	T	C	.	T	A	.
S.smaris	Hap 3	T	A	T	.	C	.	A	.	.	A	G	G	.	.	T	.	G	.	.	A	T	.	.	.
S.smaris	Hap 4	T	A	T	.	C	.	A	.	.	A	G	G	.	.	.	.	G	G	.	A	T	.	.	.
S.smaris	Hap 5	T	A	T	.	C	.	A	.	.	A	G	G	.	.	.	.	G	.	.	A	T	.	.	.

Table 3.12. Parsimony informative sites of total *Spicara spp*. based on 355 bp long cyt-b region sequences (cont.).

		Parsimony Informative Sites of cyt-b. region																							
Species name	Hap Numbers	188	194	197	198	203	209	215	227	233	245	263	272	275	278	305	308	325	335	336	338	341	344	350	353
S.cantharus	Hap 7	A	A	C	C	T	G	A	A	A	A	C	A	A	A	T	C	C	C	C	C	C	A	T	C
S.flexuosa	Hap 1	.	G	.	.	C	A	G	.	.	G	.	.	C	.	.	T	.	.	T	A	T	C	C	.
S.flexuosa	Hap 2	.	G	.	.	C	A	G	.	.	G	.	.	C	.	.	T	.	.	T	A	T	C	C	.
S.flexuosa	Hap 9	.	G	.	.	C	A	G	.	.	G	.	.	C	.	.	T	.	.	T	A	T	C	C	.
S.flexuosa	Hap 10	.	G	.	.	C	A	G	.	.	G	.	.	C	.	.	T	.	.	T	A	T	C	C	.
S.flexuosa	Hap 11	.	G	.	.	C	A	G	.	.	G	.	.	C	.	.	T	.	.	T	A	T	C	C	.
S.maena	Hap 6	.	.	.	T	.	A	G	.	G	.	.	.	C	.	C	.	T	T	.	G	.	G	.	T
S.maena	Hap 8	.	.	.	T	.	A	G	.	G	.	.	.	C	.	C	.	T	T	.	G	.	G	.	T
S.smaris	Hap 3	G	.	T	T	.	.	.	G	.	.	G	G	T	G	.	.	.	.	.	A	.	G	C	.
S.smaris	Hap 4	.	.	T	T	.	.	.	G	.	.	G	G	T	G	.	.	.	.	.	A	.	G	C	.
S.smaris	Hap 5	G	.	T	T	.	A	.	G	.	.	G	G	T	G	.	.	.	.	.	A	.	G	C	.

Among the three species, a total of 77 (21 %) nucleotide sites were variable of which 48 (13.5 %) positions were parsimony informative. A total of 16 positions were parsimony informative for *S. flexuosa* and *S. maena*, 14 for *S. smaris* and *S. flexuosa*, and 14 for *S. maena* and *S. smaris* (Table 3.12).

Considering evolutionary divergence times and phylogenetic separation of the three species, the molecular clock hypothesis was not rejected among the three species. Total number of mutations and pairwise sequence divergences, and divergence times among the three species are also given in Table 3.13. As can be seen in Table 3.13., total number of mutations and sequence divergences were approximately equal among the three species; hence they seem to be more or less equally different from each other, as observed in the numbers of parsimony informative sites, as well. Unlike the CO1 gene, the estimated divergence times of three *Spicara* species showed that the three species began to diversify from each other in the late Miocene.

Table 3.13. Total number of mutations (below diagonal), pairwise sequence divergences and divergence times (above diagonal), based on the cyt-b gene among the three *Spicara* species.

Species name	*S. maena*	*S. flexuosa*	*S. smaris*
S. maena		9.007 % -5.84 Mya	9.353 % -5.97 Mya
S. flexuosa	35		9.227 % - 6.03 Mya
S. smaris	36	37	

3.1.3.1. Mitochondrial population structure of cyt-b gene. Analysis of molecular variance (AMOVA) and pairwise Φ_{ST} values between two groups of *Spicara spp.* populations are given in Table 3.14. *S. smaris* individuals were not used for these analyses because of the low sample sizes (n=4). AMOVA analysis, however, was only undertaken between the Black Sea, TSS, Aegean and Mediterranean groups in *Spicara flexuosa*.

Table 3.14. Pairwise Φ_{ST} values of the cyt-b gene between three species of *Spicara spp*. Significant *P* (< 0.05) values are given in shaded cells.

			İstanbul	Çanakklale	Armutlu	İzmir	Muğla	Rize	Antalya	Mersin	Greece
Spicara flexuosa	TSS	İstanbul	0.00								
		Çanakkale	0.00	0.00							
		Armutlu	0.00	0.02	0.00						
	Aeg	İzmir	0.16	0.04	0.20	0.00					
		Muğla	0.00	-0.00	0.00	0.11	0.00				
	BlkSea	Rize	0.00	0.00	0.00	0.16	0.00	0.00			
	Med	Antalya	-0.00	0.00	0.00	0.06	-0.01	-0.00	0.00		
		Mersin	0.25	0.09	**0.30**	0.07	0.20	0.25	0.17	0.00	
S. maena		Greece	**0.99**	**0.99**	**0.99**	**0.99**	**0.99**	**0.99**	**0.99**	**0.99**	0.00

The highest and significant pairwise (Φ_{ST}= 0.99) values were observed between rather than within species. However, considering subpopulations for *S. flexuosa,* low levels of genetic structure was observed among individuals in the Turkish coastal waters. Pairwise Φ_{ST} values ranged from -0.01 to 0.30, and only one comparison (between Armutlu and Mersin) was significant among the *Spicara flexuosa* subpopulations (Table 3.14).

AMOVA analyses within *S. flexuosa* individuals showed that the distribution of variation was low among four groups in the Turkish coastal waters. No variation (-6.3 %) was found among the four different groups, 14.09 % of the variation was among populations within groups, and 92.20 % variation was within populations, similar to the CO1 results.

Codon usage analysis was also conducted for this gene. Average number of codons analyzed for this gene was 118. Relative synonymous codon usage and all codons were given in the Appendix Table B.2. As shown in this table, AUU (Isoleucine) was the most widely detected aminoacid. Stop codons were not observed for this gene, similar to the results for the CO1 gene.

3.1.4. mtDNA Analyses: CO1 and cyt-b Concatenated Data Analyses for *Spicara spp.*

The concatenated cyt-b and CO1 data consisted of an alignment of 809 bases for 116 individuals of *Spicara flexuosa*/*maena* (101 from Turkey, 15 from Greece), from 10 sampling locations (Appendix Table F.1). In addition, one set of sequences from the closely related outgroup species, *Spondylisma cantharus*, and another set of *Spicara smaris* (GenBank Accession numbers for CO1: KT883623 and cyt-b: KT883672) sequences were added to our data. Phylogenetic relationships between haplotypes were determined by maximum likelihood (Figure 3.19), neighbour-joining (Figure 3.20), maximum parsimony (Figure 3.21) and Bayesian trees (Figure 3.22).

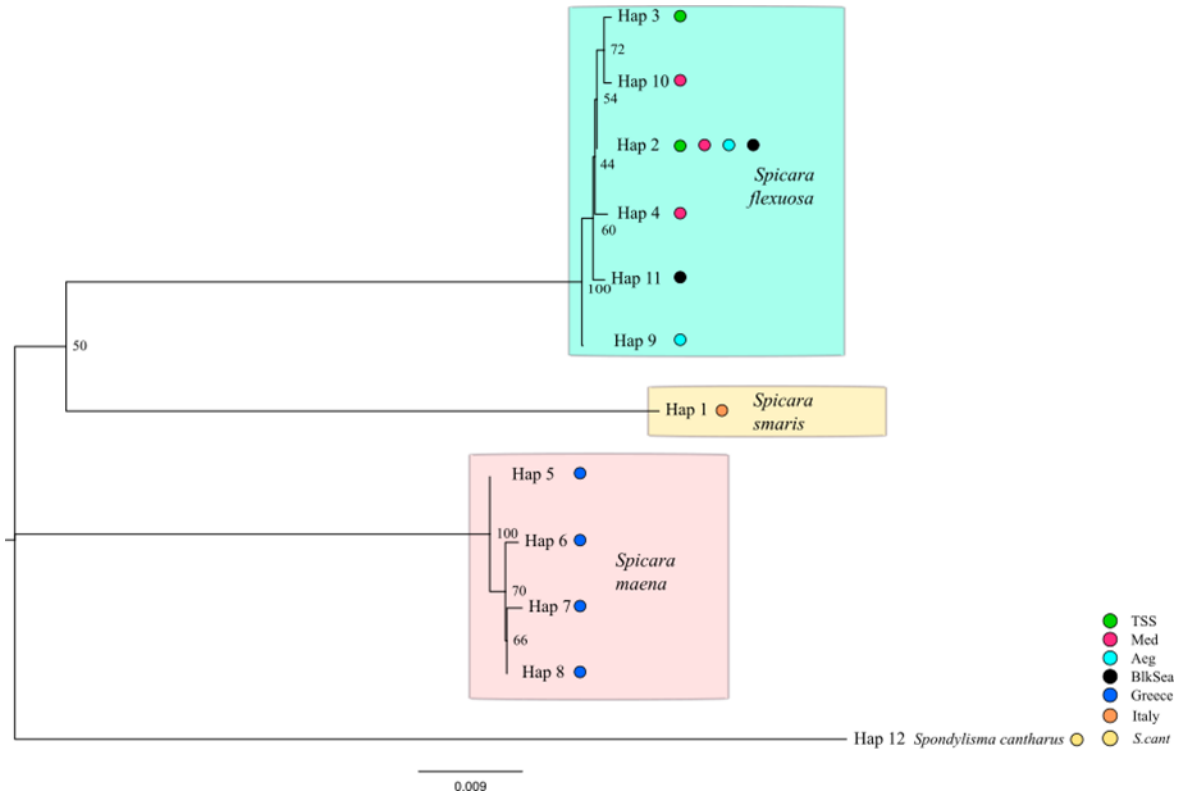


Figure 3.19. Maximum likelihood tree based on cyt-b+CO1 concatenated data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

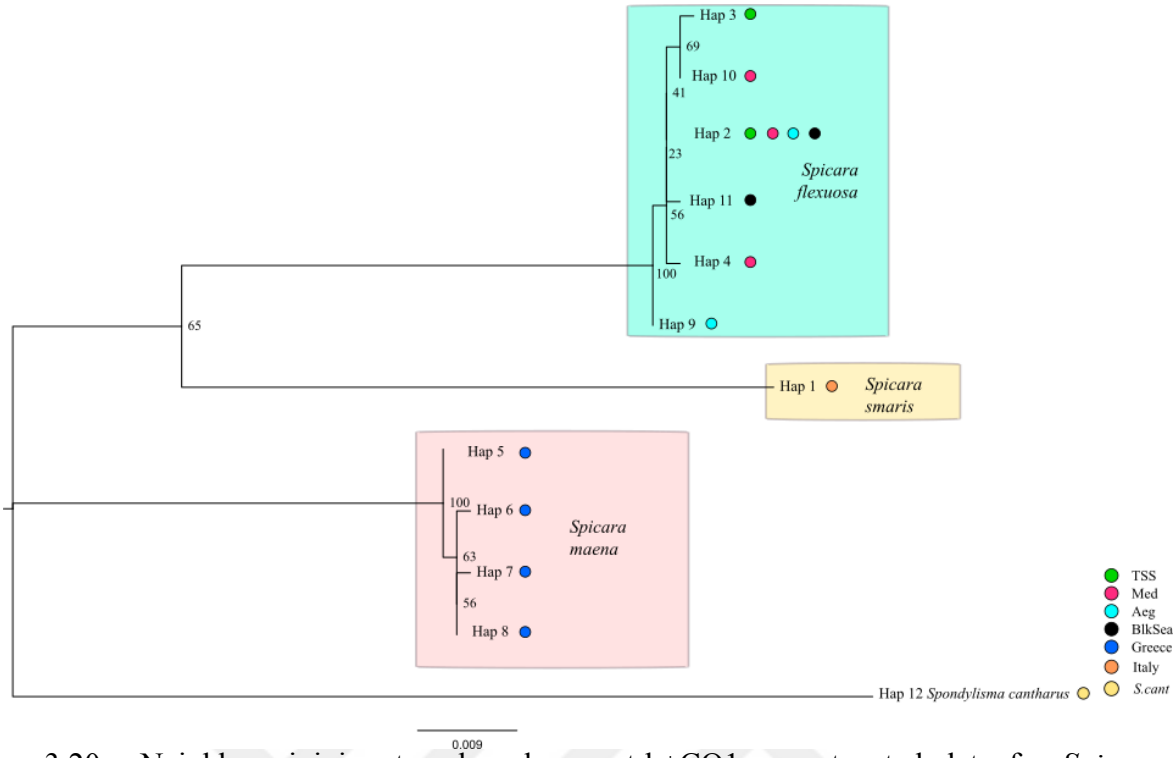


Figure 3.20. Neighbour-joining tree based on cyt-b+CO1 concatenated data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

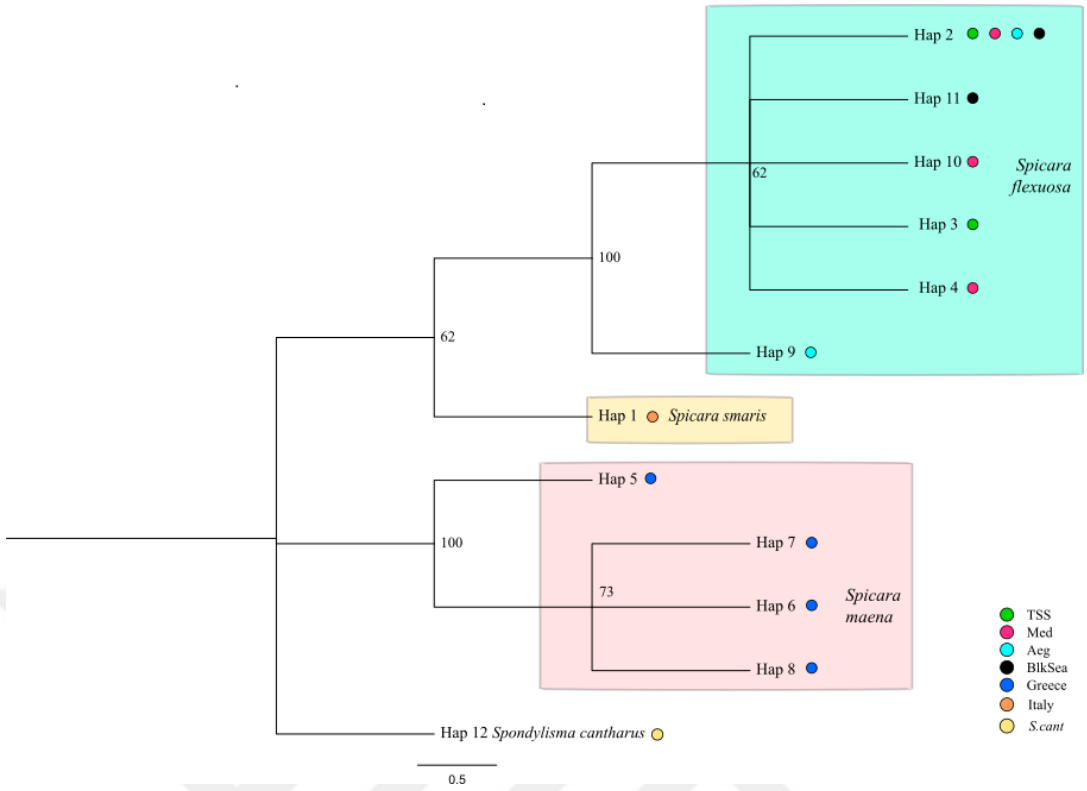


Figure 3.21. Maximum parsimony tree based on cyt-b+CO1 concatenated data for *Spicara spp.* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

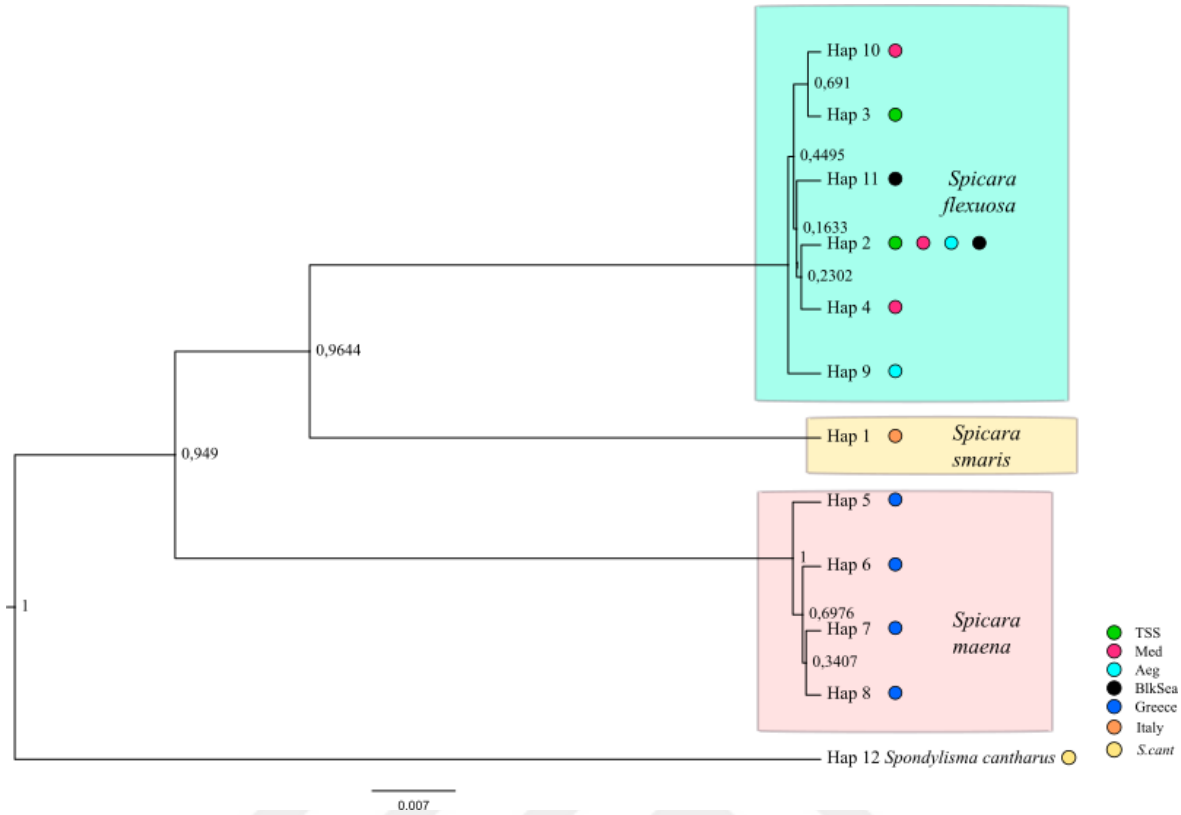


Figure 3.22. Bayesian tree based on cyt-b+CO1 concatenated data for *Spicara spp.* populations. The node values correspond to posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

All of the phylogenetic trees, constructed by the maximum likelihood, neighbour-joining, maximum parsimony, and Bayesian methods revealed the same topology with high bootstrap values. *Spicara flexuosa*, *Spicara maena* and *S. smaris* haplogroups were clearly differentiated from each other. As can also be seen in the CO1 gene results in the previous section, *Spicara flexuosa* and *S. smaris* were closely related to each other when compared to *S. maena*. Close phylogenetic relationship between *S. flexuosa* and *S. smaris* was also described in the molecular phylogeny of the cyt-b gene from Sparid fishes in the literature (Chiba et al., 2009).

3.1.5. Nuclear DNA (IRBP gene)

We also sequenced a partial fragment of the IRBP gene, to investigate the nuclear genetic make-up of samples we collected from Turkish coastal waters and Greece. In these analyses, based on the agreement of the mitochondrial DNA (mtDNA) genes (16S rRNA, CO1, cyt-b), we accepted our samples from Turkey and Greece to belong to *S. flexuosa* and *S. maena*, respectively. For this gene,

a 380 bp fragment was amplified in 195 individuals in 10 sampling locations (174 from Turkey, 21 from Greece). As some IRBP gene sequences had ambiguities (due to heterozygous individuals), the corresponding ambiguity codes were identified as two different set of sequences in all analyses. A total of 60 sequences with ambiguities were detected, due to heterozygosity (the sequences are provided in Appendix C), and these 60 sequences were recorded as 120 unambiguious sequences. On the other hand, a total of 135 individuals had no ambiquity codes, representing homozygote individuals. A total of 255 sequences were detected in 10 sampling locations (233 from Turkey and 22 from Greece) (Appendix Table F.1). After these steps, a total of 38 haplotypes were found in 195 individuals from 10 sampling locations, after addition of one outgroup species (*Spondylisma cantharus*) and one *S. maena* sample from France/GenBank (Lautredue et al. (2013) (JX627893.1) (Figure 3.23). The samples collected from Greece (with H2, H6 and H35), were grouped with *S. maena* (Lautredeu et al. (2013) from GenBank (France), separately from the other *S. flexuosa* samples (Figures 3.23, 3.24).

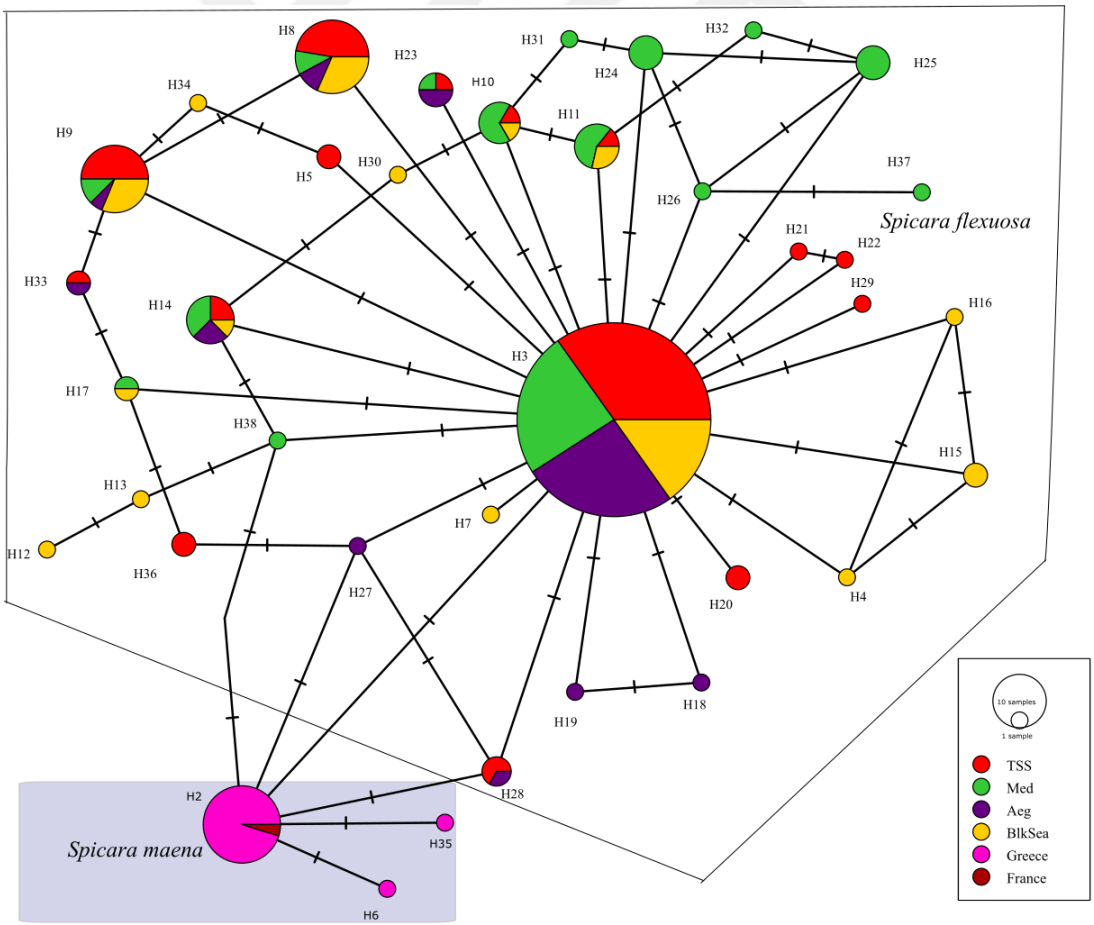


Figure 3.23. The haplotype network of IRBP gene of *S. flexuosa* and *S. maena*. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Each of the lines between haplotypes represents a single mutational change.

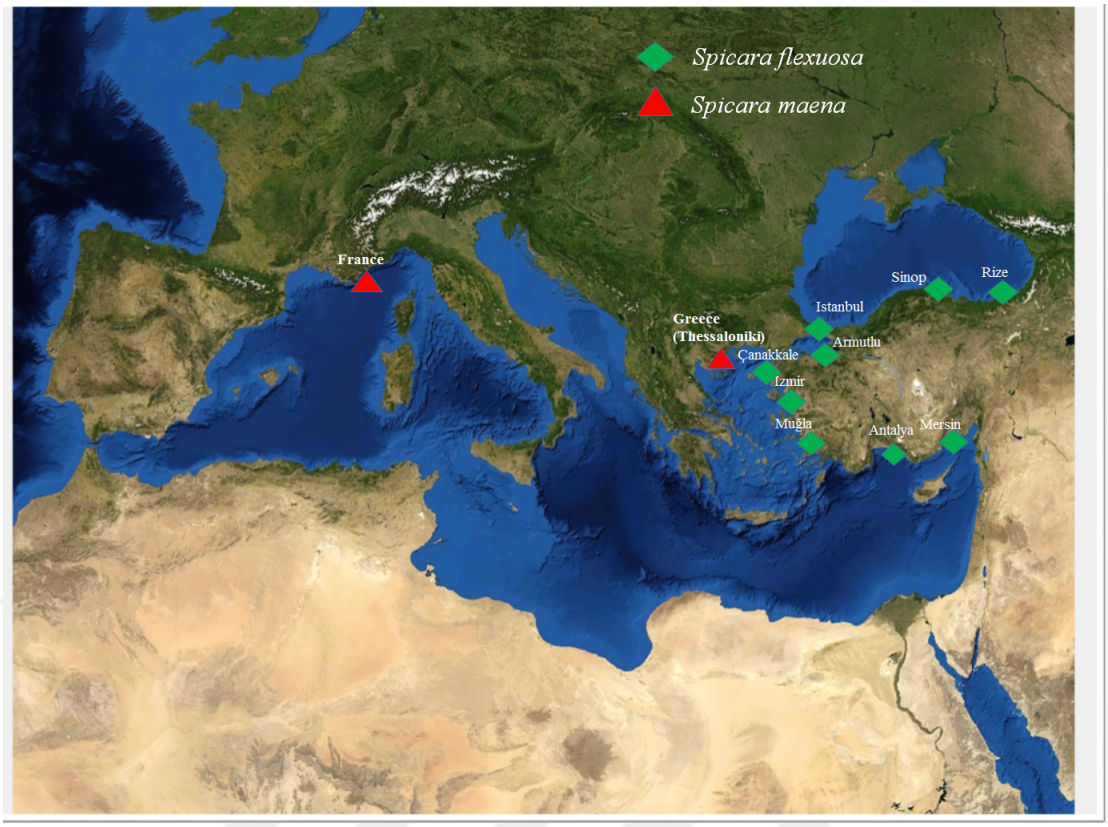


Figure 3.24. Sampling locations of two *Spicara* species, based on the IRBP gene, in the Mediterranean region.

Phylogenetic relationships between haplotypes were determined by maximum likelihood (Figure 3.25), neighbour-joining (Figure 3.26), maximum parsimony (Figure 3.27), and Bayesian trees (Figure 3.28).

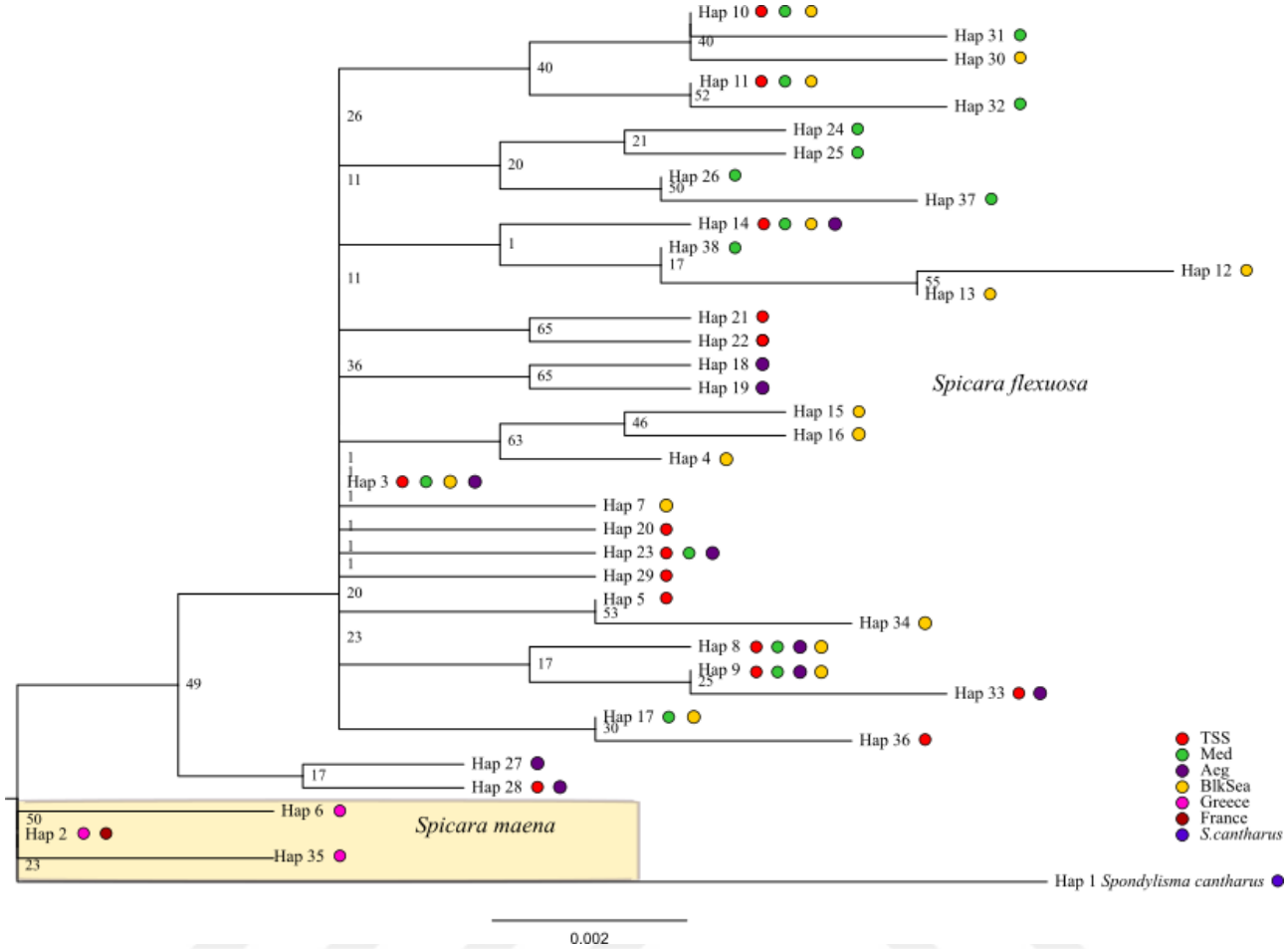


Figure 3.25. Maximum likelihood tree based on IRBP data for *S. flexuosa* and *S. maena* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

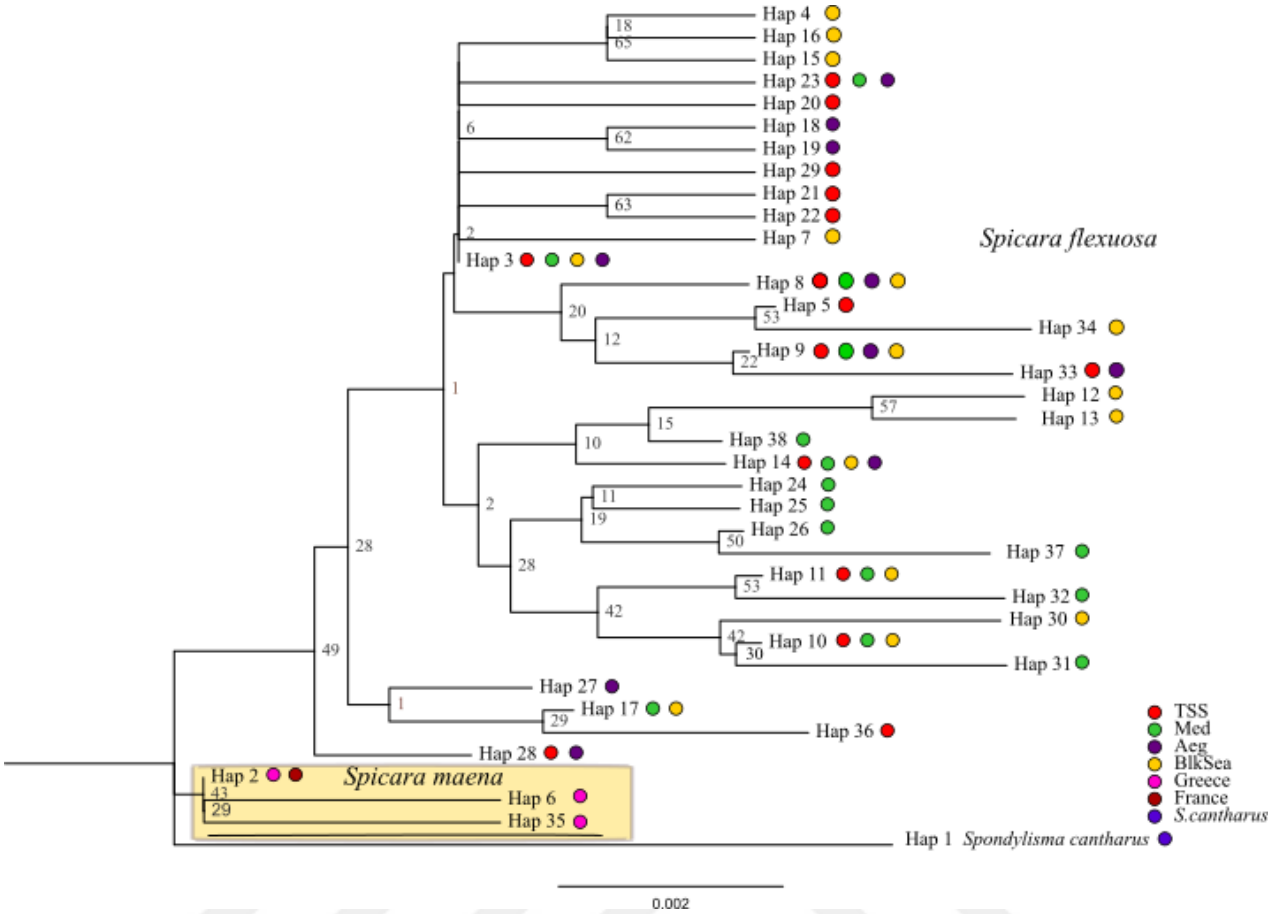


Figure 3.26. Neighbour-joining tree based on IRBP data for *S. flexuosa* and *S. maena* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

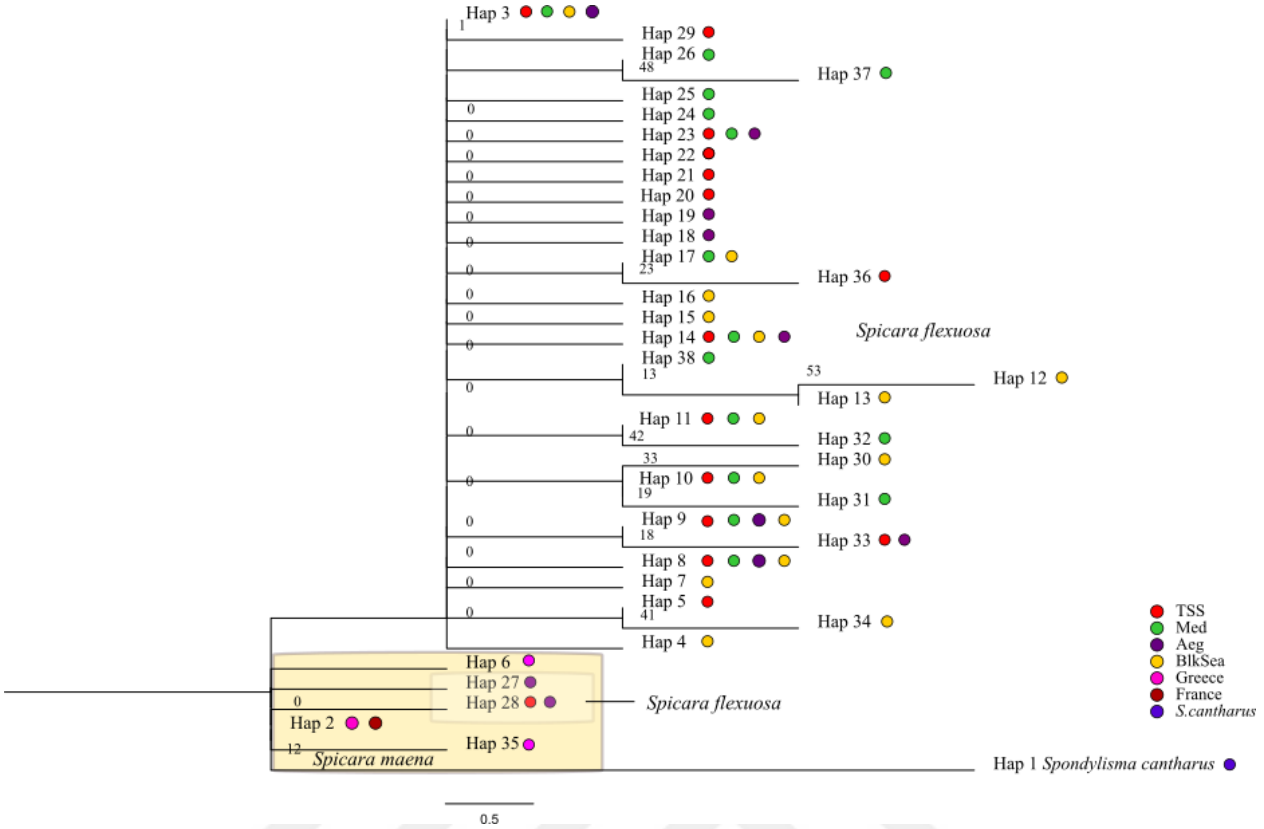


Figure 3.27. Maximum parsimony tree based on IRBP data for *S. flexuosa* and *S. maena* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

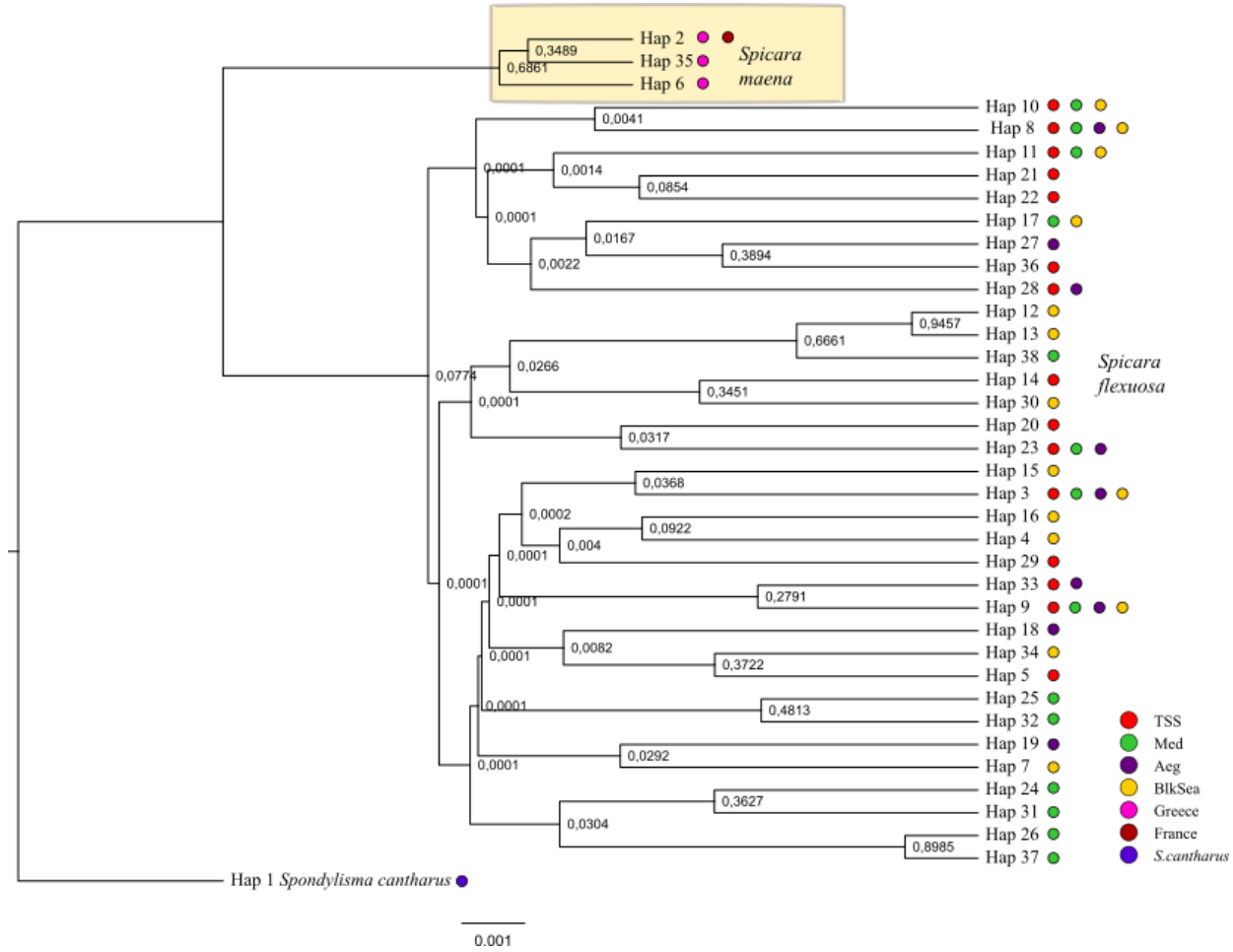


Figure 3.28. Bayesian tree based on IRBP data for *S. flexuosa* and *S. maena*. The node values correspond to posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

The phylogenetic trees obtained by maximum likelihood (Figure 3.25), neighbour-joining (Figure 3.26), and Bayesian methods (Fig 3.28) revealed the same pattern, of two monophyletic clades. However, reciprocally monophyletic clades were not seen in the maximum parsimony (Figure 3.27) trees. In general, low bootstrap values were observed in all of the phylogenetic trees constructed, potentially due to the starlike network around the most common haplotype (Hap 3), and low mutation rates of the IRBP gene.

Molecular diversity indices of IRBP gene of *S. maena* and *S. flexuosa* populations and parsimony informative sites are shown in Table 3.15 and Table 3.16. As shown in Table 3.15, *Spicara flexuosa* had higher mean haplotype and nucleotide diversities (h=0.666; π= 0.00238) than *Spicara maena* populations (h=0.170; π=0.00046). Considering *Spicara flexuosa* along the Turkish coasts, the Black Sea population had the highest cumulative haplotype and nucleotide diversities by region (h=0.781;

π=0.00307), and specifically Sinop had the highest haplotype and nucleotide diversities (h=0.830; π=0.00333) when compared to other sites. Singleton variable and parsimony informative sites of *Spicara flexuosa* were also higher than those in *S. maena*.

Table 3.15. Molecular diversity of *Spicara flexuosa* and *S. maena* based on IRBP gene sequences. N: Number of sequences; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations are given in parantheses.

	Sampling sites	**N**	**Nh**	**Np**	**h**	**π**	**SS**	**PIS**
Spicara flexuosa	İstanbul	22	4	3	0.333 (0.124)	0.00093 (0.00038)	2	1
	Çanakkale	31	12	7	0.789 (0.072)	0.00317 (0.00050)	3	4
	Armutlu	27	4	2	0.655 (0.065)	0.00181(0.00023)	1	1
	Rize	20	8	5	0.742 (0.096)	0.00284 (0.00061)	4	1
	Sinop	25	12	7	0.830 (0.068)	0.00333 (0.00054)	5	2
	İzmir	27	8	5	0.558 (0.112)	0.00187(0.00048)	3	2
	Muğla	19	4	2	0.298 (0.133)	0.00082 (0.00038)	2	0
	Antalya	35	12	5	0.820 (0.057)	0.00297(0.00037)	2	3
	Mersin	27	7	4	0.556 (0.111)	0.00196 (0.00049)	2	2
	Total *S. flexuosa*	**233**	**34**	**15**	**0.666 (0.035)**	**0.00238 (0.00018)**	**4**	**11**
	TSS	**80**	**15**	**10**	**0.651 (0.057)**	**0.00223 (0.00028)**	**4**	**6**
	Black Sea	**45**	**15**	**8**	**0.781 (0.057)**	**0.00307 (0.00042)**	**3**	**5**
	Mediterranean	**62**	**15**	**6**	**0.722 (0.060)**	**0.00256 (0.00031)**	**2**	**4**
	Aegean	**46**	**10**	**6**	**0.455 (0.092)**	**0.00145 (0.00035)**	**3**	**3**
S. maena	Greece, France	23	3	2	0.170 (0.102)	0.00046 (0.00028)	2	0
	Total *S. maena*	**23**	**3**	**2**	**0.170 (0.102)**	**0.00046 (0.00028)**	**2**	**0**

Table 3.16. Parsimony informative sites of total *S. maena* and *S. flexuosa* based on 380 bp long IRBP region sequences.

		Parsimony Informative Sites of IRBP gene							
Species name	Hap Numbers	35	44	134	243	326	330	361	368
S. cantharus	Hap 1	G	T	C	T	T	G	A	C
S.meana	Hap 2	.	C	.	.	.	.	.	.
S.meana	Hap 6	.	C	.	.	.	.	.	.
S.meana	Hap 35	.	C	.	.	.	.	.	.
S.flexuosa	Hap 3	.	C	.	C	.	.	.	.
S.flexuosa	Hap 4	.	C	.	C	.	.	.	.
S.flexuosa	Hap 5	.	C	.	C	.	.	.	A
S.flexuosa	Hap 7	.	C	.	C	.	.	.	.
S.flexuosa	Hap 8	C	C	.	C	.	.	.	.
S.flexuosa	Hap 9	T	C	.	C	.	.	.	.
S.flexuosa	Hap 10	.	C	A	C	.	.	.	.
S.flexuosa	Hap 11	.	C	G	C	.	.	.	.
S.flexuosa	Hap 12	.	G	.	C	.	.	G	.
S.flexuosa	Hap 13	.	.	.	C	.	.	G	.
S.flexuosa	Hap 14	.	A	.	C	.	.	.	.
S.flexuosa	Hap 15	.	C	.	C	.	.	.	.
S.flexuosa	Hap 16	.	C	.	C	.	.	.	.
S.flexuosa	Hap 17	.	C	.	C	A	.	.	.
S.flexuosa	Hap 18	.	C	.	C	.	.	.	.
S.flexuosa	Hap 19	.	C	.	C	.	.	.	.
S.flexuosa	Hap 20	.	C	.	C	.	.	.	.
S.flexuosa	Hap 21	.	C	.	C	.	.	.	.
S.flexuosa	Hap 22	.	C	.	C	.	.	.	.
S.flexuosa	Hap 23	.	C	.	C	.	.	.	.
S.flexuosa	Hap 24	.	C	.	C	.	C	.	.
S.flexuosa	Hap 25	.	C	.	C	.	T	.	.
S.flexuosa	Hap 26	.	C	.	C	.	A	.	.
S.flexuosa	Hap 27	.	C	.	A	.	.	.	.
S.flexuosa	Hap 28	.	C	.	G	.	.	.	.
S.flexuosa	Hap 29	.	C	.	C	.	.	.	.
S.flexuosa	Hap 30	.	A	A	C	.	.	.	.
S.flexuosa	Hap 31	.	C	A	C	.	C	.	.
S.flexuosa	Hap 32	.	C	G	C	.	T	.	.
S.flexuosa	Hap 33	T	C	.	C	A	.	.	.
S.flexuosa	Hap 34	T	C	.	C	.	.	.	A
S.flexuosa	Hap 36	.	C	.	A	A	.	.	.
S.flexuosa	Hap 37	.	G	.	C	.	A	.	.
S.flexuosa	Hap 38	.	.	.	C	.	.	.	.

As can be seen in Table 3.16, a total of 20 nucleotide sites were variable of which eight (2.1 %) positions were parsimony informative. Unlike mtDNA, none of the positions were parsimony informative for grouping *S. maena* and *S. flexuosa* separately from each other, except one position

(243). The total number of mutations and pairwise sequence divergence between *S. flexuosa* and *S. maena* were 29 and 0.00740, respectively.

3.1.5.1. Population structure of the IRBP gene. An analysis of molecular variance and pairwise F_{ST} values are given in Table 3.17. Since haplotype network and phylogenetic trees revealed two haplogroups in the IRBP gene, pairwise F_{ST} values were calculated based on these values. F_{ST} values were also calculated taking data from all sampling locations (subpopulations) in Turkish coastal waters for *S. flexuosa.*

Table 3.17. Pairwise F_{ST} values of IRBP genes between *S. maena* and *S. flexuosa.* Significant *P* (< 0.05) values are indicated in shaded cells.

			İstanbul	Çanakkale	Armutlu	İzmir	Muğla	Rize	Sinop	Antalya	Mersin	Greece
Spicara flexuosa	TSS	**İstanbul**	0.00									
		Çanakkale	**0.04**	0.00								
		Armutlu	**0.20**	0.04	0.00							
	Aeg	**İzmir**	0.02	-0.01	**0.07**	0.00						
		Muğla	-0.00	**0.03**	**0.20**	0.02	0.00					
	BlkSea	**Rize**	**0.07**	0.00	-0.00	0.00	**0.07**	0.00				
		Sinop	0.03	0.00	0.02	0.00	**0.03**	0.00	0.00			
	Med.	**Antalya**	**0.05**	**0.04**	**0.11**	**0.04**	**0.05**	**0.04**	**0.03**	0.00		
		Mersin	0.03	**0.04**	**0.19**	**0.05**	**0.04**	**0.05**	**0.03**	**0.02**	0.00	
S. maena		**Greece**	**0.79**	**0.54**	**0.71**	**0.66**	**0.81**	**0.64**	**0.58**	**0.58**	**0.68**	0.00

As can be seen in Table 3.17, high and significant pairwise F_{ST} values were observed between *Spicara flexuosa* and *S. maena* individuals; the pairwise F_{ST} values ranged from 0.54 to 0.81 between the two species. Considering *S. flexuosa* subpopulations, pairwise F_{ST} values were low to moderate, and ranged from 0.00 to 0.20. Significant *P* values were observed between İstanbul and Armutlu, between Armutlu and Muğla, and between Antalya, Mersin and all other sampling locations in Turkish coasts. AMOVA results of *S. flexuosa* populations showed that distribution of the variation

was the lowest (1.71 %) among four different groups in Turkish coasts and within groups (3.32 %), and the highest within populations (94.97 %).

3.1.6. Demographic Analyses of *Spicara flexuosa* and *S. maena* – mtDNA and Nuclear DNA Results

Mismatch analyses were done separately, based on the mtDNA and nuclear genes for *S. maena* and *S. flexuosa* populations, as well as four different geographical locations (the TSS, the Black Sea, the Mediterranean, and the Aegean) along the Turkish coasts. Specifically, two neutrality tests (Tajima's D, Fu's F_S) and raggedness statistic (rg) were used to reconstruct the population demographic history for *S. maena* and *S. flexuosa*, and their values are presented for two mtDNA genes (CO1 and cyt-b) and one nuclear (IRBP) gene (Table 3.18). Mismatch distributions for populations of *S. maena* and *S. flexuosa*, under a sudden demographic expansion model, are shown in Figure 3.29, 3.30 and Figure 3.31 for the CO1, cyt-b and IBRP genes, respectively.

Table 3.18. Neutrality test results for *S. flexuosa*/*maena* N: Number of samples; D: Tajima's D; F_S: Fu's F_S; and rg: raggedness statistic. Significant values ($P< 0.05$) are indicated in bold.

	Sampling sites	N	D	F_S	rg
CO1 gene	**Total *S. flexuosa***	197	**-1.62461**	**-7.882**	0.7790
	TSS	58	-	-	-
	Black Sea	32	-1.14244	-1.265	0.7695
	Aegean	41	-1.12187	-1.471	0.8168
	Mediterranean	45	-1.11315	-1.548	0.8321
	Total *S. maena*	27	-0.72749	-1.495	0.1987
Cyt-b gene	**Total *S. flexuosa***	143	**-1.68994**	**-7.616**	0.7493
	TSS	62	-1.08044	-1.816	0.8762
	Black Sea	25	**-**	**-**	**-**
	Mediterranean	30	**-1.25553**	**-1.669**	0.4211
	Aegean	22	-1.16240	-0.957	0.6777
	Total *S. maena*	21	-1.16356	-0.919	0.6644
IRBP gene	**Total *S. flexuosa***	233	**-2.16433**	**-47.183**	0.1163
	TSS	80	**-1.97546**	**-13.281**	0.1399
	Black Sea	45	**-1.96294**	**-12.706**	0.1492
	Mediterranean	62	**-1.76650**	**-12.939**	0.1606
	Aegean	46	**-2.07732**	**-9.507**	0.1186
	Total *S. maena*	23	**-1.51496**	**-2.027**	0.4672

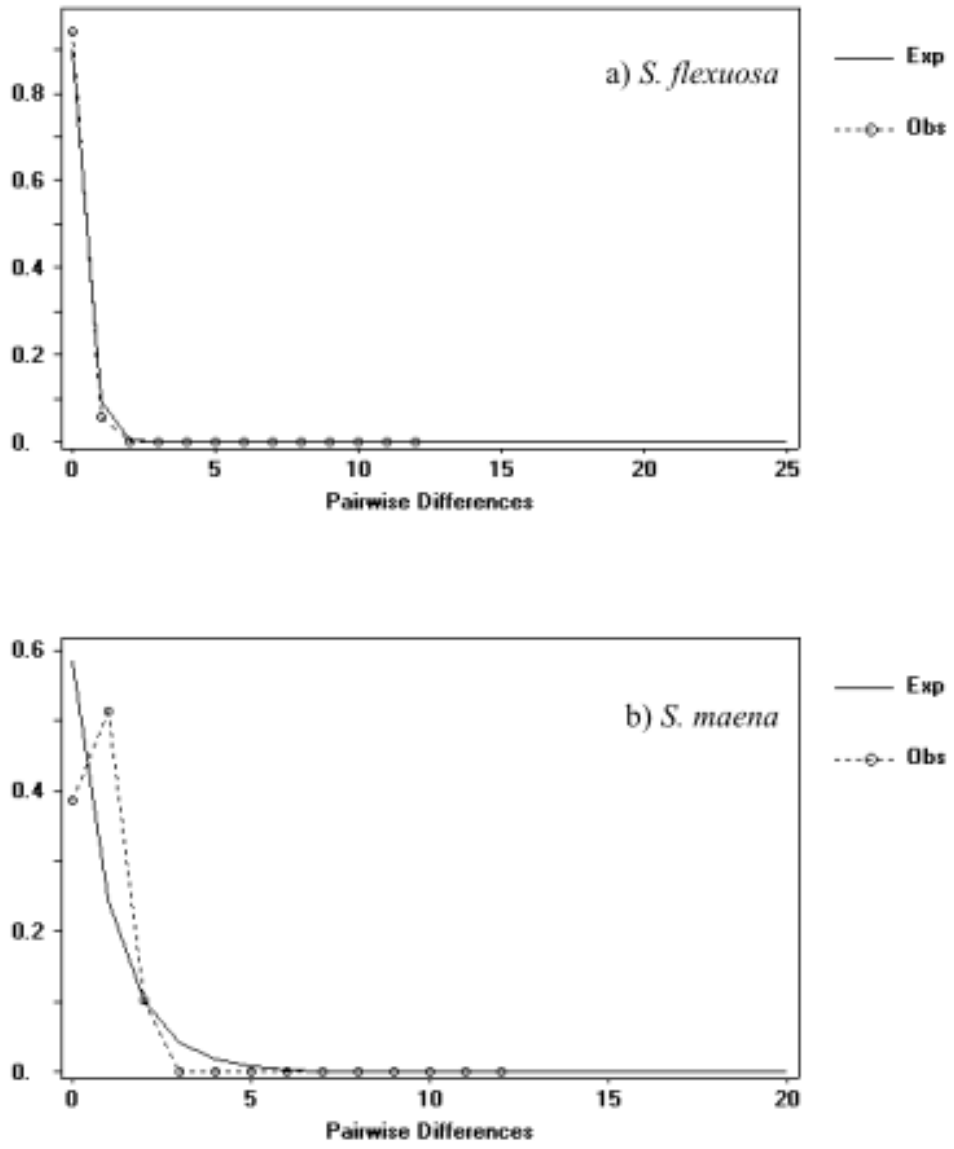


Figure 3.29. Mismatch distributions for the CO1 gene for a) S. *flexuosa*, b) *S. maena*. Solid lines represent expected distribution and dotted lines represent observed distribution under the sudden expansion model. Y-axis represents the relative haplotype frequencies.

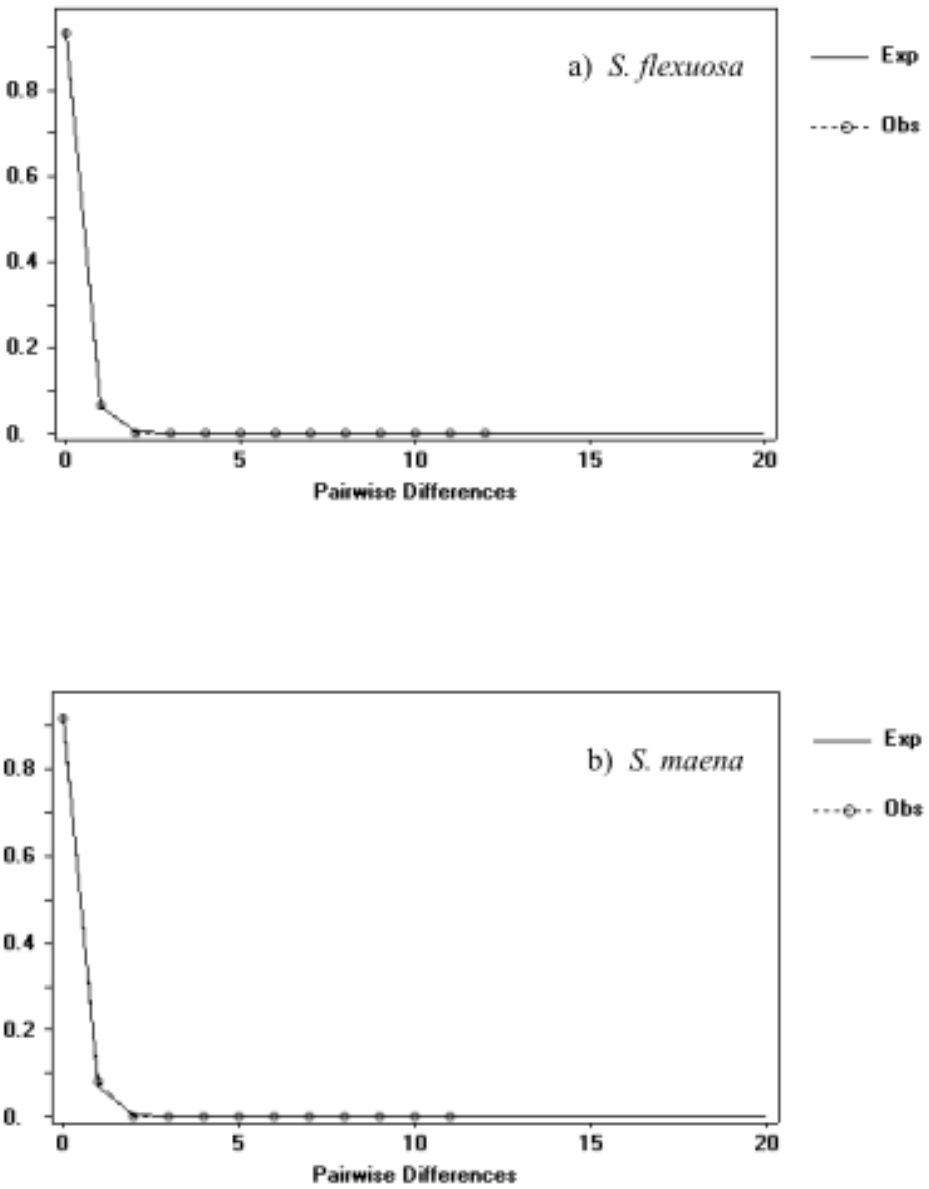


Figure 3.30. Mismatch distributions for the cyt-b gene for a) *S. flexuosa*, b) *S. maena*. Solid lines represent expected distribution and dotted lines represent observed distribution under the sudden expansion model. Y-axis represents the relative haplotype frequencies.

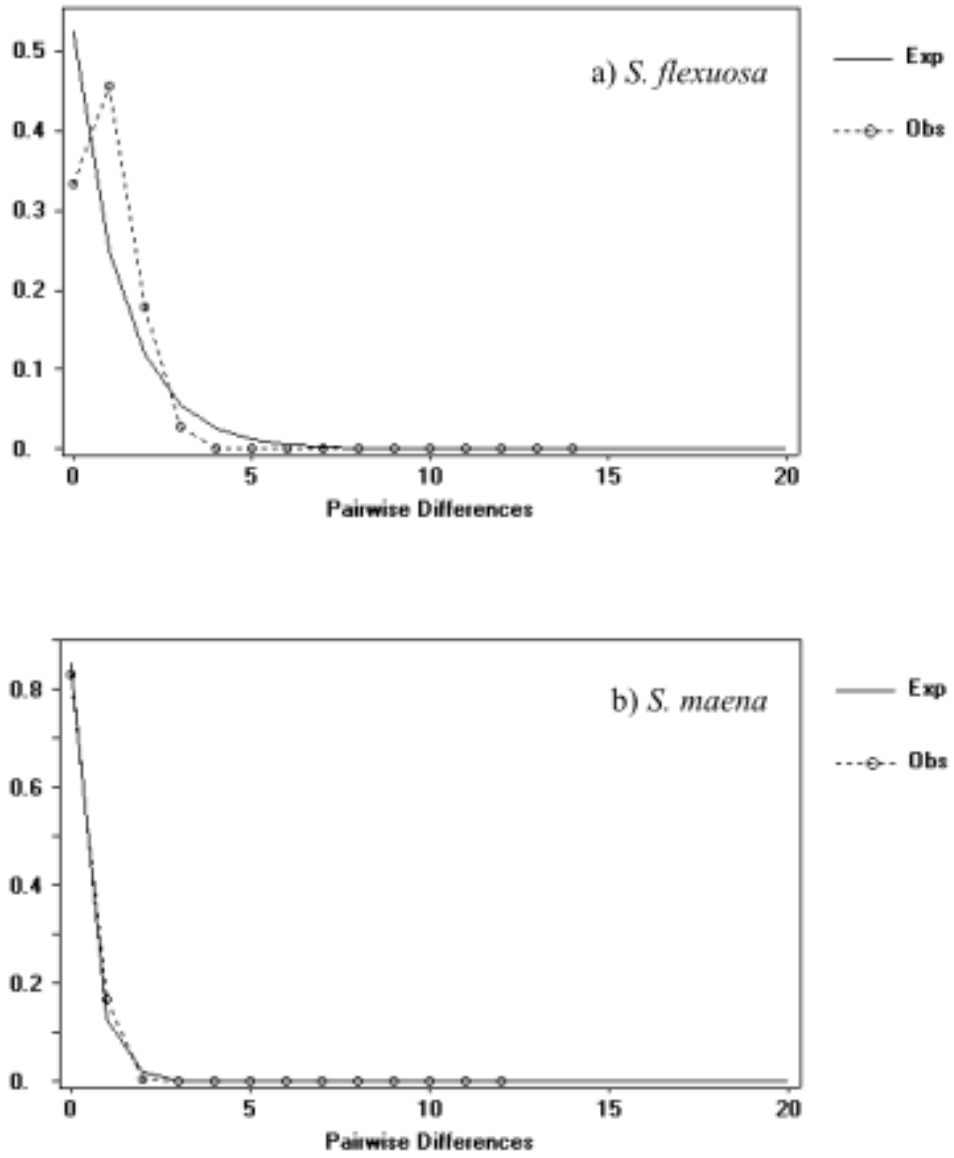


Figure 3.31. Mismatch distributions for the IRBP gene for a) *S. flexuosa*, b) *S. maena.* Solid lines represent expected distribution and dotted lines represent observed distribution under the sudden expansion model. Y-axis represents the relative haplotype frequencies.

Overall, values of neutrality tests of three different genes showed that *Spicara flexuosa* populations had unimodal mismatch distributions (Figure 3.29a, 3.30a, 3.31a) and experienced recent demographic expansion, which was also indicated by negative and significant F_S and D values (Table 3.18). Unimodal mismatch distributions suggesting recent demographic expansion were also observed in *S. maena* populations for the three genes (Figure 3.29b; 3.30b, 3.31b). Neutrality test values (F_S and D) were all negative, and also supported the population expansion model. In addition to this, raggedness statistic (rg) values were all non-significant, and hence not rejecting the population expansion model for both species (Table 3.18).

Demographic analysis of *S. flexuosa* populations in the eastern Mediterranean based on the Bayesian skyline analysis is shown in Figure 3.32. Population of *S. flexuosa* remained relatively constant from 30 Kya to 15Kya. However, recent population expansion (before 15 Kya) among the *S. flexuosa* was detected in the skyline method after the end of the Last Glacial Maximum (LGM).

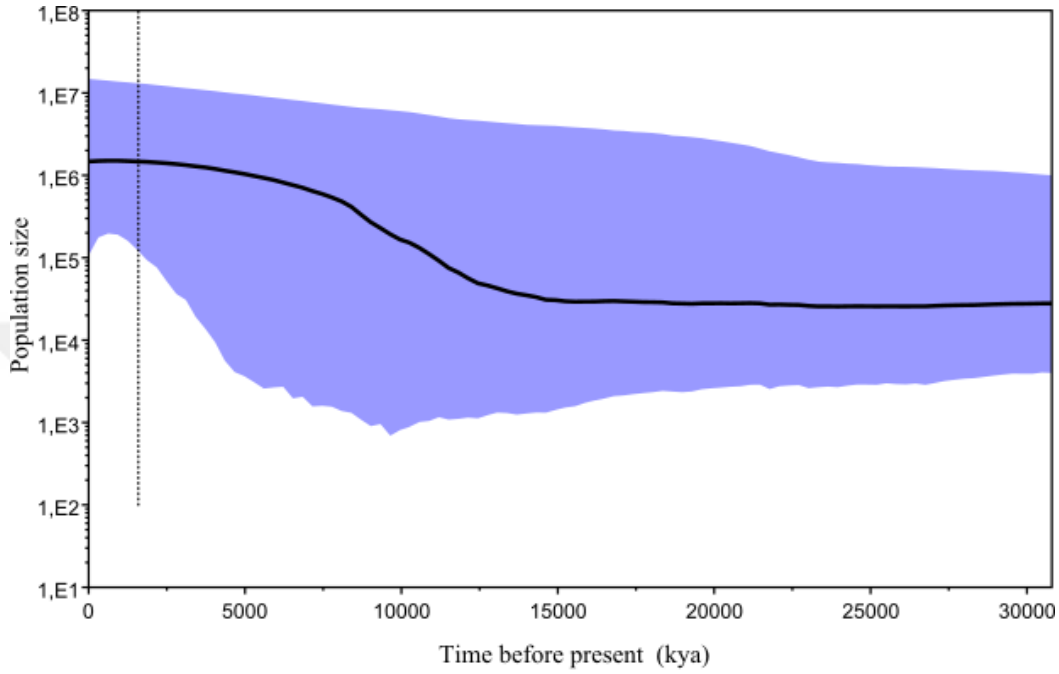


Figure 3.32. Bayesian skyline plot for *S. flexuosa* reflecting changes effective population size against time in thousands of years (Kya) before present.

3.1.7. Morphometric Measurements

The baseline data based on Krey et al. (HL (Head length), BD (Body depth)) were used to define two *Spicara* species (*maena*, *flexuosa*) collected from İstanbul. Box plots of measurements of two variables (HL, BD) and standard deviations of *maena* and *flexuosa* based on baseline data and genetic data results from İstanbul, are presented in Figure 3.33.

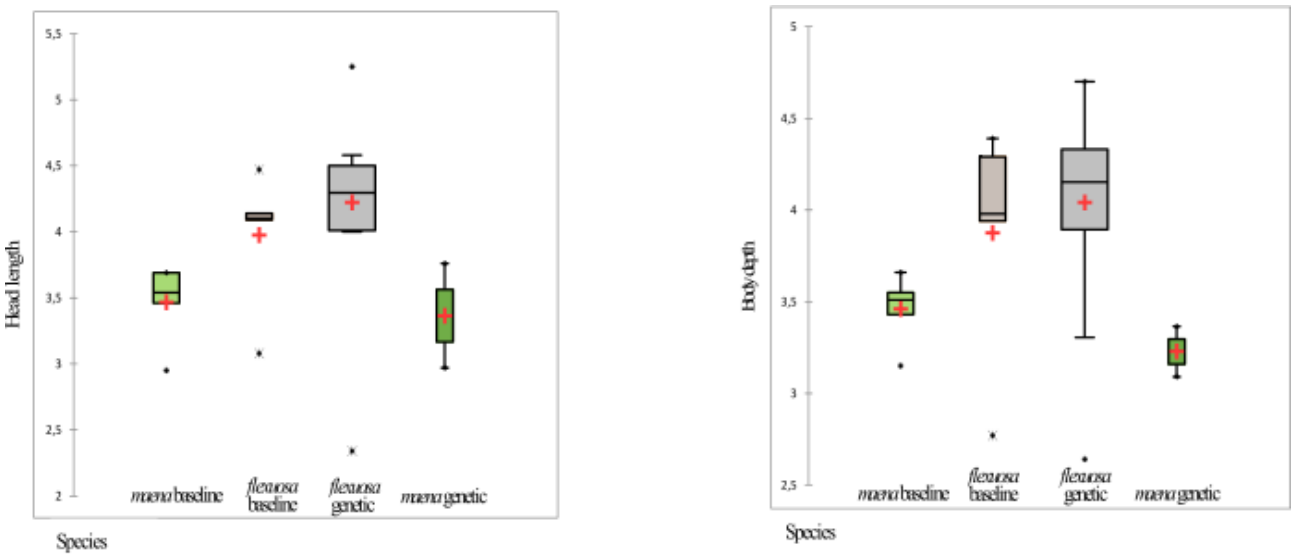


Figure 3.33. Box plots of measurements of each variable investigated, with baseline data for *S. flexuosa* and *S. maena* from Greece, and genetic data results from İstanbul.

As indicated in Figure 3.33, measurements from baseline data (Greece) and genetic data results (İstanbul) were relatively parallel to each other, based on these three characters. All measurements of *S. flexuosa* were found to be higher than those in *S. maena*. Morphometric measurements (HL, BD) of baseline *S. flexuosa* and *S.maena* (Greece) and genetic data results from İstanbul are given in Table 3.19.

Table 3.19. Morphometric measurements (HL, BD) of baseline *S. flexuosa* and *S. maena* collected from Greece, and *Spicara maena* and *S. flexuosa* samples collected from İstanbul (cm).

Samples	**HL values (cm.)**	**BD values (cm.)**
maena baseline 1	3.69	3.66
maena baseline 2	3.54	3.51
maena baseline 3	3.46	3.43
maena baseline 4	2.95	3.15
maena baseline 5	3.69	3.55
maena genetic İst 1	3.76	3.36
maena genetic İst 2	2.97	3.09
flexuosa baseline 1	4.47	4.39
flexuosa baseline 2	3.08	2.77
flexuosa baseline 3	4.10	3.94
flexuosa baseline 4	4.09	4.29
flexuosa baseline 5	4.14	3.98
flexuosa genetic İst 3	2.34	2.64
flexuosa genetic İst 4	5.25	4.15
flexuosa genetic İst 5	4.58	4.70
flexuosa genetic İst 6	4.09	4.21
flexuosa genetic İst 7	4.24	4.08
flexuosa genetic İst 8	4.47	3.86
flexuosa genetic İst 9	4.50	4.24
flexuosa genetic İst 10	4.50	4.15
flexuosa genetic İst 11	4.00	4.35
flexuosa genetic İst 13	4.41	4.09
flexuosa genetic İst14	4.35	4.45
flexuosa genetic İst 15	4.01	3.76
flexuosa genetic İst 19	4.55	4.32
flexuosa genetic İst 20	4.01	3.90
flexuosa genetic İst 22	4.24	4.40
flexuosa genetic İst 23	4.00	3.30

The results from morphological mesaurements of *S. flexuosa* and *S. maena* showed that *S. maena* samples were smaller than 3.76 cm, (range from 2.95 to 3.76 cm) and *S. flexuosa* samples were larger than 4.00 cm (range from 4.00 cm to 5.25 cm) based on the HL values. Considering BD values, *S. maena* samples were smaller than 3.66 cm (range from 3.09 to 3.66 cm) and *S. flexuosa* samples were larger than 3.76 cm (range from 3.76 to 4.70 cm) (Table 3.19). Only two individuals of genetically assigned *S. flexuosa* baseline 2 and *S. flexuosa* İstanbul 3 were smaller than *S. maena,* based on both

head length and body depth measurements. On the other hand, all *S. maena* baseline and *S. maena* genetic İstanbul samples were correctly classified based on both measurements.

3.1.8. Discussion

3.1.8.1. Genetic differentiation of the three Spicara species: Spicara maena, S. flexuosa and S. smaris. The taxonomy of the genus *Spicara* has been problematic around the Mediterranean region (Pollard and Pichot, 1971; İlkyaz et al., 2007; Imsiridou et al., 2011; Minos et al., 2013). Multiple studies (Tortonose, 1975; Heemstra (1981, 1990); Quero et al., 2003; Eschmeyer, 2010; Froese and Pauly, 2017) suggest that *S. maena* and *S. flexuosa* should be considered conspecific, whereas others (Tortonose, 1986; Fischer et al., 1987; Costa, 1991; Louisy, 2002; Golani et al., 2006; Vasileva, 2007; Bilecenoğlu et al., 2014) support the distinction of the two species. More recent studies based on both genetic (Chiba et al., 2009; Imsiridou et al., 2011; Georgiadis et al., 2014; Bektaş et al., 2018) and morphological data (Minos et al., 2013) also support the latter conclusion.

The main problem with the genus *Spicara* is the difficulty to distinguish between *S. maena* and *S. flexuosa* morphologically. For instance, the samples analyzed for this study were at first assumed to belong to *S. maena*, because according to FishBase and World Register of Marine Organisms (WORMS) databases, *S. maena* was the common *Spicara* species that was found along Turkish coasts, as well as the Mediterranean region. Hence the samples were analyzed genetically to investigate their taxonomic status. The distinction of the three taxa (including *S. smaris*) under *Spicara* were made with the help of three different mtDNA genes, which are based on four independent studies. These genes were 16S rRNA (Imsiridou et al., 2011; Georgiadis et al., 2014), CO1 (Cannas et al. retrieved from GenBank, 2010), and cyt-b (Krey et al. retrieved from GenBank, 2007). The phylogenetic analyses using data from these three genes, and neighbour-joining, maximum likelihood, maximum parsimony and Bayesian algorithms, and the resulting haplotype networks, show three units with different evolutionary histories, corresponding to *S. maena, S. flexuosa* and *S smaris.* For the 16S rRNA gene, sequences submitted by Imsiridou et al. (2011) and Georgiadis et al. (2014), which were retrieved from GenBank, support the differentiation of these three species. Regarding the CO1 and cyt-b genes, classification of three *Spicara* species was done by the sequences submitted by Cannas et al. (2010), and Krey et al. (2007) to GenBank, respectively. Considering CO1, the sequences for the three *Spicara* species, which have recently been submitted to GenBank (Yokeş retrieved from GenBank, 2016) also supported the identification of *Spicara* species parallel to this study, and corroborated our CO1 sequences results. In this study, a total of 217

samples (194 from Turkey and 23 from Greece) were evaluated genetically to determine their species designation as *S. flexuosa* or *S. maena.* Based on the genetic analyses, 192 out of 194 samples collected from Turkey were *S. flexuosa,* and two were *S. maena.* On the other hand, all 23 samples collected from Greece turned out to be *S. maena*, again based on the genetic analyses. In several former studies, *S. flexuosa* was reported to be present in the Turkish coastal waters (Tortonose, 1986; Salekhova, 1979; Livadas, 1986; Mytilineou, 1987; Fischer et al., 1987; Heemstra, 1990; Nelson, 1994; Ragonese et al., 2004; Golani et al., 2006; İlkyaz et al., 2007; Turan et al., 2007; Turan, 2011; Bektaş et al., 2018). On the other hand, in other studies on the Turkish coast, *S. flexuosa* and *S. maena* were considered to be conspecific (Karakulak et al., 2006; Soykan et al., 2010; Keskin et al., 2011; Saygılı et al., 2016a; Froese and Pauly, 2017).

According to the genetic analyses carried out in this study, and the criteria described above, some sequences submitted to GenBank and BOLD, as well as some from the literature seem to be incorrectly classified. For the 16S gene, one sequence from Italy (Orell and Carpenter, 2004), previously identified as *S. maena*, should be classified as *S. flexuosa*. Focusing on the CO1 gene, a total of six sequences from Portugal (Atlantic) (Costa et al., 2012; Landi et al., 2014), one sequence from Italy and 13 sequences from Israel (that were previously identified as *S. maena* in GenBank), should also be classified as *S. flexuosa*. Landi et al. (2014) found two distinct lineages, previously identified as *Spicara maena*, in the phylogenetic trees, based on barcoding (CO1) sequences. Since *S. flexuosa* is considered as a synonym of *Spicara maena* in the FishBase, they indicated that one of the lineages of the *Spicara maena* could probably be *S. flexuosa*. Regarding the cyt-b gene, two sequences from Italy, previously identified as *S. maena,* actually belong to *S. flexuosa*. Consequently, the main problem of the studies on the species of the *Spicara* genus in the Mediterranean region seems to be the morphological similarity between *S. flexuosa* and *S. maena*. As a result, not only was *S. flexuosa* misclassified as *S. maena* in the Mediterranean, including its Turkish coasts. This similarity also indicates the possibility of cryptic species of *Spicara,* which are morphologically very closely related. Sexual dimorphism and protogynous hermaphroditism are the main specific characteristics of this genus and they could have invoked this misidentification (Zei, 1941; Pollard and Pichot, 1971; İlkyaz et al., 2007). For instance, male individuals of *S. smaris* were described as a separate species, and male *S. flexuosa* were considered to be female *S. smaris*, and these results may have led to uncertainties in age determination of *S. smaris* in the Adriatic Sea (Dulcic et al., 2003). Thus the results indicate that the precise classification of these three species may not be easily made morphologically. Still, Minos et al. (2013) developed new tools to evaluate similarity/dissimilarity of the *S. maena* and *S. flexuosa* using multivariate analysis of morphometric characters such as head

length to standard length, head height to head length, head height to standard length and the ratio of the two body heights. Their discriminant analysis showed that 83.2 % of the examined individuals were classified correctly by this method. Minos et al. (2013) also reported that 13 % of non-discrimination could be related to biological aspects, such as sexual dimorphism and protogynous hermaphroditism.

Misidentification problem of the three *Spicara spp.* was also observed in another study that made use of the 16S rRNA marker (Turan, 2011). The result of this study showed that samples assigned to the three different species by Turan (2011) are in fact members of the same species, namely *S. flexuosa*. Turan (2011) found no genetic differences between *S. maena* and *S. smaris*, and genetic divergence between *S. maena* and *S. flexuosa* was found to be low (0.5 %). However, the values reported in this study are higher (4.5- 5.8 %) than those recorded by Turan (2011). In the same study, morphological analyses of these *Spicara spp.* were also performed, and the result was congruent with the genetic results. Systematic confusion due to variation in coloration results from sexual and seasonal dimorphism, which is reinforced by sex inversion, chromatic and morphological modifications during reproductive period, cryptic nature of the three *Spicara* species, and similarity between males of *S. flexuosa* to females of *S. maena* due to protogynous hermaphroditism (Zei, 1941; Pollard and Pichot, 1971; Arculeo et al., 1996; Vidalis and Tsimenidis, 1996; İlkyaz et al., 2007; Imsiridou et al., 2011; Froese and Pauly, 2017) could be some of the possible reasons for morphological misidentification as observed by Turan (2011).

Initially, in this study, prior to any morphological measurements, sequences for a total of 199 samples of *Spicara* were obtained. 176 and 23 of these samples were found to belong to *S. flexuosa* and *S. maena*, respectively. As a next step, to confirm the morphological identification of the specimens, an additional 23 *Spicara* individuals were collected from İstanbul (for 18 of which genetic species assignment was possible). Considering discrimination between two species based on the HL values, *S. maena* samples were smaller than 3.76 cm, (range from 2.95 to 3.76 cm) and *S. flexuosa* samples were larger than 4.00 cm (range from 4.00 cm to 5.25 cm). Considering BD values, *S. maena* samples were smaller than 3.66 cm (range from 3.09 to 3.66 cm) and *S. flexuosa* samples were greater than 3.76 cm (range from 3.76 to 4.70 cm). Thus, morphological measurements indicated that, *S. maena* samples are smaller than *S. flexuosa* samples based on the both values. These findings of *S. flexuosa* being larger than *S. maena* is contrary to some of the previous findings in the literature (Akşiray, 1954, Tortonose, 1986; Lythgoe and Lythgoe, 1992). However, more recent morphological analyses from Minos et al. (2013) showed that *S. maena* samples were characterized by shorter heads

than *S. flexuosa* as shown in this study, and most of the total length measurements values were nearly identical between the two species.

In terms of the distribution of *S. flexuosa* and *S. maena*, of all the genetically analyzed samples in this study, a total of 192 out of the 194 samples (98.9 %) were identified as *S. flexuosa*, and only two samples were determined as *S. maena* in the Turkish coastal waters, showing that the *S. flexuosa* mtDNA is by far much more dominant than *S. maena* along the Turkish coasts. Based on the molecular classification scheme, and the subsequent re-evaluation, the distribution of the three *Spicara* species based on the three mtDNA genes and one nuclear (IRBP) genes in the literature and in this study is given in Figure 3.34. As can be seen in this figure, overlapping distribution of the three species was only seen in Greece and Sardinia. *S. flexuosa* was recorded in all sampling locations in the Mediterranean, except Spain, France, Corsica and Malta. On the other hand, *S. maena* was found in İstanbul, Spain, France, Corsica, Malta, Sardinia and Greece coasts. *S. smaris* was observed at all sampling locations except Malta, France and Corsica.

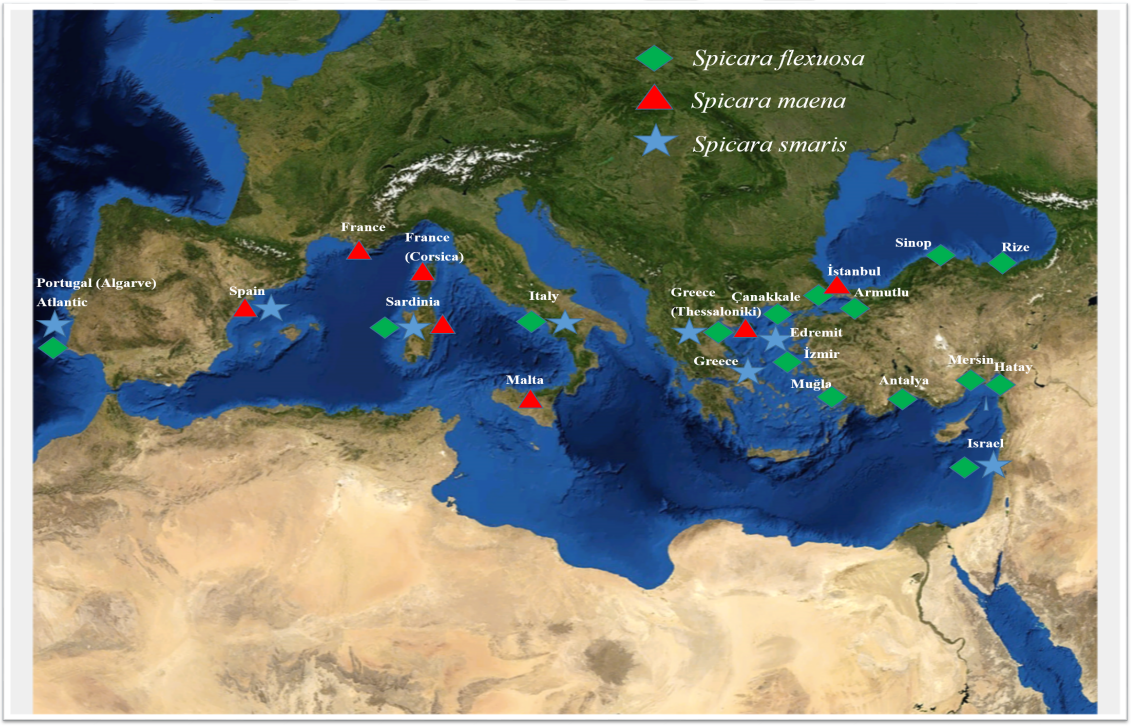


Figure 3.34. Sampling locations of three *Spicara* species, based on the 16S, CO1, cyt-b and the IRBP genes, and the taxonomic evaluations made in previous studies in the Mediterranean ((Imsiridou et al., 2011; Georgiadis et al., 2014; Cannas et al retrieved from GenBank, (2010); and Krey et al retrieved from GenBank, (2007)).

Sequence divergences and calculated divergence times based on 16S rRNA, CO1 and cyt-b genes (2.6 to 6.03 Mya), point out to the high, but similar levels of distinction among the three *Spicara* species. So even though each of the three species are very distinct genetically, they seem to be more or less equally distinct from each other. In addition, the number of parsimony informative sites that group two of the three species in any combination were approximately equal, based on 16S, CO1 and cyt-b genes. The phylogenetic tree analyses made based on the three different mtDNA genes reflect this pattern. For example, the phylogenetic trees based on CO1 gene, and the concatenated CO1 and cyt-b data set indicated that *Spicara smaris* and *Spicara flexuosa* were the most closely related to each other, as can be seen in molecular phylogeny of the Sparid family members including *Spicara* species based on cyt-b data (Chiba et al., 2009). On the other hand, trees based on the cyt-b gene only showed that *Spicara flexuosa* and *Spicara maena* were the two most closely related species among the three, in parallel with the results of Bektaş et al. (2018). As opposed to these, *Spicara smaris* and *S. maena* were more closely related to each other, than either is to *Spicara flexuosa* based on the 16S rRNA gene. However, this relatively unexpected result could be due to us having used a relatively short 16S fragment (155 bp) in this study.

Previous biogeographical analysis among the Sparid family members showed that the common ancestor of Sparid clades has undergone adaptive radiation in order to take advantage of various environmental conditions in the Central Tethys Sea during the Early Paloecene (Orell and Carpenter, 2004; Chiba et al., 2009; Santini et al., 2014). At that time, diversification of the clades occurred simultaneously, and as a result many species have evolved and were distributed worldwide in a short time period, as observed for other fish species, such as coral reef fish (Rocha et al., 2007; 2008) and chiclids (Sugawara et al., 2002). This feature is now reflected in the northeastern Atlantic and the Mediterranean distribution of the Sparid family members, and twentyfour fish species of this family were reported along these coasts (Bauchot and Hureau, 1986). Adaptive radiation could be the process that could have invoked cryptic speciation, and thus it might be the underlying reason of the common morphological misidentification of Sparid species (Chiba et al., 2009).

Estimated divergence times among the three species indicated that they began to diversify during the late Pliocene (2.9-2.6 Mya), early or middle Pliocene (3.83-3.33 Mya), or late Miocene (6.03-5.84 Mya), based on the analyses carried out using the 16S, CO1 and cyt-b markers, respectively. As a relatively longer fragment was used, we decided to use the divergence estimate from the CO1 gene for our biogeographic discussion, which dates the divergence to after the Messinian Salinity Crisis that took place around 6 Mya (Por, 1989). In this perspective, we propose different scenarios

regarding the diversification of *S. maena* and *S. flexuosa* throughout Atlantic, Mediterranean and the Black Sea. In the first scenario, the Black Sea is considered as the possible place of origin for *Spicara flexuosa*, and the species might have subsequently expanded to the Aegean and the Mediterranean, as was observed for other species in this region, such as anchovy (Magoulos et al., 1996), *Mytilus galloprovincialis* and *Paleomon elegans* (Kalkan, 2013). According to this scenario, *S. flexuosa* would have originated in the Black Sea during the early or middle Pliocene, in the Paratethys. During this time, the Paratethys, which covered much of the present southeastern Europe, got divided into a number of brackish lakes, including the present day Black Sea (Rogl and Steininger, 1983). In the early Pliocene, most of the Mediterranean fish species entered the region, when the connection of Atlantic was re-established through the Strait of Gibraltar, ca. 5 Mya (Sara, 1985). At that time, *S. maena* was most likely isolated from ancestral *Spicara flexuosa* in the western Mediterranean, as can be seen in its predominant distribution in this basin. Subsequently, it is likely that it partially expanded its distribution to the eastern Mediterranean basin. During these periods, the salinity of the system changed multiple times, due to the opening and closing of the connection between the Black Sea and the Mediterranean (Zenkevitch, 1963; Degens and Ross, 1972; Hsü, 1978; Por, 1989) and *S. flexuosa* was probably able to adapt to brackish or estuarine waters like that in the Black Sea. The expansion of *S. flexuosa* from Black Sea to Mediterranean might have occured via the surface current of the Bosphorus and the species might have expanded into the Mediterranean basin after the last glaciation by planktonic larval gene flow, as pelagic larvae can disperse massively via hydrological processes. However, *S. maena* larvae might not have been able to colonize the Black Sea due to the direction of the current from the Black Sea towards the Aegean.

However, it should also be noted that, in this study, Black Sea populations showed low haplotype diversities (in some cases zero, e.g. for the cyt-b gene), and the highest haplotype diversities were found in the Mediterranean populations for the cyt-b gene, which suggests that a second scenario could be more plausible. According to this second scenario, Atlantic-Mediterranean Sea is the possible origin of the *S. flexuosa,* and the species might have expanded from Atlantic to the Mediterranean and Black Sea regions. In several other studies, an Atlantic–Mediterranean origin was detected for some marine species, including *Pagrus pagrus* and *Dentex dentex* (Bargelloni et al., 2003); *Sprattus sprattus* (Debes et al., 2008); *Crangon crangon* (Luttikhuizen et al., 2008); *Paleomon elegans* (Reuschel et al., 2010); zooplanktonic organisms (*Sagitta setosa)* (Peijenburg et al., 2004); *Sygnatus* pipe fish (Wilson and Veraguth, 2010), *Psetta maxima* (Suziki et al., 2004); anchovy (Magoulas et al., 2006) and the mullet (Durand et al., 2013). This second scenario assumes that during the early or middle Pliocene (5.00-3.50 Mya) two important events took place: isolation of the

Mediterranean Sea from the Atlantic Ocean and the cessation of the connection between the Mediterranean and the Black Sea (Moraitou-Apostolopoulou and Kiortsis, 1985). The Messinian Salinity crisis was the main reason for fish population bottlenecks in the Mediterranean and the Black Sea during the late Miocene (5.59-5.33 Mya) (Krijgsman et al., 1999). During the Messinian Salinity Crisis, the Mediterranean Sea level decreased, and the Black Sea was also affected. It seems probable that differentiation of the *Spicara* species took place in the post Messinian period (in the early or middle Pliocene) in this closed Mediterranean region, with ancestral *S. maena* and *S. flexuosa* populations differentiation happening in the western and eastern Mediterranean basins, respectively. An eastern Atlantic origin of *S. maena* is also possible. During the Pleistocene, with each glacial and interglacial cycle, *S. flexuosa* could have gradually expanded into the Black Sea, but might not have been able to tolerate the unfavorable conditions. After the Last Glacial Maximum however, the final opening of the Dardanelles and Bosphorus straits, (approximately 10 Ky BP) caused the interchange of the marine waters between Atlantic and Mediterranean Sea, and led to the establishment of marine conditions in the Black Sea (Zaitsev and Mamaev, 1997). The expansion of *S. flexuosa* from Mediterranean to Black Sea might have been initiated approximately 15 Kya (the estimated time for the onset of population expansion based on the Bayesian skyline analysis) during the post glacial warming period after the end of the last glacial maximum. During this period, there was ouflow from Black Sea into Aegean Sea through the Bosphorus and Dardanelles. The population expansion from Mediteranean to Black Sea might have occurred via the lower layer current of the Dardanelles (approximately 10 Ky BP). Since *S. flexuosa* has different physiological requirements (e.g. it is better able to tolerate the lower salinity and higher H_2S concentrations in the Black Sea), it might have colonized and adapted to the brackish conditions in the Black Sea more easily than *S. smaris*, which is less tolerant of the conditions in the Black Sea (Banarascu, 1964; FAO, 1973). Similar to *S. smaris, S. maena* is not able to tolerate low salinity in the Black Sea, but is generally found in the high salinity conditions, e.g., 37-38 ‰. (EOL, 2016). The recent study from Bektaş et al. (2018) also supported this second scenario, where *S. maena* was not detected genetically in the Black Sea. Further and extensive sampling in eastern Atlantic and western Mediterrenean, similar to that undertaken around Turkish coasts in this study, is necessary for a better understanding of the origins of *S. flexuosa* and *S. maena*.

3.1.8.2. Connectivity of the Turkish Spicara flexuosa populations. Low values of mtDNA molecular diversity indices, and absence of genetic structure between subpopulations, and star shaped haplotype networks indicate the connectivity of the *Spicara flexuosa* populations from Turkey, suggesting that the TSS is a biological corridor (sensu Öztürk and Öztürk, 1996), rather than a barrier to gene flow,

for this species. Considering overall molecular diversity of *S. flexuosa* populations, we see that the two lowest haplotype (h=0.062, h=0.073) and nucleotide diversities (π= 0.00014, π= 0.00021) were found in the CO1 and cyt-b genes, respectively. Geographically speaking, the highest haplotype and nucleotide diversities values were found in Mersin (h=0.389, π=0.0010) based on the cyt-b gene. On the other hand, certain regions showed haplotype diversities that were zero, such as Black Sea region for cyt-b and TSS for CO1. Low levels of genetic variability were observed in other fish populations such as *Psetta maxima* in isolated eastern basins, including the Aegean, the Sea of Marmara, Black Sea and the Azov Sea (Suziki et al., 2004). Suziki et al. (2004) found two distinct lineages in the western Mediterranean and eastern Mediterranean for *P. maxima*, and suggested that the eastern Mediterranean lineage showed lower genetic variability than the western lineage, potentially due to a population bottleneck in the last glacial period that took place in the Black Sea as well as in the eastern Mediterranean. It seems possible that the low levels of genetic variability in *S. flexuosa* populations might be reflecting a bottleneck in the Black Sea as well.

Low levels of genetic variation might also be a result of small population sizes. A molecular genetic study on the variation of cyt-b sequences among the *Trachurus mediterraneus* (h=0.476; π=0.00225), *Trachurus trachurus* (h=0.426; π=0.00199), and *Trachurus picturatus* (h=0.652; π=0.00406) populations showed that low to moderate level of haplotype and nucleotide diversities were found among the Black Sea, Mediterranean and Aegean Sea populations of these species (Bektaş and Beldüz, 2008). Authors indicated that the haplotype frequency differences among these three populations might have resulted from the differences of the effective population sizes of these species. Low levels of genetic variation were also observed in *Sarda sarda* populations among the Turkish coastal waters (Turan et al., 2015b). Overall nucleotide diversity (π= 0.0013) in the Turkish coasts was found to be lower than that in the Ionian, Baleric and Aegean Sea samples as reported by Vinas et al. (2004) and Vinas et al. (2010). Overfishing might be another reason for the low levels of genetic variation of fish populations observed in the Turkish coastal waters. Based on this concept, Turan et al. (2015b) suggested that high fluctuations of total catch and heavy fishing pressure were probably the main reasons for population bottleneck among the Turkish coastal waters in fishes. In recent years, *Spicara flexuosa/ maena* has been widely used as a fish bait, and also has been subject to heavy fishing pressure in the Turkish coastal waters (pers. comm. with fishermen). Thus, its populations have been severely impacted, resulting in a decrease of the population numbers. Previous morphological studies indicated that behavior of the *Spicara* species during the spawning season, which includes benthic male nesting might also have contributed to the overfishing female individuals in the spawning season (Karidas et al., 2009; 2011). Other morphological studies of the three *Spicara*

species revealed that female populations were smaller in size than the male populations, due to sex reversal and protogynous hermaphroditism (Lepori, 1960; Reinboth, 1962; Salekhova, 1979; Vidalis and Tsimenidis, 1996; Dulcic et al., 2000; Karakulak et al., 2006; Saygılı et al., 2016 a, b). As a result, sex ratio and life span of female population were lower than those in males. Hence, the low level of genetic diversity might also be a result of the small population size in general, and of the female population in particular, as previously observed in the literature for other Sparid hermaphrodite family members such as *Pagellus bogavareo* (Bargelloni et al., 2003; Stockley et al., 2005), and hermaphrodite species with small female population sizes, such as the striped bass, *More saxatilis* (Waldman et al., 1998).

The absence of significant genetic structure was deduced from the generally low and insignificant pairwise Φ_{ST} values between populations of *Spicara flexuosa* along the Turkish coasts. This might have been affected by the life histories of the species (e.g. pelagic larval phase) and the TSS not being a hydrological barrier for the species. The dispersal of pelagic larvae as a result of hydrological processes may enhance the gene flow between *Spicara flexuosa* populations from different seas, as was previously observed for other Sparid fish (Vigliola et al., 1998). A lack of genetic structure was also detected for *Trachurus mediterraneus, Trachurus trachurus,* and *Trachurus picturatus* populations, based on the cyt-b gene analysis (Bektaş and Beldüz, 2008) along the Turkish coastal waters. These authors revealed that gene flow was the main mechanism for the observed genetic homogeneity of the Turkish *Trachurus* populations. Moreover, lack of genetic structure was also observed among the Atlantic, Aegean and Mediterranean *Trachurus* populations based on 16S rRNA data. Authors revealed that migratory behavior of fish and lack of barriers were the main mechanisms that affect genetic differentiation (Karaiskou et al., 2003). In another study, Debes et al. (2008) reported the absence of the genetic structure (average Φ_{ST}=-0.018) between the Black and Bosphorus populations of the European sprat. Although four phylogeographically distinct groups (Atlantic, Adriatic, Mediterranean, Black Sea) were defined, based on the mtDNA control region analysis, no phylogeographical breaks were observed between the Black Sea and the Bosphorus populations. Wilson and Veraguth (2010) also detected low levels of genetic structuring (Φ_{ST}=-0.01) among *Syngnathus* pipefish populations, between the Black Sea and the Sea of Marmara. They suggested that a historical population bottleneck resulting from strong Pleistocene glaciations was the underlying reason for the low genetic structure, as well as low level of genetic diversity in the eastern Mediterranean basin. Durand et al. (2013) investigated *Mugil cephalus* populations from the Atlantic to the Black Sea using the cyt-b gene, and detected only a single clade across this region. They also suggested that Pleistocene glaciations was the main reason for the demographic expansion,

especially in the Black Sea region. In this study, star-shaped haplotype networks of the mtDNA genes of *Spicara flexuosa* populations indicate that the species might have also undergone a population expansion in the past, as has been previously observed for other protoygnous hermaphrodite fish (*Pagellus bogavareo)* (Bargelloni et al., 2003; Stockley et al., 2005), protandrous hermaphrodite fish *(Diplodus puntazzo*) (Bargelloni et al., 2005) and fresh to brackish water fish species *Coregonus clupeaformis* (Bernatchez et al., 1989).

Neutrality test results (significantly negative F_S and Tajima D values), unimodal mismatch distribution and BSP also support the scenario of sudden population growth (15 Kya) of *S. flexuosa* populations after a potential bottleneck in the eastern Mediterranean. Wilson and Veraguth (2010) also detected unimodal mismatch distributions among *Syngnathus* pipefish populations from the Black Sea and the Sea of Marmara. As opposed to these results, Debes et al. (2008) found ragged mismatch distribution among the European sprat populations between the Black Sea and the Bosphorus, not supporting a scenario of demographic expansion after having a small population sizes. Hence along the Turkish coasts and TSS, the presence/absence of bottleneck and demographic expansion scenarios can be species-specific in fish, and for *S. flexuosa* we see support for their presence.

Recently, genetic differentiation of the three species of *Spicara* and intraspecific sub-structure of *S. flexuosa* were investigated in the Turkish coastal waters by using partial 16S rRNA (1021 bp), and whole cytochrome b gene (1141 bp) sequences (Bektaş et al., 2018). The results revealed that three *Spicara* species were distinctly separated from each other based on the phylogenetic trees. *S. flexuosa* and *S. maena* were found to be genetically more closely related to each other than to *S. smaris*, similar to our cyt-b results. However, our 16S rRNA gene results indicated that *S. maena* and *S. smaris* were more closely related to each other than *S. flexuosa*. This result is kind of unexpected, and could be due to us having used a relatively short 16S fragment (155 bp), when compared to Bektaş et al. (2018), as mentioned above. Molecular diversity indices of the same study indicated that *S. maena*, *S. flexuosa* and *S. smaris* had low levels of haplotype and nucleotide diversities for the cyt-b and 16S rRNA genes. Low levels of haplotype and nucleotide diversities and star shaped haplotype networks for *S. flexuosa* were also found in this dissertation, which supports the hypothesis of a population bottleneck in the eastern Mediterranean region during the late Pleistocene. Moreover, relatively high interspecific sequence variation among the three *Spicara* species based on the 16S (2.90-4.44 %) and cyt-b genes (7.99-9.41 %) were reported by Bektaş et al. (2018), similar to our 16S (4.5- 5.8 %) and cyt-b findings (9.01- 9.23 %). Bektaş et al. (2018) collected *S. flexuosa*, *S. smaris*

and *S. maena* along the Turkish coastal waters, however they only found *S. flexuosa* in the Black Sea. They hypothesize that *S. maena* was not able to colonize the Black Sea due to low saline conditions. Bektaş et al. (2018) also discuss that, among three of them, *S. flexuosa* was the only species that could tolerate a high range of the salinity levels (Lleonart, 2008) and was found all along the Turkish coasts. Although as far as we can see, salinity toleration for *S. flexuosa* was not reported in Llenoart (2008), *S. flexuosa* was detected across all Turkish coastal waters, which supports the tolerance idea. In this dissertation, based on genetics analyses, *S. flexuosa* was found in all Turkish coastal waters and *S. maena* was found in Greece (Thessalonaki), and two *S. maena* individuals were found in the Sea of the Marmara in İstanbul. Seasonal variation of *Spicara maena* abundance, and especially its high abundance values during the spring in the Turkish coastal waters (pers. comm., B. Yokeş,), might be the reason for the low detection rate of *S. maena* in this study. *S. maena* individuals were found in the spring season in the Thessalonaki, on the other hand *S. flexuosa* specimens were found in the winter, autumn and summer seasons in the Turkish coasts in the collection periods for this study. Thus, this unsampled period (especially late winter/early spring) might be the reason for us not having detected any *S. maena* individuals along the Turkish coastal waters, except the two (based on the genetic results) in the Bosphorus.

Nuclear DNA (nuDNA) analysis results of *Spicara flexuosa* and *Spicara maena* revealed that the previous phylogenetic analyses were in accordance with the taxonomies of the two species of *Spicara spp.*, as no haplotypes were shared between them. The genetic differentiation between the two species of *Spicara* confirm that reproductive isolation of the two species was complete. The phylogenetic trees obtained by the Bayesian, neighbour-joining and maximum likelihood revealed the same pattern of two monophyletic clades. However, reciprocally monophyletic clades were not seen in the maximum parsimony tree. Moreover, the total number of mutations and number of parsimony informative sites between two *Spicara* species were not as high as those between the mtDNA genes, suggesting that the lack of the detection could be due to the low resolution of the IBRP gene (Verma et al., 2004).

Based on the results of this study, *S. flexuosa* was detected across the eastern Atlantic, Mediterranean and Black Sea, and *S. maena* was observed in the Mediterranean, and with a limited distribution in the eastern Mediterranean (Figure 3.34). According to FishBase (Froese and Pauly, 2017), *Spicara flexuosa* is considered as a synonym of *Spicara maena*, and therefore, data on its population statistics have not been reported in the literature (Pollard et al., 2014). These problems are significant, as they can result in inaccurate determination of current stock levels for both *Spicara*

maena and *S. flexuosa*. Overall picarel populations from FAO data reported that *Spicara maena/Spicara flexuosa* populations were decreased in the last 10 years. Therefore, it is recommended that separate statistics and population studies should be used for conservation considerations of both species (Pollard et al., 2014).

3.2. *Merlangius merlangus* (Linnaeus, 1758)

3.2.1. Mitochondrial DNA (CO1 gene)

A 481 bp fragment of the CO1 region was amplified for a total of 164 individuals from Turkish coastal waters, Greece and two individuals from France (Appendix Table F.2). 22 sequences of *Merlangius merlangus* individuals from GenBank were also used. The GenBank accession numbers and locality information of individuals are given in Table 3.20. A total of 17 haplotypes were found in 187 individuals. *Melanogrammus aeglefinus* was included in all phylogenetic analyses, as an outgroup species. The most common haplotype, Hap 6 (79 %) was found in eight different locations in the Black Sea, TSS and the Aegean Sea (Figure 3.39). The maximum likelihood, neighbour joining, maximum parsimony and Bayesian analysis results, as well as the haplotype network of the CO1 gene are shown below (Figures 3.35-3.39).

Table 3.20. The accession numbers and locations of individuals for the CO1 sequences of *Merlangius merlangus* that were retrieved from GenBank.

Species	Accession codes	Location	Accession codes	Location
M. merlangus	DQ174008	France	KC500911	Turkey
M. merlangus	DQ174007	France	KC500910	Turkey
M. merlangus	JQ623954	Turkey	KC500909	Turkey
M. merlangus	KP136724	Turkey	KC500908	Turkey
M. merlangus	KP136723	Turkey	KC500907	Turkey
M. merlangus	KP975779	France	KC500906	Turkey
M. merlangus	KJ205026	North Sea	FN689176	Atlantic
M. merlangus	KJ205025	North Sea	FN688951	Atlantic
M. merlangus	KJ205024	North Sea	KC500912	Turkey
M. merlangus	KJ205395	U.K		
M. merlangus	KJ205394	U.K		
M. merlangus	KJ205393	U.K		
M. merlangus	KJ205392	U.K		

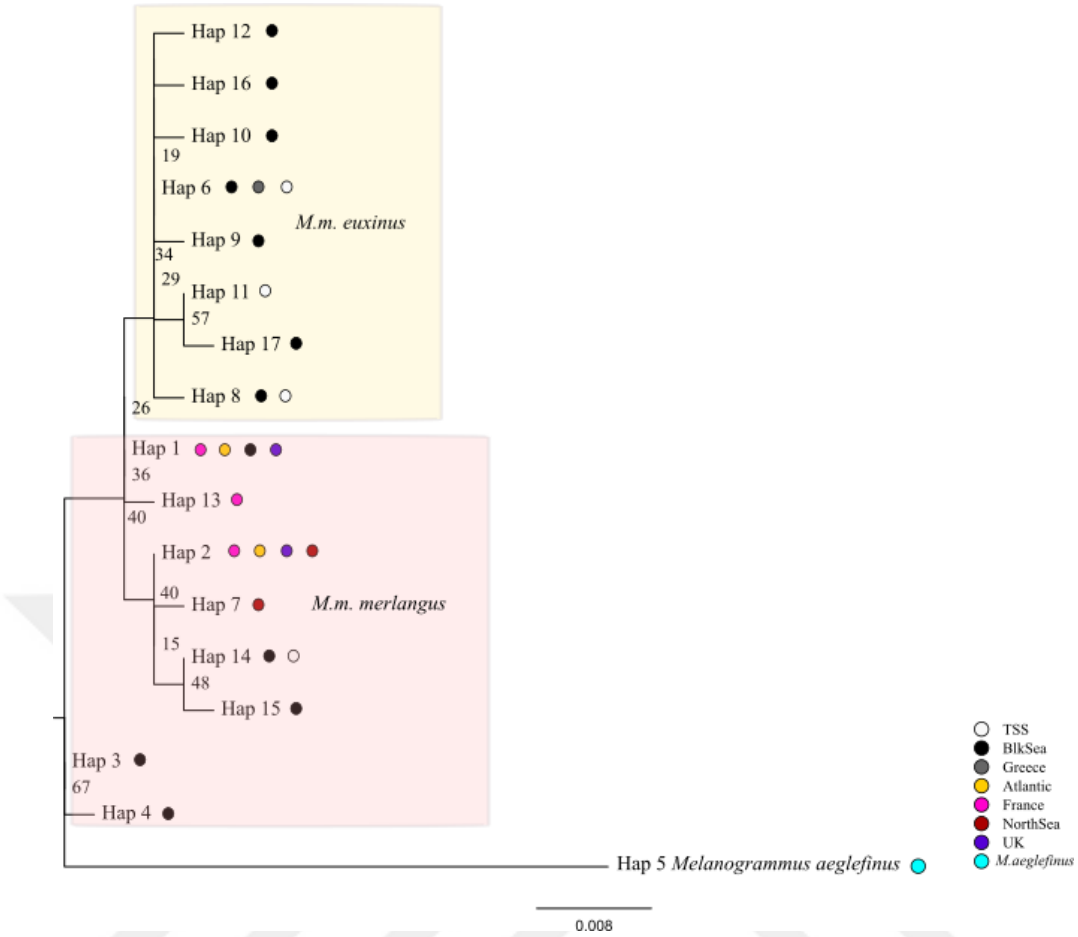


Figure 3.35. Maximum likelihood tree based on CO1 data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

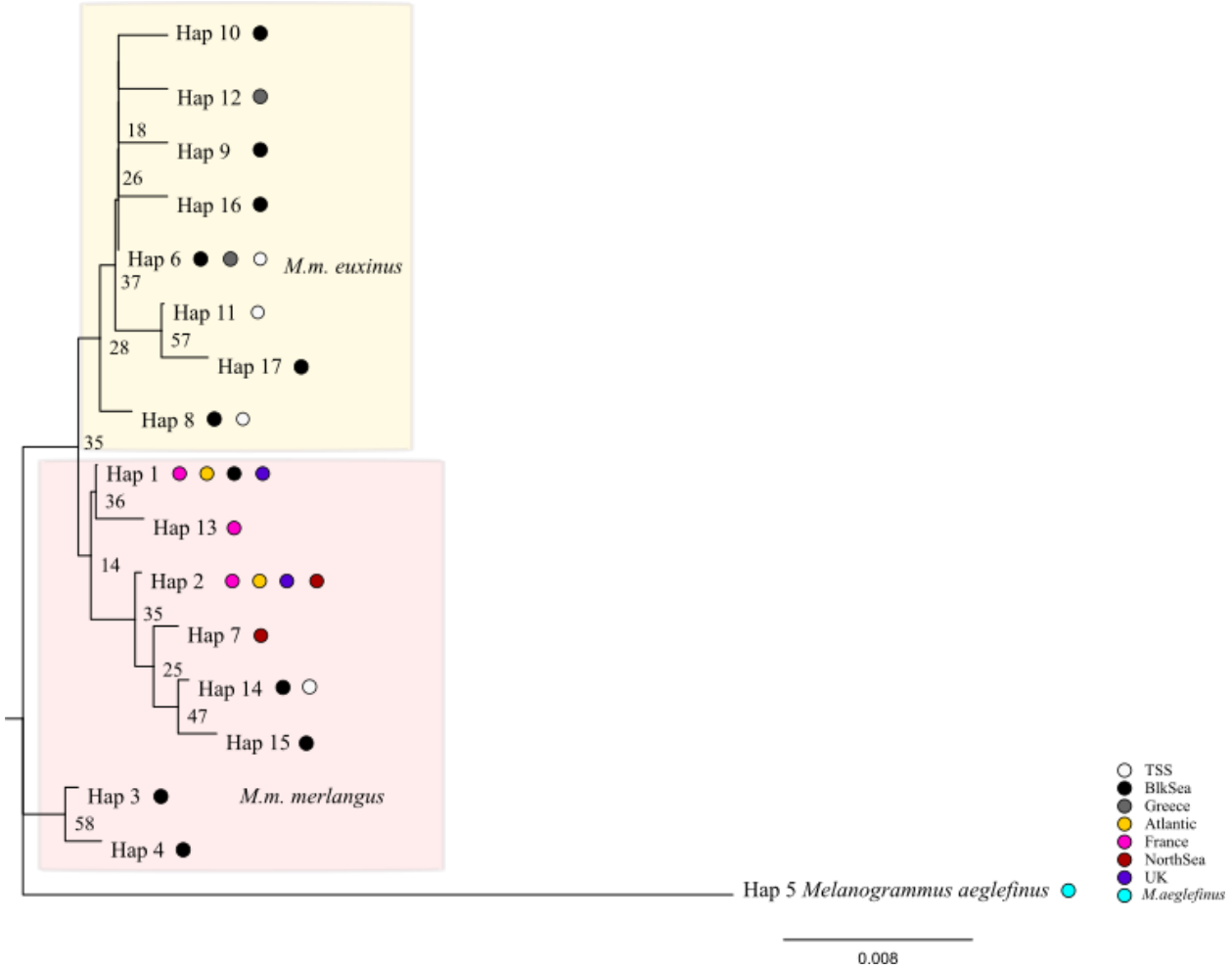


Figure 3.36. Neighbour-joining tree based on CO1 data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.



Figure 3.37. Maximum parsimony tree based on CO1 data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

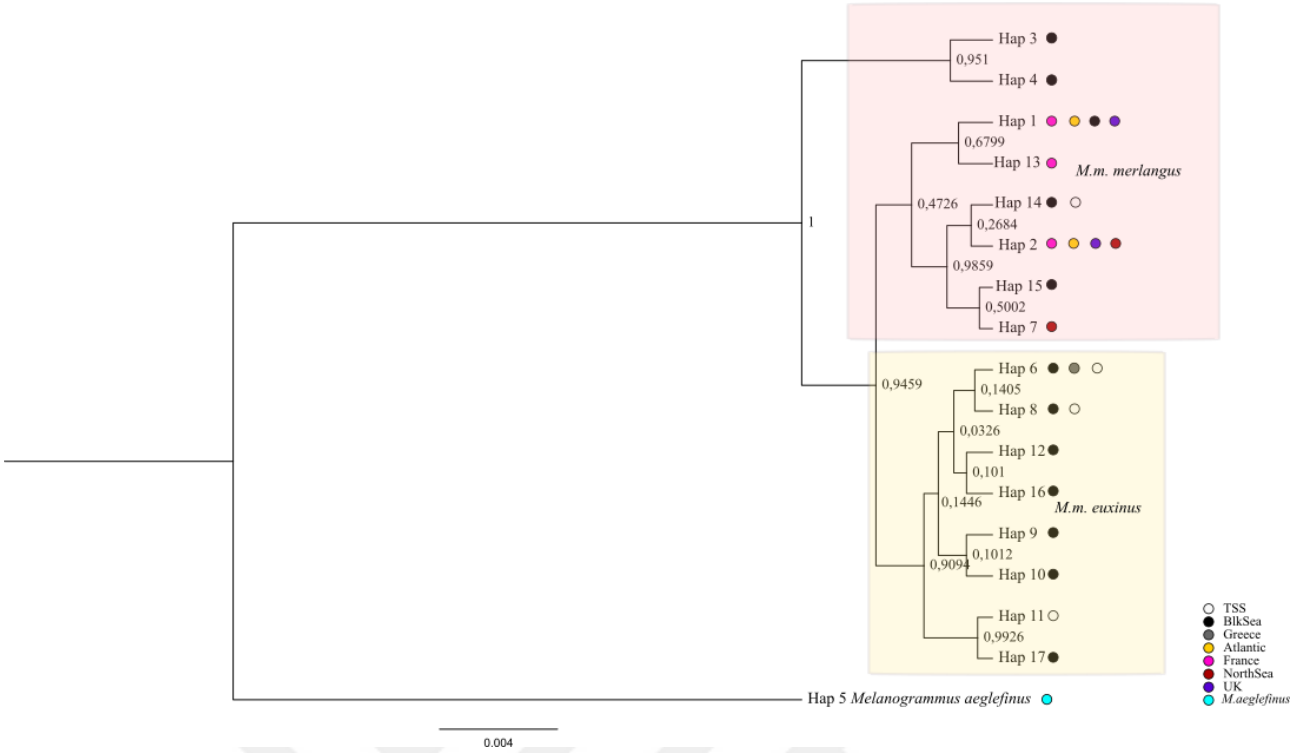


Figure 3.38. Bayesian tree based on CO1 data for *Merlangius merlangus* populations. The node values represent posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

All of the phylogenetic trees, which were built using different methods, were able to differentiate between the two different subspecies, *Merlangius merlangus merlangus* and *Merlangius merlangus euxinus*, with moderate to high bootstrap values. *Merlangius merlangus merlangus* haplotypes (Hap 1, Hap 2, Hap 3, Hap 4, Hap 7, Hap 13, Hap 14 and Hap 15) were detected in the Atlantic, France, North Sea, U.K. and Black Sea sequences that were retrieved from GenBank, and also one TSS and two Black Sea individuals collected from Turkish coastal waters in this study. On the other hand, *Merlangius merlangus euxinus* haplotypes (Hap 6, Hap 8, Hap 9, Hap 10, Hap 11, Hap 12, Hap 16, Hap 17) were found in samples collected from TSS, Black Sea and Greece, in this study.

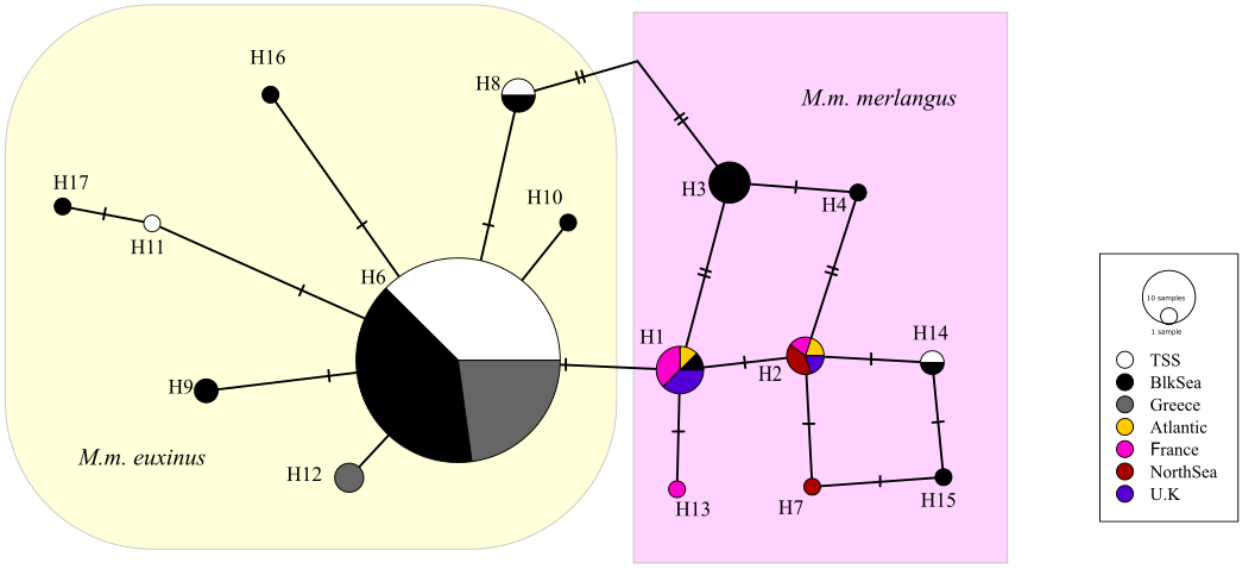


Figure 3.39. The haplotype network of the CO1 gene of *Merlangius merlangus.* Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Each line represents a single mutation.

The star-shaped haplotype network showed that haplotypes H1 and H6, the most frequent haplotypes in *Merlangius merlangus merlangus* and *Merlangius merlangus euxinus*, respectively, and were separated from each other by one mutational step (Figure 3.39). The two haplogroups/subspecies, which were detected in the haplotype network were also seen in all of the phylogenetic trees. Molecular diversity indices of the CO1 gene were calculated for each *Merlangius merlangus* population and are given in Table 3.21. As with the phylogenetic trees and the haplotype network, *Merlangius merlangus* populations were found to correspond to two subspecies. Six *Merlangius merlangus euxinus* (İnebolu) samples were excluded from this analysis due to low sample size.

Codon usage analysis was also conducted for the CO1 gene, to determine the reliability of the sequences with regard to Numts. The average number of codon positions analyzed for this gene was 160. Relative synonymous codon usage and all codons are given in the Appendix Table B.3. AUU (Isoleucine) was the most widely observed aminoacide (Appendix Table B.3) among the two *Merlangius merlangus* subspecies in the CO1 gene. As indicated in this table, stop codons were not observed for this gene.

Table 3.21. Molecular diversity of *Merlangius merlangus* based on CO1 gene sequences. N: Number of samples; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations are given in parantheses. *Includes *M. m. merlangus* samples detected in these water bodies.

Sampling sites	N	Nh	Np	h	π	SS	PIS
İstanbul	20	3	2	0.195 (0.115)	0.00042 (0.115)	2	0
Çanakkale	18	2	3	0.111 (0.096)	0.00069 (0.00060)	3	0
Bandırma	22	2	1	0.091 (0.081)	0.00019 (0.00017)	1	0
Rize	30	7	8	0.416 (0.112)	0.00174 (0.00063)	4	4
Sinop	14	2	1	0.143 (0.119)	0.00030 (0.00025)	1	0
Zonguldak	18	2	1	0.111 (0.096)	0.00023 (0.00020)	0	0
Greece	36	2	1	0.108 (0.068)	0.00022(0.00024)	0	1
Total *M .merlangus*	**158**	**10**	**10**	**0.181 (0.042)**	**0.00060 (0.00017)**	**3**	**7**
TSS*	**60**	**4**	**5**	**0.129 (0.059)**	**0.00041 (0.00023)**	**4**	**1**
Black Sea*	**62**	**8**	**9**	**0.270 (0.074)**	**0.00099 (0.00035)**	**4**	**5**
Aegean	**36**	**2**	**1**	**0.108 (0.068)**	**0.00022(0.00024)**	**0**	**1**
M. m. euxinus	**155**	**8**	**7**	**0.149 (0.039)**	**0.00035 (0.00010)**	**3**	**4**
M. m. merlangus	**25**	**8**	**6**	**0.820 (0.046)**	**0.00373 (0.00043)**	**1**	**5**

Molecular diversity indices of the CO1 gene for all *Merlangius merlangus euxinus* populations showed that variation was low among the coastal waters of Turkey and Greece. A total of eight haplotypes were found in 155 individuals. Mean haplotype and nucleotide diversities were found to be low (h=0.149; π= 0.00035). On the other hand, the highest haplotype and nucleotide diversities were found in the Rize population (h=0.416; π=0.00174). Singleton and parsimony informative sites were also the highest in Rize. A total of 42 out of 481 bp, (8 %) nucleotide sites were variable, of which eight (2 %) positions were parsimony informative, when comparisons were made using all sequences, including the outgroup. Of all the parsimony informative sites, only one position (316) was fixed for differentiating between the two subspecies. As opposed to *M. m. euxinus*, the average haplotype and nucleotide diversities were found to be higher (h=0.820, π=0.00373) in *M. m. merlangus*. The total number of mutations and percentage of pairwise sequence divergences between all *M. m. euxinus* and *M. m. merlangus* samples were found to be 13 and 4.73 %, respectively. Considering the evolutionary divergence times and phylogenetic separation of the two subspecies, a molecular clock hypothesis was not rejected. The 4.73 % divergence rate, with a molecular clock rate of 1.2 % *per* million years for the CO1 gene (Bermingham et al., 1997), corresponded to an estimated

divergence time that dated to around 125.000 Ky BP, suggesting that the two subspecies began to diversify during the late Pleistocene.

3.2.1.1. Mitochondrial population structure of Merlangius merlangus based on the CO1 gene. An analysis of molecular variance (AMOVA) and pairwise Φ_{ST} values are given in Table 3.22. Since the haplotype network and phylogenetic trees revealed two subspecies, Φ_{ST} values were calculated based on these groups. Φ_{ST} values were also calculated among *Merlangius merlangus euxinus* from Turkish coastal waters and Greece. AMOVA analyses were undertaken for the Black Sea, TSS, Aegean subpopulations, and between *M. m. merlangus* and *M. m. euxinus.*

Table 3.22. Pairwise Φ_{ST} values for the CO1 gene of *M. m. merlangus* and *M. m. euxinus*. Significant *P* values are given in shaded cells (< 0.05).

			İstanbul	Çanakkale	Bandırma	Rize	Sinop	Zonguldak	Greece	GnBnkBlk	BlkSeaTSS	Atlantic	France	NorthSea	U.K
M. m euxinus	TSS	**İstanbul**	0.00												
		Çanakkale	-0.00	0.00											
		Bandırma	-0.03	-0.01	0.00										
	BlkSea	**Rize**	-0.03	-0.00	-0.02	0.00									
		Sinop	-0.00	0.01	0.00	-0.00	0.00								
		Zonguldak	-0.00	-0.00	0.00	-0.00	-0.06	0.00							
	Aeg	**Greece**	0.01	-0.00	0.01	**0.02**	0.01	0.01	0.00						
M. m. merlangus		**GnBnkBlk**	**0.87**	**0.91**	**0.90**	**0.83**	**0.87**	**0.89**	**0.92**	0.00					
		BlkSeaTSS	**0.92**	**0.97**	**0.95**	**0.87**	**0.93**	**0.94**	**0.95**	**0.81**	0.00				
		Atlantic	**0.83**	**0.95**	**0.90**	**0.71**	**0.85**	**0.88**	0.90	0.64	0.57	0.00			
		France	**0.77**	**0.86**	**0.83**	**0.68**	**0.77**	**0.81**	**0.85**	**0.65**	**0.67**	-0.24	0.00		
		NorthSea	**0.89**	**0.96**	**0.93**	**0.81**	**0.90**	**0.92**	**0.93**	**0.75**	0.53	0.04	0.43	0.00	
		U.K.	**0.81**	**0.93**	**0.88**	**0.70**	**0.83**	**0.85**	**0.88**	**0.67**	**0.72**	-0.37	-0.19	0.47	0.00

Pairwise Φ_{ST} values between populations of *M. m. merlangus* and *M. m. euxinus* were the highest, with significant *P* values. These values ranged between 0.65 to 0.97, indicating the differentiation of the two species. According to AMOVA results, the variation was 70.35 % among *M. m. euxinus* and *M. m. merlangus* groups, 3.26 % among populations within groups, and 26.38 % within populations. For *M. m. euxinus*, pairwise Φ_{ST} values were low and non-significant among all subpopulations except between Greece and Rize (Φ_{ST}=0.02). AMOVA revealed that variation was almost non-existent among three groups (TSS, Black Sea and Greece) (0.83 %) and among populations within groups (-0.92 %), and virtually all variation was contained within populations (100.09 %). The overall results showed restricted gene flow between *M. m. euxinus* (TSS, Black Sea, Aegean) and *M. m. merlangus* (Atlantic, France, North Sea, U.K., TSS and from the Black Sea) populations.

3.2.2. The cyt-b Gene

A 612 bp fragment of the mitochondrial cyt-b gene was amplified for a total of 126 individuals from Turkish coastal waters, Greece and one individual from France (Appendix Table F.2). 10 sequences of *Merlangius merlangus* from GenBank were also used. GenBank accession numbers and locality information of individuals are given in Table 3.23. *Melanogrammus aeglefinus* was included in all phylogenetic analyses as an outgroup species. A total of 31 haplotypes were found in 137 individuals. Hap 19 turned out to be the most common haplotype that was encountered in 28 individuals (20 %), and Hap 5 and Hap 12 were the second and third most common haplotypes that were each found in 21 specimens from TSS, the Black Sea and the Aegean Sea (Figure 3.40). The haplotype network and phylogenetic trees built with the maximum likelihood, neighbour joining, maximum parsimony and Bayesian methods are shown below (Figures 3.40-3.44).

Table 3.23. The accession numbers and location of individuals for the cyt-b sequence *Merlangius merlangus* that were retrieved from GenBank.

Species	Accession numbers	Location
M. merlangus	EU224015	Atlantic
M. merlangus	EU224016	Atlantic
M. merlangus	DQ174058	Atlantic
M. merlangus	EU492299	Baltic Sea
M. merlangus	DQ174057	Atlantic
M. merlangus	EU492153	North Sea
M. merlangus	EU492152	North Sea
M. merlangus	EU492300	Baltic Sea
M. merlangus	KP644320	North Sea
M. merlangus	AJ517487	North Sea

The haplotype network revealed the clear differentiation between the populations of *M. m. merlangus* and *M. m. euxinus*. The star-shaped *M. m. euxinus* haplogroups were found exclusively in individuals from TSS, the Black Sea and Greece, whereas the *M .m. merlangus* haplogroups, which did not for a star-shaped phylogeny, were found in individuals from Atlantic, Baltic Sea, North Sea and France. These two haplogroups were separated from each other by one or two mutational steps. In addition, all phylogenetic trees revealed that the *M. m. merlangus* and *M. m. euxinus* haplogroups were clearly separated from each other. The distribution of the haplogroups in the tree also reflects the geographic separation of the samples.

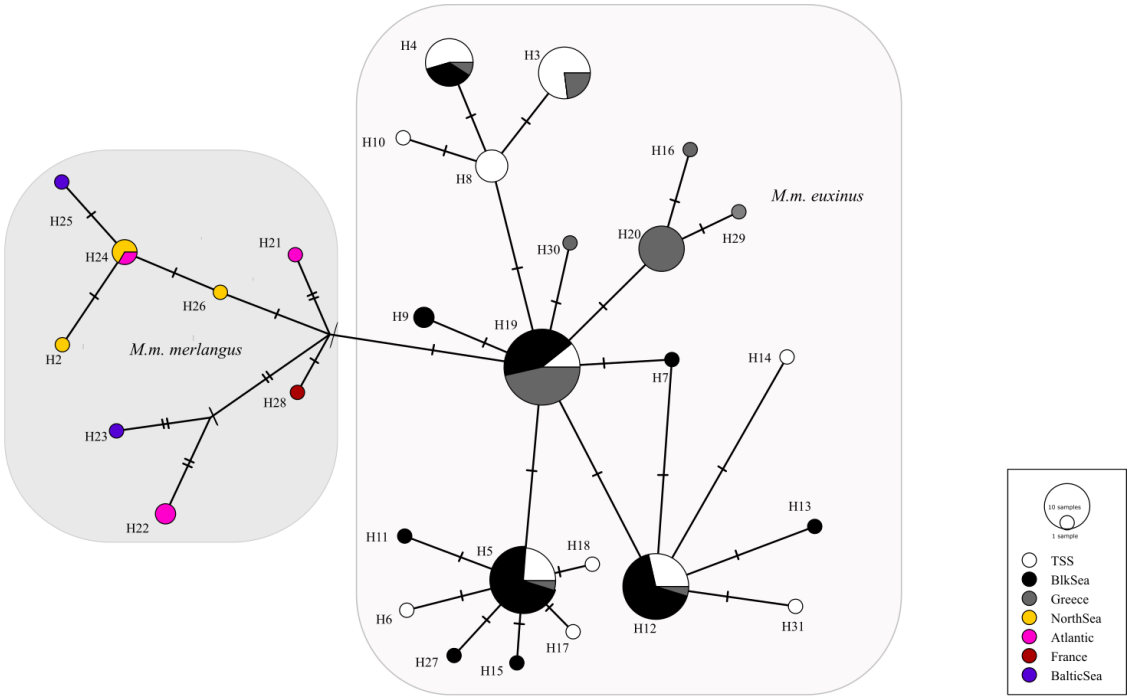


Figure 3.40. The haplotype network of the cyt-b gene of *Merlangius merlangus*. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Each of the hatches over the lines between haplotypes represents one mutational step.

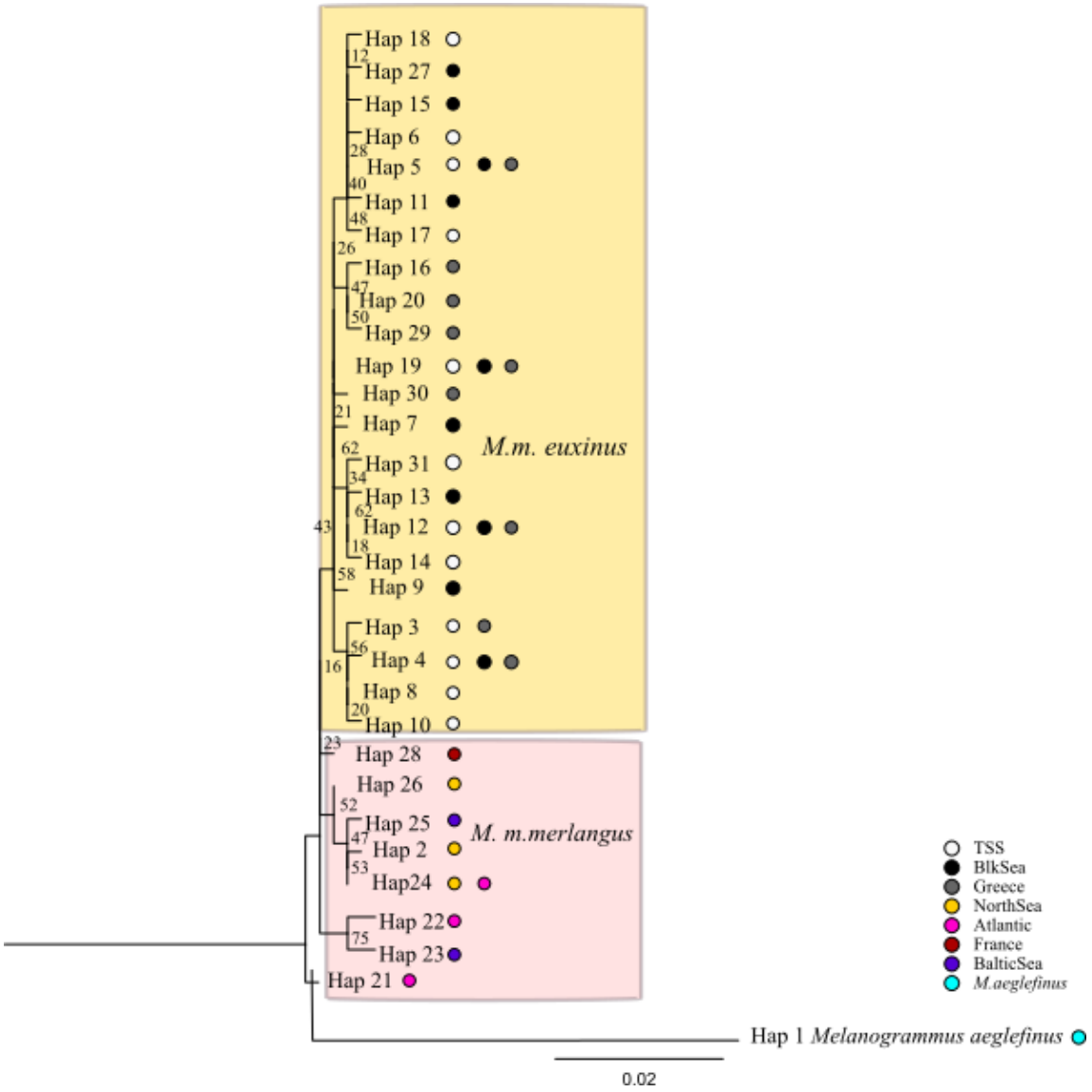


Figure 3.41. Maximum likelihood tree based on cyt-b data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

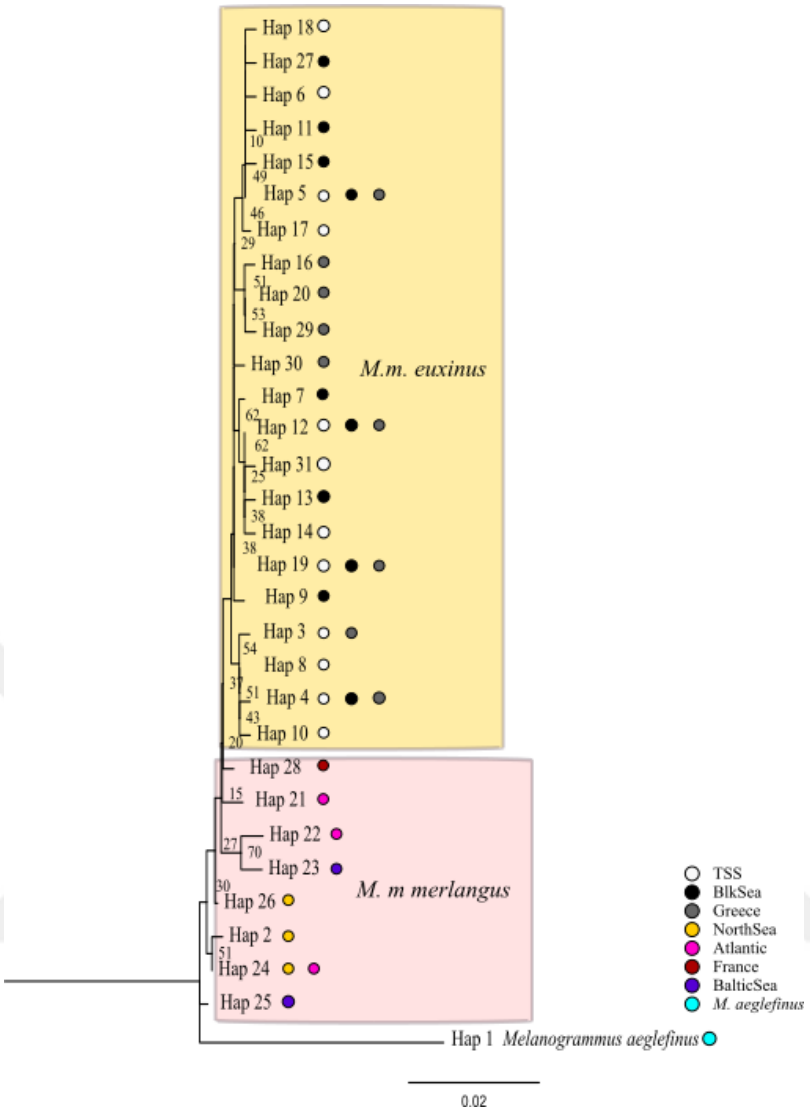


Figure 3.42. Neighbour-joining tree based on cyt-b data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

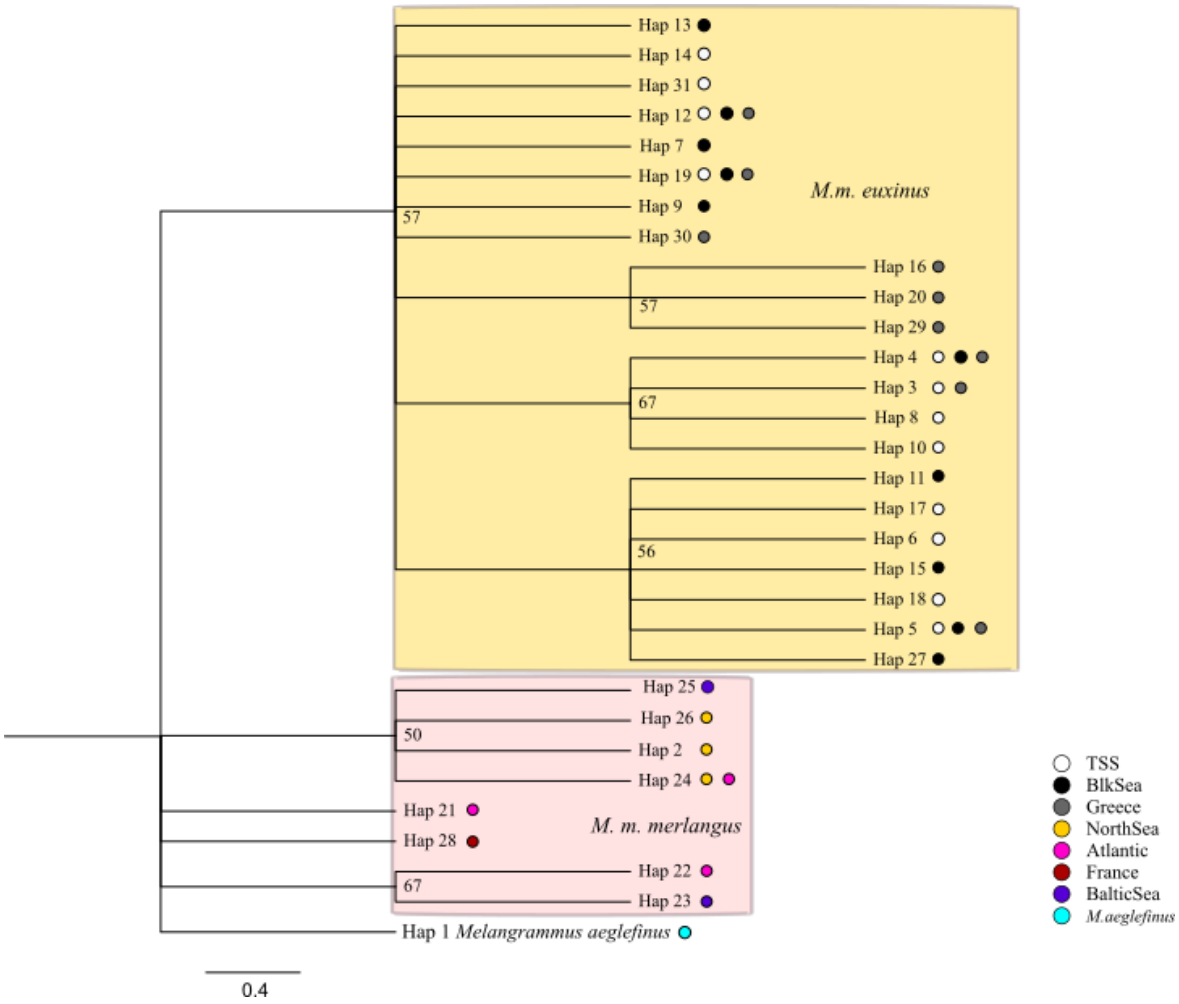


Figure 3.43. Maximum parsimony tree based on cyt-b data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

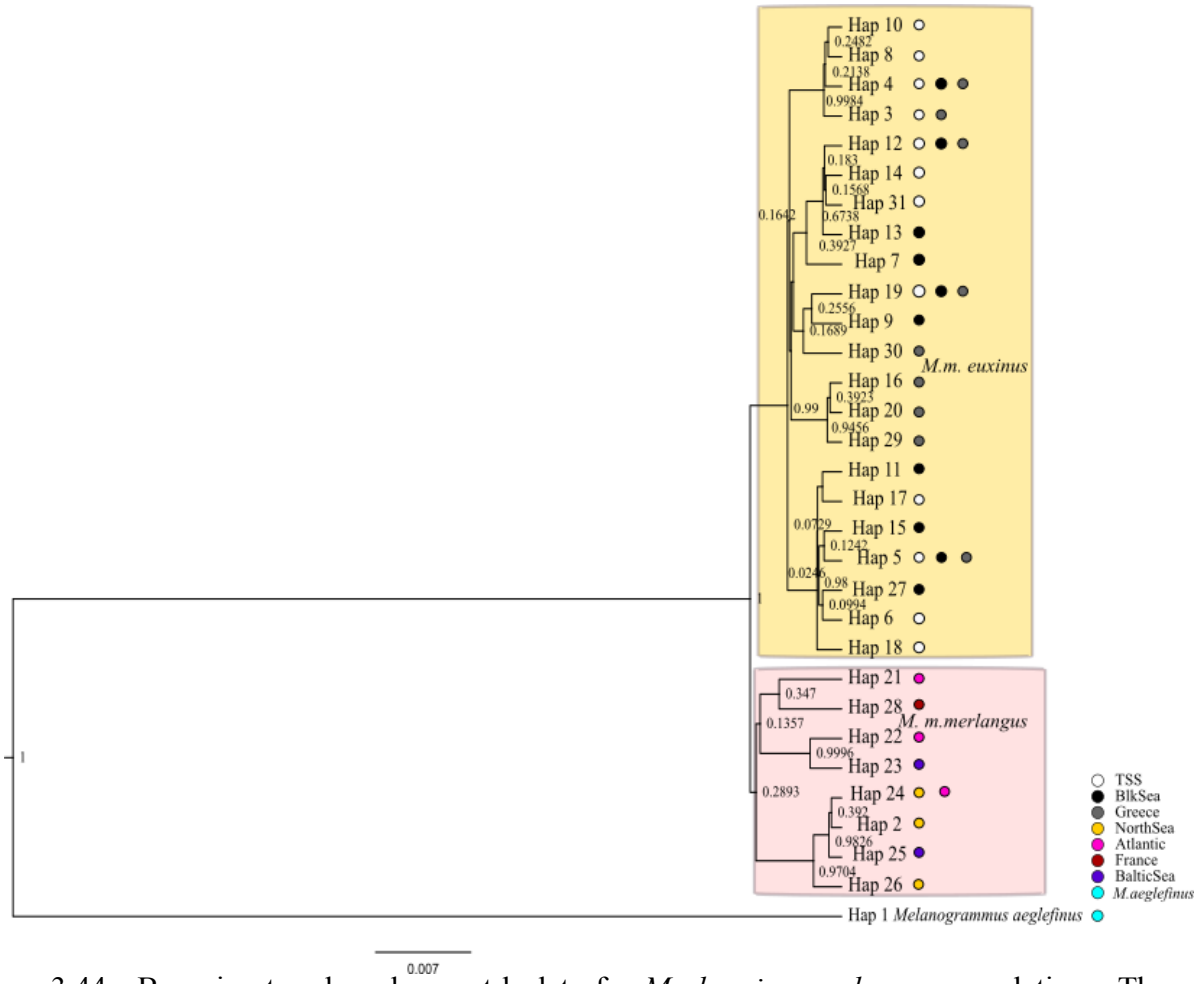


Figure 3.44. Bayesian tree based on cyt-b data for *Merlangius merlangus* populations. The node values represent posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

Molecular diversity indices of the cyt-b gene for all *Merlangius merlangus* populations from each sampling location, as well as the two subspecies, were calculated and are given in Table 3.24.

Table 3.24. Molecular diversity of *Merlangius merlangus* based on cyt-b gene sequences. N: Number of samples; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations are given in parantheses.

Sampling sites	**N**	**Nh**	**Np**	**h**	**π**	**SS**	**PIS**
İstanbul	10	6	5	0.889 (0.075)	0.00309 (0.00046)	2	3
Çanakkale	16	7	8	0.825 (0.0441)	0.00323 (0.00058)	4	4
Bandırma	15	8	7	0.895 (0.053)	0.00314 (0.00041)	3	4
Rize	18	4	4	0.791 (0.037)	0.00249 (0.00033)	0	4
Sinop	10	5	3	0.800 (0.100)	0.00225 (0.00036)	1	2
Zonguldak	8	3	2	0.679 (0.122)	0.00128 (0.00032)	1	1
İnebolu	16	7	6	0.850 (0.060)	0.00260(0.00034)	3	3
Greece	32	9	9	0.746 (0.055)	0.00206 (0.00033)	6	3
M .m. euxinus	**125**	**22**	**19**	**0.873 (0.013)**	**0.00294 (0.00015)**	**11**	**8**
TSS	**41**	**12**	**11**	**0.880 (0.025)**	**0.00340 (0.00024)**	**6**	**5**
Black Sea	**52**	**10**	**9**	**0.797 (0.028)**	**0.00233 (0.00022)**	**4**	**5**
Aegean	**32**	**9**	**9**	**0.746 (0.055)**	**0.00206 (0.00033)**	**6**	**3**
M. m. merlangus	**11**	**8**	**13**	**0.927 (0.066)**	**0.00637(0.00103)**	**7**	**6**

Overall, the genetic diversity of *Merlangius merlangus euxinus* was the highest along the Turkish coastal waters. Bandırma samples had the highest haplotype and nucleotide diversities (h=0.895, π=0.00314). The lowest haplotype and nucleotide diversities, on the other hand, were found in Zonguldak samples (h=0.679; π=0.0128). Among the three defined regions (Black Sea, TSS and Aegean), the highest number of haplotypes (12), as well as haplotype and nucleotide diversities, and singleton and parsimony informative sites were found in the TSS. However, considering *M. m. euxinus* and *M. m. merlangus* populations together, the highest mean haplotype and nucleotide diversities were detected in *M. m. merlangus* (in the Atlantic region) (h=0.927, π=0.00637). A total of 89 nucleotide sites were variable, of which 15 (2 %) positions were parsimony informative when comparisons were made using the outgroup, and only one position (110) was informative for differentiating between the two subspecies. Total number of mutations and percentage of pairwise sequence divergences between *M. m. euxinus* and *M. m. merlangus* were found to be 34 and 7.37 %, respectively.

Average number of codon positions analyzed for this gene was 204. Stop codons (UAG, UAA, AGA, AGG) were observed in the cytb gene for the original sequences as well as sequences where

one and two bases were deleted (Appendix Table B.4, B.5, B.6), and the reverse complement sequences. Moreover, stop codons were also detected in the complete cyt-b sequences from GenBank, e.g. (DQ020496. 1) (Roques et al., 2006); as well as partial cyt-b gene sequences (FN688361.1, FN683362.1) (Kochzius et al., 2010); (KC128878.1) (Lago et al., 2013). In addition, UUU (Phenylalanine), UUG (Leucine), CUU (Leucine) aminoacids were the most widely observed in the original sequences, as well as those where one and two bases were deleted (Appendix Table B.4, 5, 6). Since there was indication for the presence of Numts (due to stop codons) for the cyt-b fragment analyzed, a divergence time was not estimated based on this gene for the two subspecies.

3.2.2.1. Mitochondrial population structure of Merlangius merlangus based on the cyt-b gene. The AMOVA results and pairwise Φ_{ST} values are given in Table 3.25. AMOVA analysis was undertaken for the regional groups of Black Sea, TSS, and the Aegean. Φ_{ST} values were calculated between the two subspecies, as well as among *Merlangius merlangus euxinus* populations from Turkish coastal waters and Greece.

Table 3.25. Pairwise Φ_{ST} values for the cyt-b gene of *M. m. merlangus* and *M. m. euxinus*. Significant *P* values are given in shaded cells (< 0.05).

			İstanbul	Çanakkale	Bandırma	Rize	Sinop	Zonguldak	İnebolu	Greece	Atlantic	BalticSea	NorthSea
M. m. euxinus	TSS	**İstanbul**	0.00										
		Çanakkale	0.04	0.00									
		Bandırma	0.02	**0.17**	0.00								
	BlkSea	**Rize**	0.07	**0.19**	-0.02	0.00							
		Sinop	**0.17**	**0.35**	0.01	0.04	0.00						
		Zonguldak	0.12	**0.32**	0.03	0.02	-0.00	0.00					
		İnebolu	**0.15**	**0.34**	0.04	0.05	-0.03	-0.04	0.00				
	Aeg	**Greece**	**0.18**	**0.31**	**0.15**	**0.14**	**0.24**	**0.14**	**0.21**	0.00			
M. m. merlangus		**Atlantic**	**0.44**	**0.51**	**0.47**	**0.53**	**0.50**	**0.50**	**0.52**	**0.58**	0.00		
		BalticSea	**0.56**	**0.61**	**0.59**	**0.65**	**0.64**	0.68	**0.64**	**0.70**	-0.08	0.00	
		NorthSea	**0.64**	**0.66**	**0.64**	**0.69**	**0.71**	**0.77**	**0.68**	**0.72**	0.23	0.17	0.00

The results showed that the highest level of significant genetic structuring was observed between *M. m. merlangus* and *M. m. euxinus*. Significant pairwise Φ_{ST} values ranged from 0.44 to 0.77 between the two subspecies. AMOVA results showed that percentage of variation was 13.16 % among groups (TSS, Black Sea and Aegean), 5.73 % among populations within groups, and 81.1 % within populations. Considering *M. m. euxinus* subpopulations, high and significant Φ_{ST} values were observed between Çanakkale and Sinop (Φ_{ST}= 0.35), İnebolu and Çanakkale (Φ_{ST}= 0.34), and Zonguldak and Çanakkale populations (Φ_{ST}= 0.32). Moreover, pairwise Φ_{ST} values between Greece and all other locations were found to be significant and had moderate to high values (0.14 to 0.31).

3.2.3. CO1 and cyt-b Concatenated Data for *M. merlangus*

The concatenated cyt-b and CO1 data consisted of 1093 aligned bases of mitochondrial sequences of 82 individuals of *Merlangius merlangus* (58 from Turkey, 23 from Greece and one individual from France), from eight sampling locations (Appendix Table F.2). In addition, sequences from one closely related outgroup species (*Melanogrammus aeglefinus*) from GenBank were used in the analyses. A total of 25 haplotypes were found in 83 individuals. The concatenated data set for *M. m. merlangus* comprised a single unique sequence as only a single sample from France had sequences for both CO1 and cyt-b genes. Phylogenetic relationships between haplotypes were determined by maximum likelihood (Figure 3.45), neighbour-joining (Figure 3.46), maximum parsimony (Figure 3.47), and Bayesian trees (Figure 3.48).

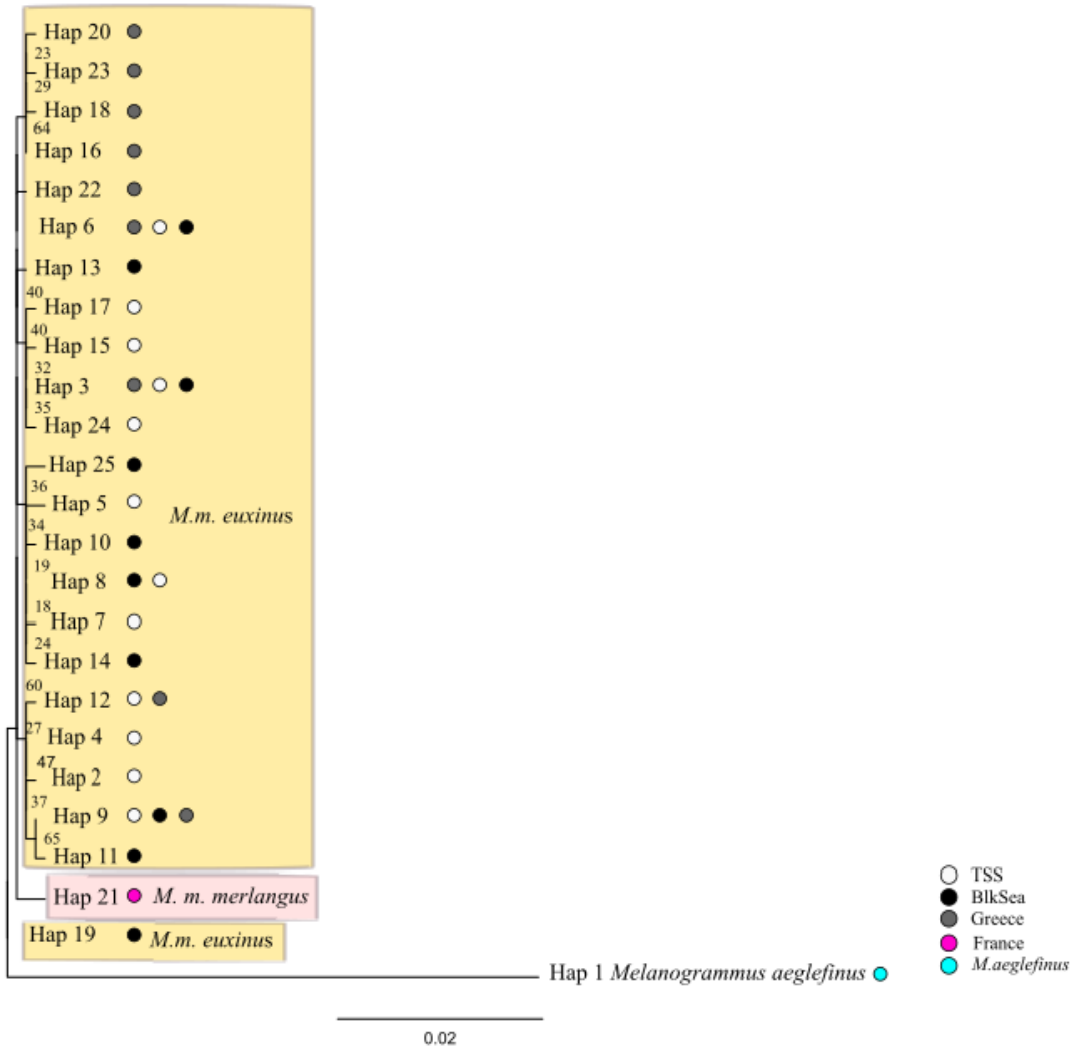


Figure 3.45. Maximum likelihood tree based on cyt-b+CO1 concetanated data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

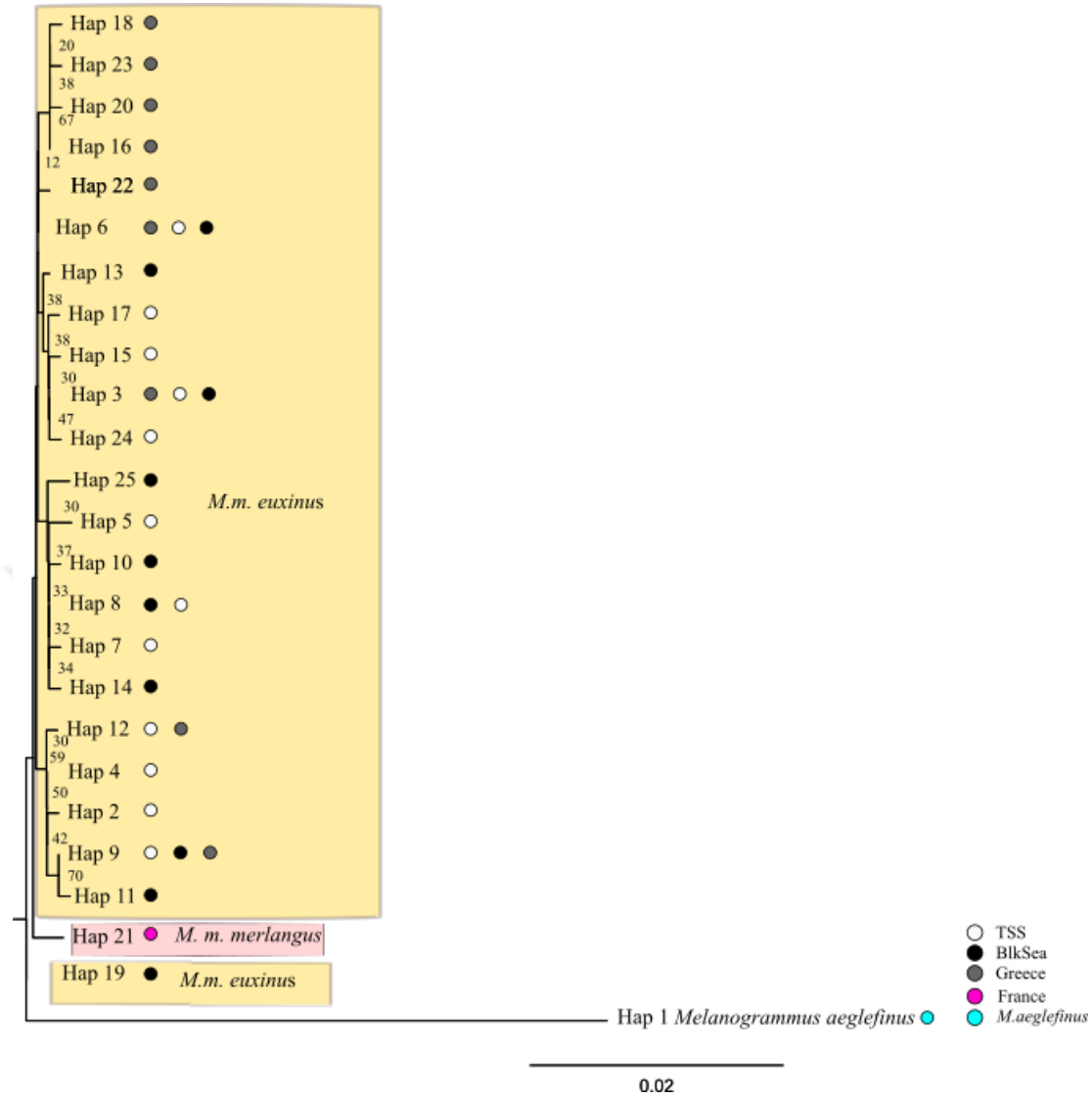


Figure 3.46. Neighbour-joining tree based on cyt-b+CO1 concatenated data for *Merlangius merlangus* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

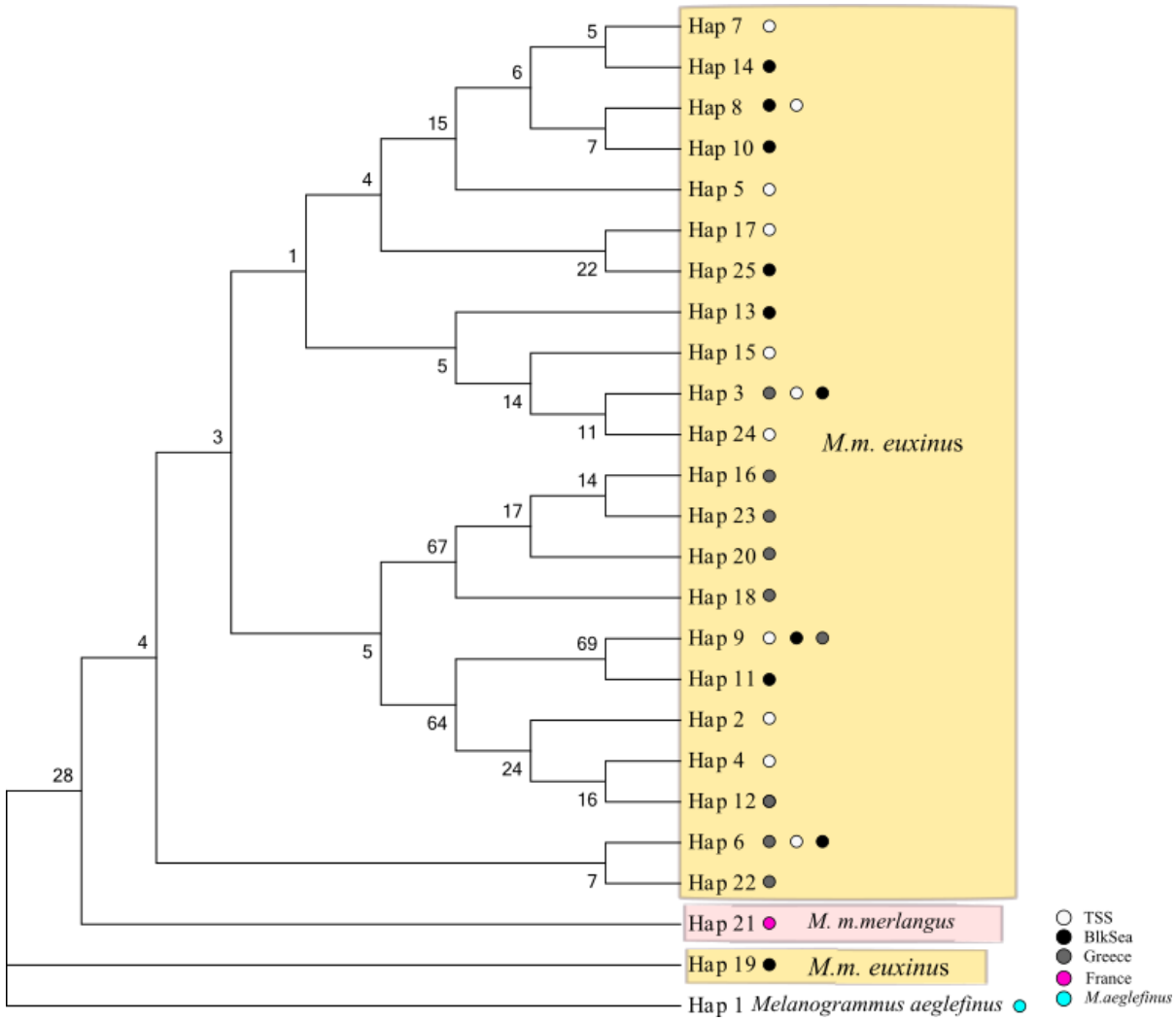


Figure 3.47. Maximum parsimony tree based on cyt-b+CO1 concatenated data for *Merlangius merlangus* populations. The node values correspond to bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

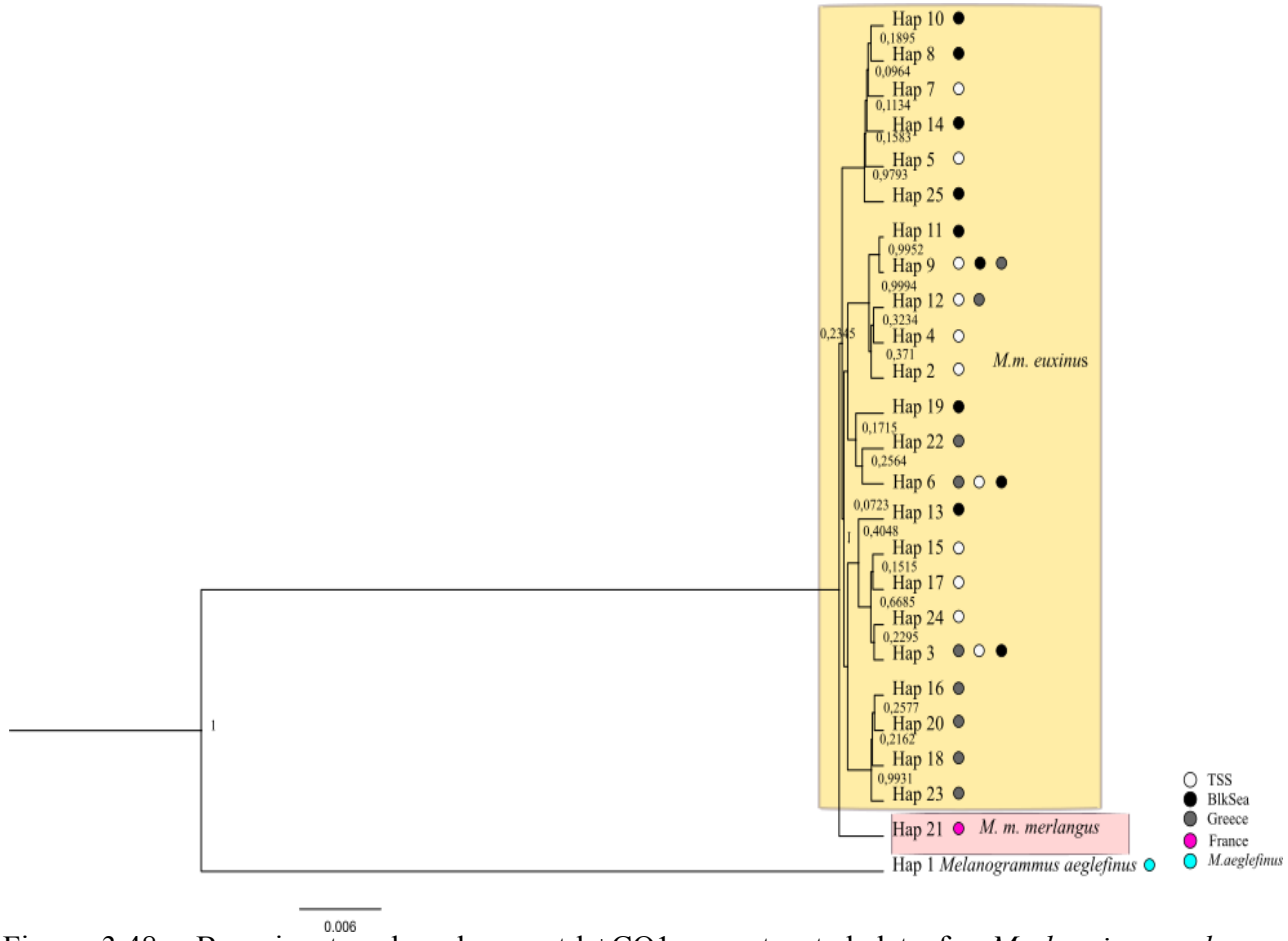


Figure 3.48. Bayesian tree based on cyt-b+CO1 concatenated data for *Merlangius merlangus* populations. The node values correspond to posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

As shown all the phylogenetic trees, *M. m. euxinus* haplogroups from Turkey and Greece were clearly differentiated (except Hap 19, see below) from the *M. m. merlangus* haplotype from France, in a parallel manner to the CO1 and cyt-b partial fragments, analyzed above. In the maximum likelihood (Figure 3.45), neighbour joining (Figure 3.46) and maximum parsimony (Figure 3.47) trees, Hap 19 (a sample from Black Sea/İnebolu) separated from the other Turkish *Merlangius merlangus euxinus* populations. On the other hand, as can be seen in the Bayesian tree (Figure 3.48), Hap 19 was clustered with *Merlangius merlangus euxinus* haplogroups from Turkey and Greece. The results of the Bayesian tree make more sense from a geographical perspective, with regards to the clustering of Hap 19.

3.2.4. Nuclear DNA (RAG1 gene)

A 346 bp fragment of the RAG1 gene was amplified in a total of 173 individuals from the Turkish coastal waters and Greece, and two individuals from France (Appendix Table F.2). Two sequences of RAG1 ((KP644393.1; Atlantic, (Owens, 2015) and (FJ215265.1; Atlantic, Varon and Orti, 2009)) from GenBank were also used. *Melanogrammus aeglefinus* was used in all phylogenetic analyses as an outgroup.

In total, 23 haplotypes were found in 176 individuals. Phylogenetic relationships between the haplotypes are presented as a haplotype network (Figure 3.49), as well as maximum likelihood, neighbour-joining, maximum parsimony, and Bayesian trees (Figures 3.50-3.53). As opposed to mtDNA results of *Merlangius merlangus,* discrimination between Atlantic-France and the Black Sea-Greece-TSS populations was not observed in the star-shaped haplotype network of the RAG1 gene (Figure 3.49). The network shows that the most common haplotype (Hap 1, found in 70 % of all individuals) was found in all of the investigated populations. Low to moderate bootstrap and posterior probability values were observed in the phylogenetic trees obtained by maximum likelihood, neighbour-joining, maximum parsimony and Bayesian analyses, as the most common haplotypes were dispersed among populations, and also possibly due to the low mutation rates of the RAG1 gene.

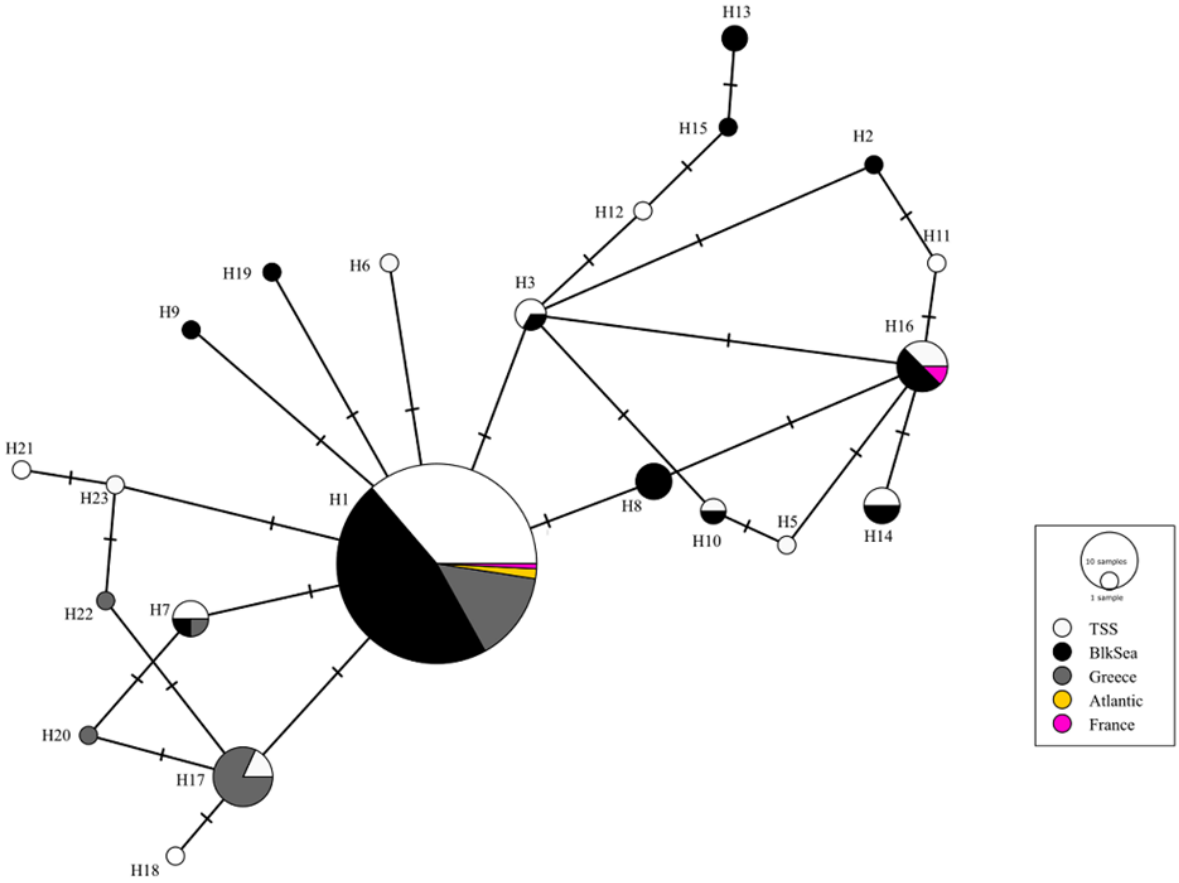


Figure 3.49. The haplotype network for the RAG1 gene of *Merlangius merlangus*. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend. Each line represents a single mutational change.



Figure 3.50. Maximum likelihood tree based on RAG1 data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

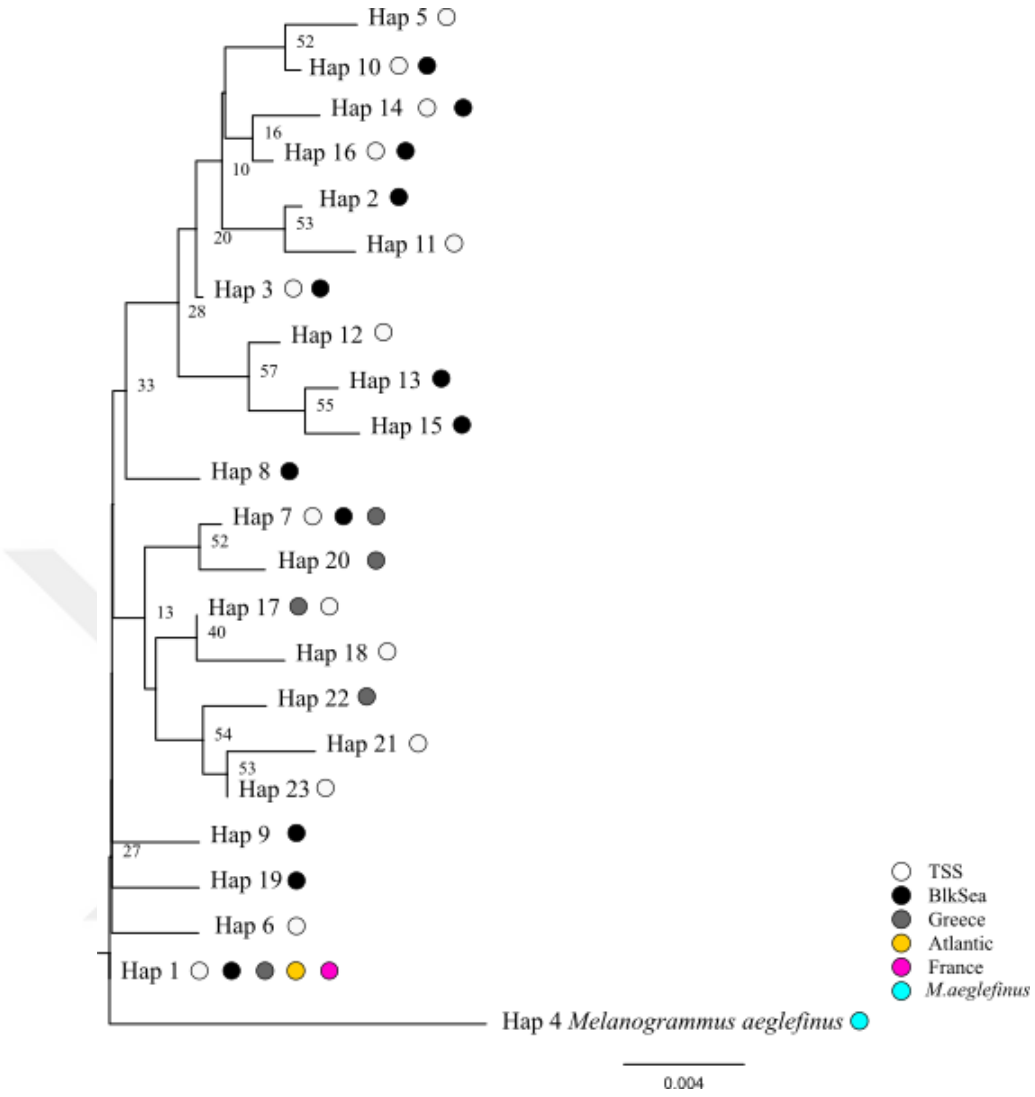


Figure 3.51. Neighbour-joining tree based on RAG1 data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

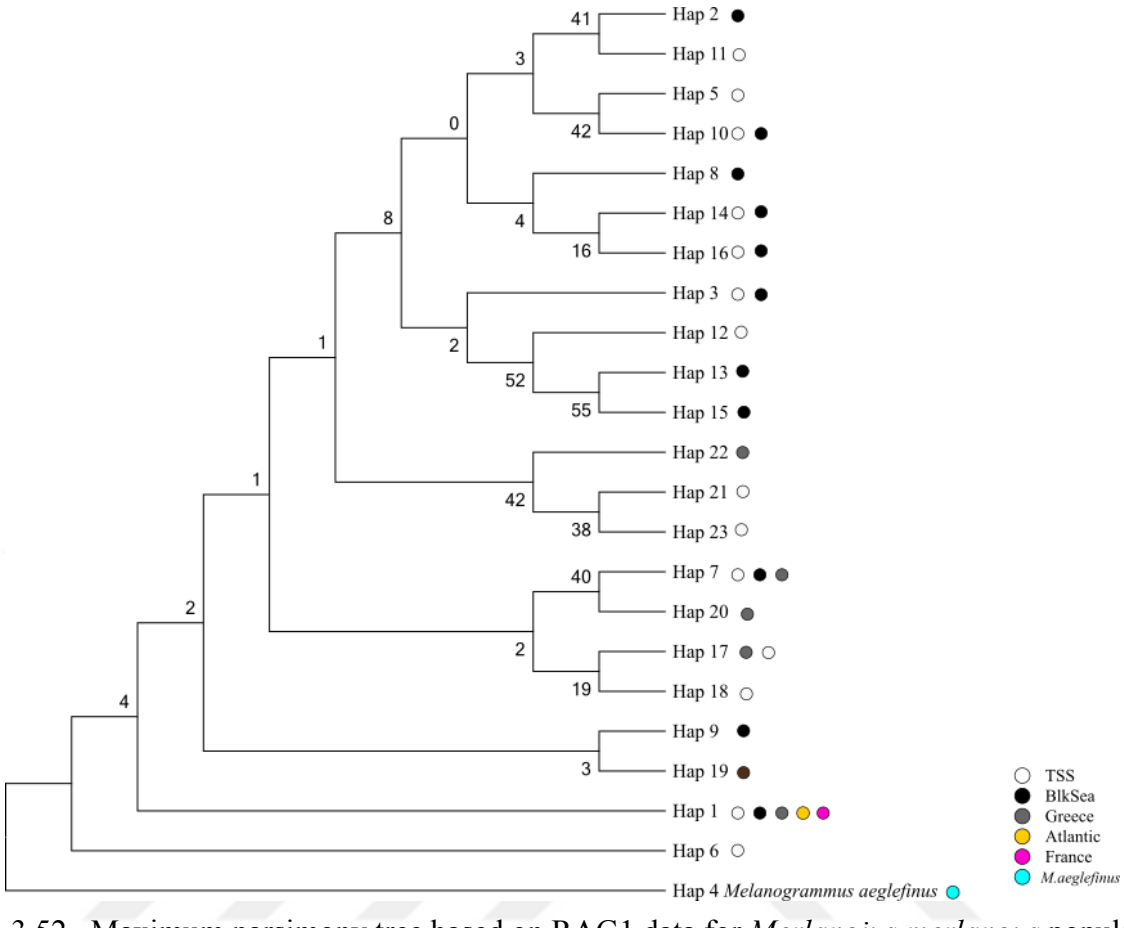


Figure 3.52. Maximum parsimony tree based on RAG1 data for *Merlangius merlangus* populations. The node values represent bootstrap support. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.



Figure 3.53. Bayesian tree based on RAG1 data for *Merlangius merlangus* populations. The node values represent posterior probabilities of support for the branches. Locations of the different haplotypes are given as circles, with each sampling locality indicated by different colors as given in the legend.

Molecular diversity indices of the RAG1 gene for all *Merlangius merlangus* populations are given in Table 3.26. Low to moderate mean haplotype and nucleotide diversities of *Merlangius merlangus euxinus* were found in eight sampling locations (h=0.494; $\pi = 0.00254$). On the other hand, the highest haplotype and nucleotide diversities were detected in the İnebolu population (h=0.705; π=0.00400) from the Black Sea. The Aegean Sea (Greece) population had the highest haplotype and nucleotide diversities, when compared to other Turkish coastal water populations (h=0.566; π=0.00195). As opposed to the mtDNA, haplotype and nucleotide diversities of the nuclear DNA (nuDNA) of the Atlantic and France populations were relatively low (h= 0.5; π= 0.00289), possibly due to the low population sizes of Atlantic and France populations (n=4), and again the slower evolution rate of the RAG1 gene when compared to that of the mtDNA markers. Considering parsimony informative sites, a total of 27 (7.8 %) nucleotide sites were variable, of which nine (2.6

%) positions were parsimony informative, when compared including the outgroup. The highest number of singleton and parsimony informative sites were found in the TSS.

Table 3.26. Molecular diversity of *Merlangius merlangus* based on the RAG1 gene sequences. N: Number of samples; Nh: Number of haplotypes; Np: Number of polymorphic sites; h: Haplotype diversity; π: Nucleotide diversity; SS: Singleton variable sites; PIS: Parsimony informative sites. Standard deviations are given in parantheses.

Sampling sites	N	Nh	Np	h	π	SS	PIS
İstanbul	20	8	7	0.647 (0.120)	0.00380 (0.00095)	3	4
Çanakkale	24	5	5	0.438 (0.121)	0.00186 (0.00063)	2	3
Bandırma	20	5	4	0.442 (0.133)	0.00281 (0.00091)	1	3
Rize	25	2	1	0.080 (0.072)	0.00023 (0.00021)	1	0
Sinop	19	4	4	0.380 (0.134)	0.00199 (0.00076)	2	2
Zonguldak	20	6	6	0.637 (0.115)	0.00368 (0.00096)	1	5
İnebolu	13	5	5	0.705(0.122)	0.00400(0.00101)	3	2
Greece	30	5	3	0.566 (0.074)	0.00195 (0.00034)	1	2
M. m. euxinus	**171**	**22**	**14**	**0.494 (0,047)**	**0.00254 (0,00031)**	**5**	**9**
TSS	**64**	**14**	**11**	**0.505 (0.077)**	**0.00282 (0.00053)**	**5**	**6**
Black Sea	**77**	**12**	**10**	**0.430 (0.071)**	**0.00229 (0.00045)**	**5**	**5**
Aegean	**30**	**5**	**3**	**0.566 (0.074)**	**0.00195 (0.00034)**	**1**	**2**

3.2.4.1. Population structure of Merlangius merlangus according to the RAG1 gene. Pairwise F_{ST} values were calculated among *M .m. euxinus* subpopulations from the Turkish coastal waters and Greece (Table 3.27), since the two subspecies of *M. merlangus* were not separated from each other, neither in the haplotype network nor the phylogenetic trees. In addition, AMOVA was undertaken for the Black Sea, TSS and Aegean subpopulations. F_{ST} values were low to moderately high among populations from the Black Sea, TSS and Greece. The highest and significant F_{ST} values were detected between İnebolu (Black Sea) and Greece, and between İnebolu and Rize (Black Sea) populations (F_{ST}= 0.25). Moreover, significant F_{ST} values were also observed between İstanbul (TSS) and Rize (F_{ST}= 0.13), Bandırma (TSS) and Rize (F_{ST} = 0.12), and Çanakkale (TSS) and İnebolu (F_{ST}= 0.07) populations. Pairwise F_{ST} values between Greece and all other locations were found to be significant and ranged from 0.11 to 0.25. These low to moderately high, but significant values of F_{ST} in comparisons with the TSS suggest that the system cause some partial restriction to gene flow. However, AMOVA results showed that distribution of variation was low among groups (4.19 %) and among populations within groups (4.4 %), whereas the variation within populations was 91.35 %.

Table 3.27. Pairwise F_{ST} values of RAG1 gene for *M. m. euxinus*. Significant *P* values are given in shaded cells (< 0.05).

		İstanbul	**Çanakkale**	**Bandırma**	**Rize**	**Sinop**	**Zonguldak**	**İnebolu**	**Greece**
TSS	**İstanbul**	0.00							
	Çanakkale	0.04	0.00						
	Bandırma	0.03	0.01	0.00					
BlkSea	**Rize**	**0.13**	0.01	**0.12**	0.00				
	Sinop	-0.00	-0.01	0.00	**0.07**	0.00			
	Zonguldak	0.01	0.01	-0.01	**0.10**	-0.00	0.00		
	İnebolu	-0.01	**0.07**	0.03	**0.25**	0.02	-0.02	0.00	
Aeg	**Greece**	**0.18**	**0.12**	**0.05**	**0.23**	**0.18**	**0.11**	**0.25**	0.00

3.2.5. Demographic Analyses of *Merlangius merlangus* mtDNA and Nuclear DNA

Mismatch analyses were carried out based on the mtDNA and nuDNA of *Merlangius merlangus* populations, and for three different geographical locations along the Turkish coasts. Two neutrality tests (Tajima's D, Fu's F_S) and raggedness statistic (rg) were used to reconstruct the population demographic history of *Merlangius merlangus*. These neutrality test parameter values of two mtDNA genes (CO1 and cyt-b) and one nuclear gene (RAG1) for each *Merlangius merlangus* population are presented in Table 3.28. Mismatch distributions for inferred populations of *Merlangius merlangus* under a sudden demographic expansion model are also given in Figures 3.54-3.56.

Table 3.28. Neutrality test results for *M. m. euxinus* and *M. m. merlangus*. N: Number of samples; D: Tajima's D; F_S: Fu's F_S; and rg: raggedness statistic. Significant values ($P< 0.05$) are indicated in bold.

	Sampling sites	N	D	F_S	rg
CO1 gene	**Total *M. merlangus***	**164**	**-2.01481**	**-11.618**	0.5059
	TSS	**60**	**-1.90219**	**-2.889**	0.6123
	Black Sea	**68**	**-2.03782**	**-6.463**	0.3715
	Aegean	**36**	-0.81338	-0.597	0.6265
	M. m. euxinus	**161**	**-1.90397**	**-10.943**	0.5447
	M. m. merlangus	**25**	0.38462	-2.047	**0.0356**
Cyt-b gene	***M. m. euxinus***	**125**	-1.44897	**-13.305**	0.0720
	TSS	**41**	-0.57616	**-4.087**	0.0587
	Black Sea	**52**	-1.00958	-3.412	0.0614
	Aegean	**32**	-1.33597	**-4.023**	0.0620
	M. m. merlangus	**11**	-0.53992	-2.173	0.0476
RAG1 gene	***M. m. euxinus***	**171**	**-1.64338**	**-22.814**	0.0940
	TSS	**64**	**-1.62288**	**-10.992**	0.1064
	Black Sea	**77**	**-1.62542**	**-8.831**	0.1740
	Aegean	**30**	-0.25296	-1.666	0.1349

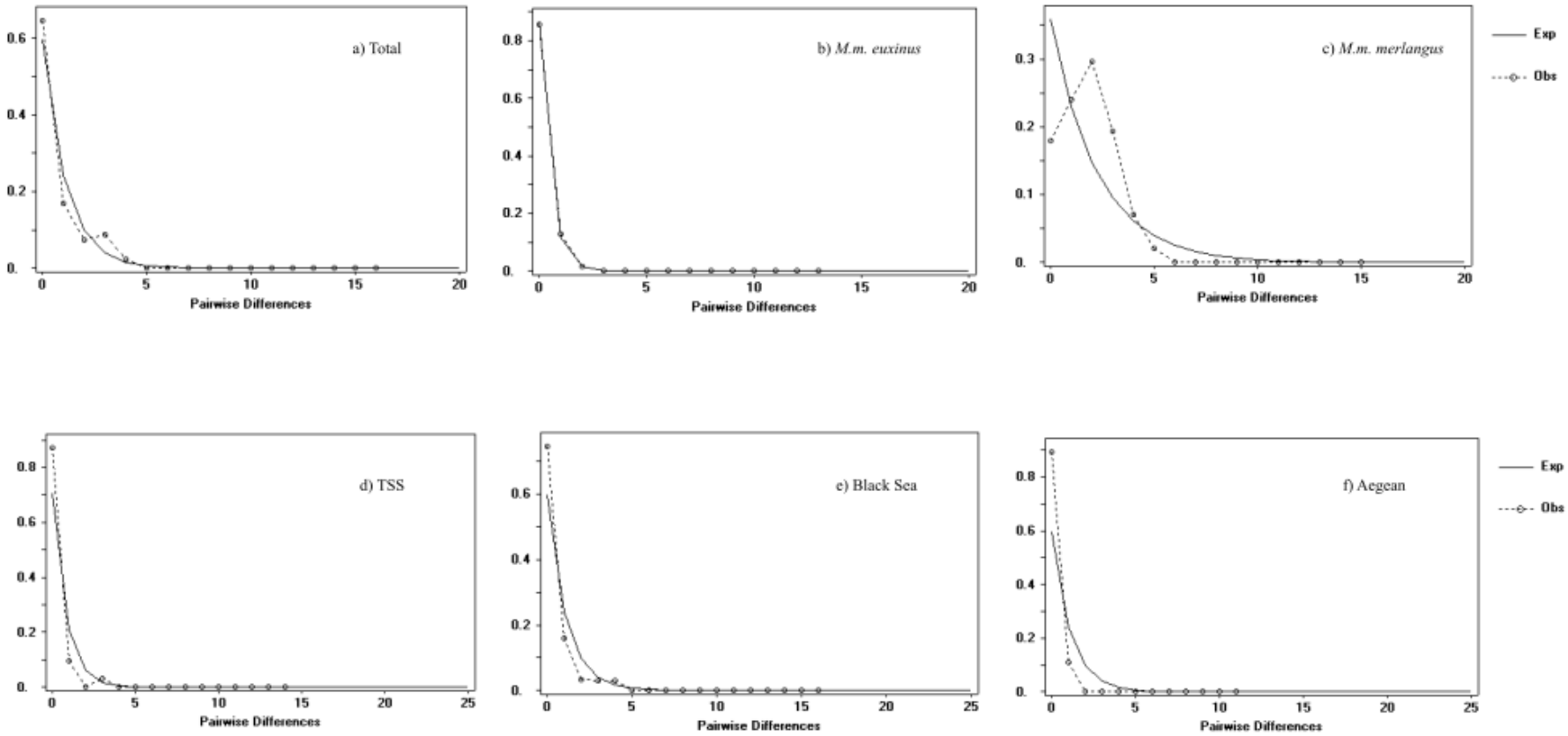


Figure 3.54. Mismatch distributions based on CO1 gene for a) Total, b) *M. m. euxinus*, c) *M. m. merlangus*, d) TSS, e) Black Sea, f) Aegean populations. The solid lines represent the expected distribution and the dotted lines represent the observed distribution, under the sudden expansion model. Y-axis represents the relative haplotype frequencies.

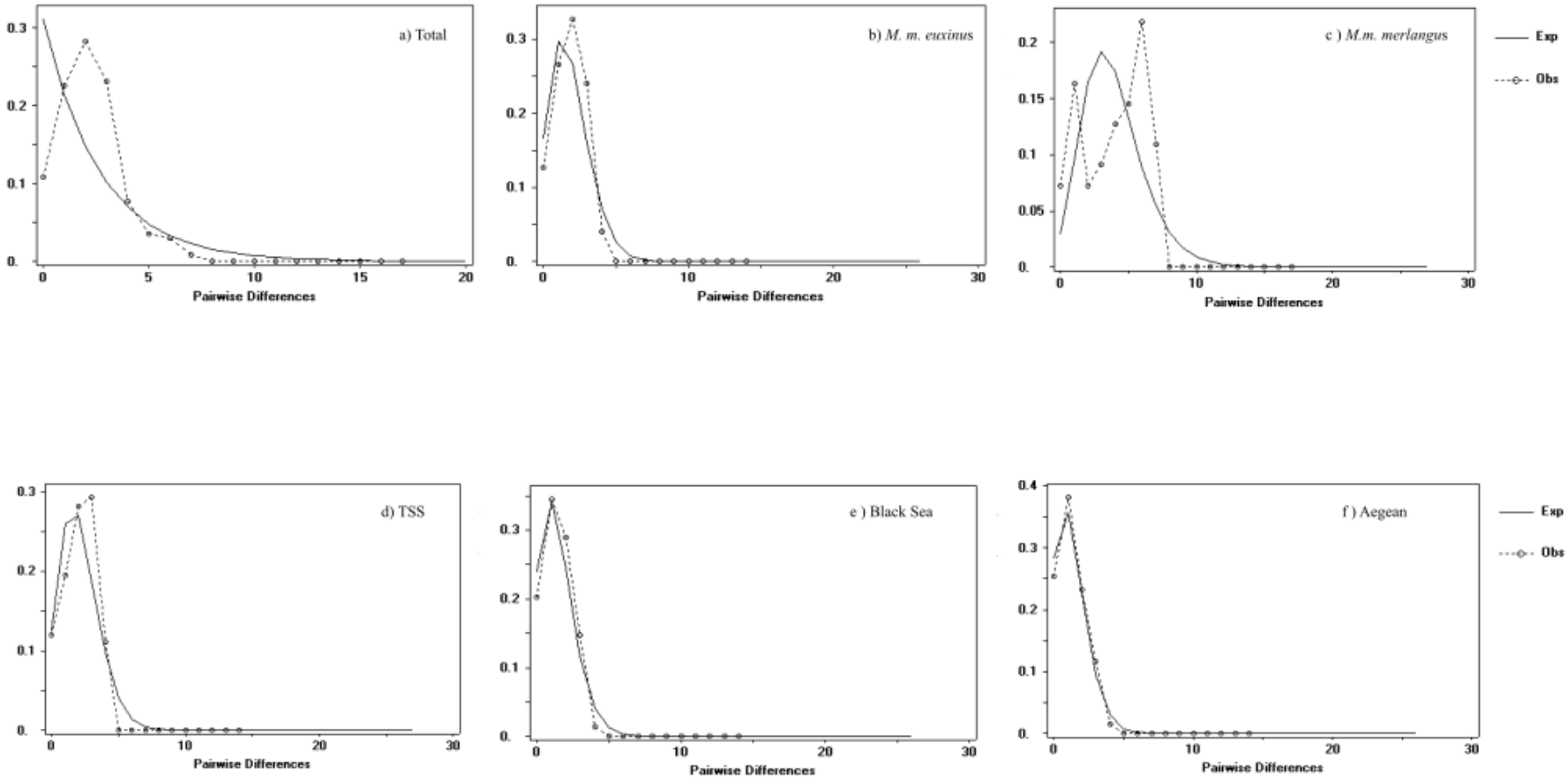


Figure 3.55. Mismatch distributions based on cyt-b gene for a) Total, b) *M. m. euxinus,* c) *M. m. merlangus,* d) TSS, e) Black Sea, f) Aegean populations. The solid lines represent the expected distribution and the dotted lines represent the observed distribution, under the sudden expansion model. Y-axis represents the relative haplotype frequencies.

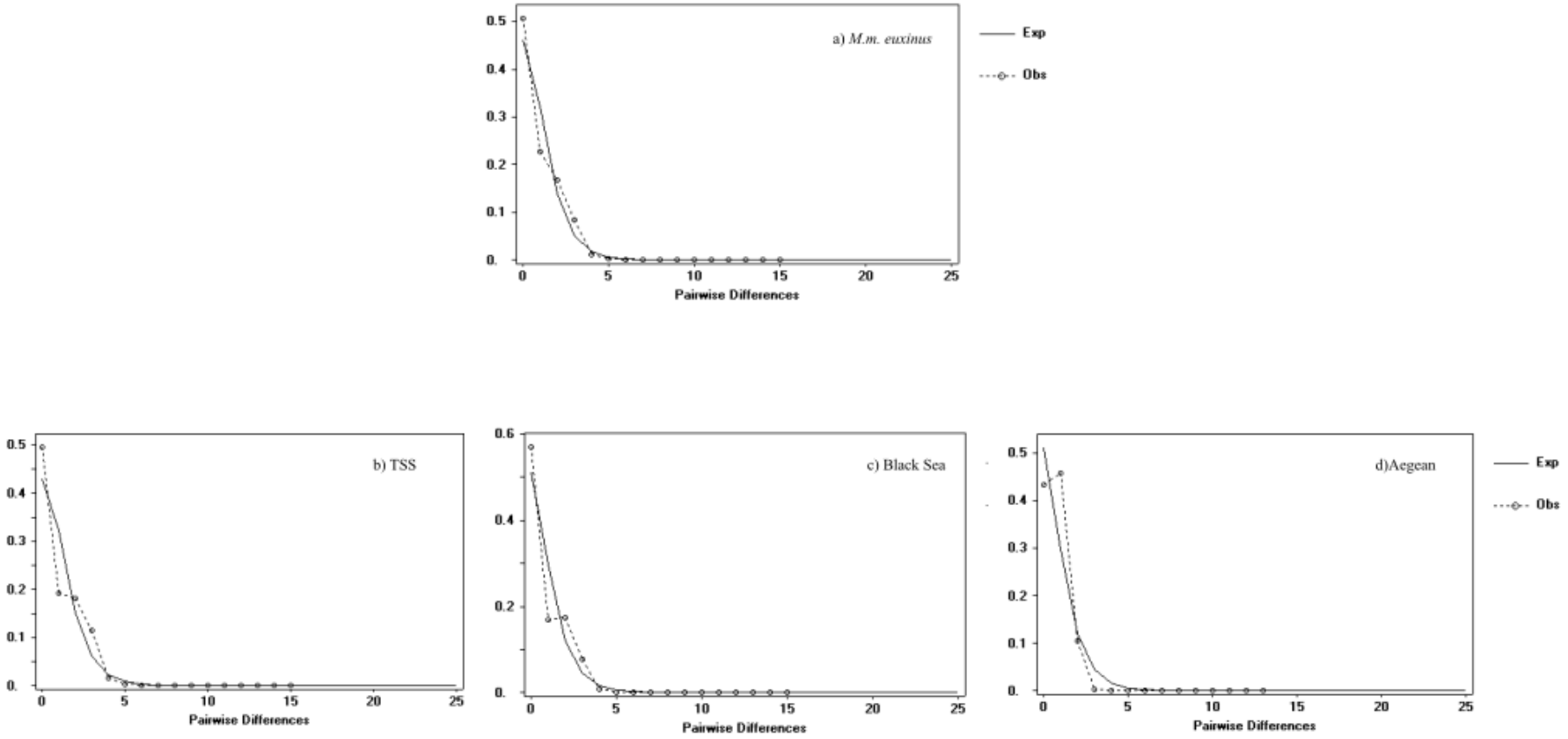


Figure 3.56. Mismatch distributions based on RAG1 gene for a) Total, b) *M. m. euxinus,* c) TSS, d) Black Sea, e) Aegean populations. The solid lines represent the expected distribution and the dotted lines represent the observed distribution, under the sudden expansion model. Y-axis represents the relative haplotype frequencies.

Overall values of neutrality tests of the three different genes showed that *M. m. euxinus* populations had unimodal mismatch distributions (Figure 3.54b, 3.55b, 3.56a), and experienced a recent demographic expansion which was also indicated by negative and significant F_S and D values (Table 3.28). In addition, TSS, Black Sea, and Aegean sub-populations of *M. m. euxinus* had clearly uni-modal mismatch distributions (Figures 3.54, 3.55 d, e, f, 3.56 b, c, d). For these subpopulations, neutrality test values (F_S and D) were all negative, and also supported the population expansion model (Table 3.28). However, considering *M. m. merlangus*, multi-modal or ragged mismatch distributions were observed for CO1, cyt-b, suggesting stable populations (Figure 3.54c, 3.55c). The value for Tajima's D was positive for the CO1 gene, supporting the results of the mismatch distributions (Table 3.28). For *M. m. euxinus*, raggedness statistic (rg) values were all non-significant, and the population expansion model was not rejected (Table 3.28). For *M. m. merlangus* populations, however, the significant raggedness statistic (rg) detected for the CO1 gene helps reject the population expansion model. Considering the cyt-b gene, however, a non-significant raggedness statistic (rg) value was observed, and the population expansion model was not rejected for *M. m. merlangus.*

Demographic analysis of *M. m. euxinus* populations based on the Bayesian skyline analysis is shown in Figure 3.57. As can be seen in Figure 3.57, the total population seems to have remained relatively constant from 40 Kya to 30 Kya. However, a population expansion for *M. m. euxinus* started around 30 Kya in the eastern Mediterranean.

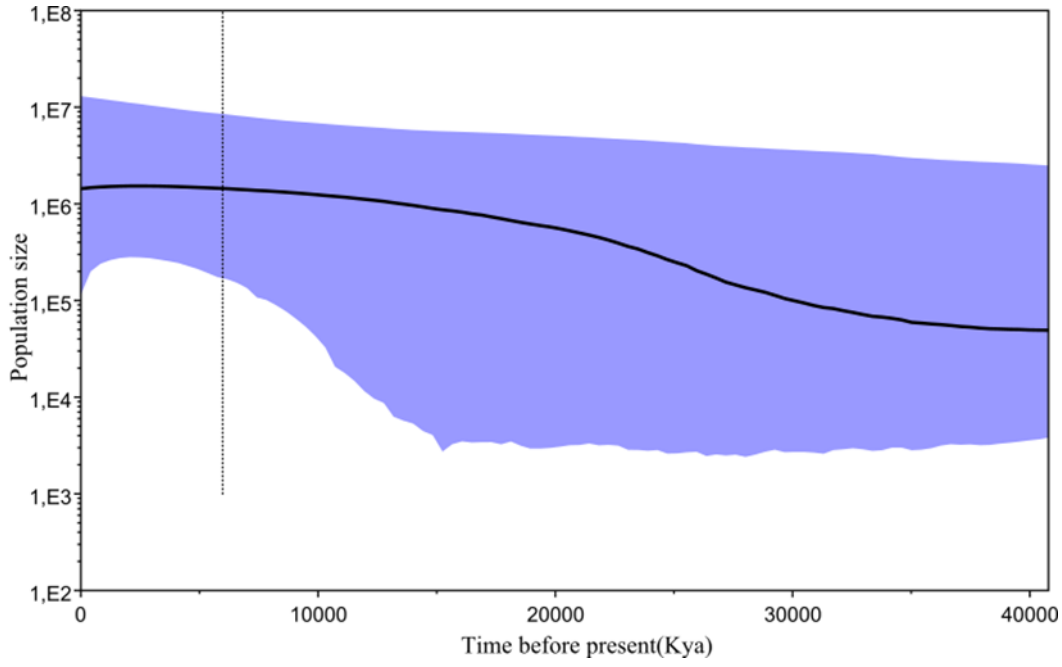


Figure 3.57. Bayesian skyline plot for *M. m. euxinus* reflecting changes effective population size against time in thousands of years (Kya) before present.

3.2.6. Discussion on Mitochondrial Phylogeography and Nuclear DNA Structure of *Merlangius merlangus*

In the present study, the phylogeography of the whiting, *Merlangius merlangus* (a Gadoid fish) (sensu lato) was investigated with data from the Turkish coastal waters, Aegean Sea and the Atlantic region. As mentioned above, *M. merlangus* is widely distributed in the Atlantic and adjacent waters, and is represented by two subspecies: *M. m. euxinus* in the Adriatic, Mediterranean and Black Sea, and *M. m. merlangus* in the eastern Atlantic and with a restricted distribution in the western Mediterranean (Svetnovidov, 1986; Quero and Vayne, 1997). Fishes from the Gadidae family are probably the most studied group for the investigation of interconnected roles of geophysical barriers and physiological tolerance in allopatric diversification (Coulson et al., 2006). Gadoid fish are euryhaline and eurythermal organisms that can adapt to different ecological conditions, but they mostly occur in sub-arctic and temperate waters (Cohen et al., 1990). Low level of genetic differentiation was observed in Gadoid fish such as hake, *Merluccius merluccius* (Lundy et al., 1999) in the Atlantic waters, findings consistent with a high level of transport of pelagic eggs and larvae. On the other hand, genetic structure was previously detected in *M. merlangus* due to geophysical barriers and oceanographic conditions in the Atlantic (Rico et al., 1997; Charrier et al., 2007).

Phylogenetic trees obtained with four approaches, and stand-alone and concatenated CO1 and cyt-b data revealed that *M. m. euxinus* from Black Sea and Aegean, and *M. m. merlangus* from Atlantic were separated from each other, and no haplotypes were shared between the two subspecies. However, phylogenetic trees from concatenated CO1 and cyt-b data (maximum likelihood (Figure 3.45), neighbour-joining (Figure 3.46), maximum parsimony (Figure 3.47)) showed that Hap 19 (a sample from Black Sea/İnebolu) separated from the other Turkish *Merlangius merlangus euxinus* populations. On the other hand, as observed in the Bayesian tree (Figure 3.48), Hap 19 was clustered with *Merlangius merlangus euxinus* haplogroups from Turkey and Greece. The results suggest that Bayesian tree might have performed better than the other methods for the phylogenetic reconstructions. Some previous studies (e.g. on dwarf boas (Wilcox et al., 2002), land plants (Karol et al., 2001) and some mammalian genes (Suziki et al., 2002)) also indicated that phylogenetic reconstructions using the Bayesian method performed better than other methods, such as maximum likelihood, neighbour-joining and maximum parsimony analyses, and here we see another example of the greater reliability of Bayesian phylogenetics inference.

Mitochondrial DNA analyses also showed the different mutational dynamics, and exhibited past isolation of *M. merlangus* resulting in the two distinct subspecies. Significant genetic differentiation and reciprocally monophyletic clades of sand goby (*Pomatoschistus minutus*) populations were also found between Atlantic, western and eastern Mediterranean (Adriatic) populations by using mt DNA (D-loop sequences) (Steffani and Thorley, 2003), and between eastern Atlantic and western Mediterrenean populations in the bathyal bony fish (*Coryphaenoides mediterraneus)* based on CO1 sequences (Catarino et al., 2017). Moreover genetic differentiation was also observed between several other fish species between Atlantic, western and eastern Mediterranean, such as sea bass (*Dentex dentex*) (Bargelloni et al., 2003); striped seabream (*Lithognathus mormyrus*) (Bargelloni et al., 2003), Pacific chub mackerel (*Scomber japonicus*), Atlantic mackarel (*Scomber scomborus*) (Zardoya et al., 2004*),* sword fish (*Xiphias gladius*) (Alvarado Bremer et al., 1995; Kotoulas et al., 1995; Rosel and Block, 1996) and other marine organisms such as, common shrimp (*Crangon crangon*) (Luttikhuizen et al., 2008) and common prawn (*Paleomon serratus*) (Weiss et al., 2017), lagoon cockle (*Cerastoderma glaucum*) (Nikula and Vainola, 2003) and Black Sea harbour porpoise (*Phocoena phocoena*) (Viaud-Martínez et al., 2007; Viaud- Martinez et al., 2008; Fontaine et al., 2007). All of the studies mentioned above showed that Strait of Gibraltar played a barrier role for the phylogeographical differentiation of these species. Here, we see another example the Strait of Gibraltar acting as a phylogeographic break for the differentiation between *M. m. euxinus* and *M. m. merlangus.*

A total of 13 and 34 mutations were observed between two subspecies in the CO1 and cyt-b genes, respectively. However, nucleotide substitution values of CO1 (Dxy=0.0047); and cyt-b genes (Dxy=0.0073) were relatively low between the two subspecies, an observation consistent with the findings of distribution of genetic diversity within subspecies of other marine fish, e.g., *Mullus spp.,* (Keskin and Can, 2009), and within other fish species, e.g. *Trachurus spp*., (Bektaş and Beldüz, 2008), *Alosa spp.,* (Turan et al., 2015a), and *Thunnus spp*. (Seyhan and Turan, 2016). Moreover, two per cent genetic distance based on the barcoding data (CO1 gene) was reported as a threshold value for differentiation at the species level (Avise, 2000; Ratnasigham and Habert, 2007). In the present study, genetic distance of about 0.3 % was found between the two subspecies of *Merlangius merlangus* as previously observed between subspecies of *Diplodus sargus* (Karahan et al., 2017). In the same study, distances among other species such as *Trachurus*, *Sardinella* and *Fistuleria* were found to be smaller than the 2 % threshold value. Zang and Hanner (2011) found 0.3 % of genetic distances of CO1 identification among conspecifics. Therefore, the genetic distance of 0.3 % supports

the idea that the *Merlangius* taxa sampled in this study belong to the same, rather than different, species.

Stop-codons in the cyt-b sequences indicate that Numts were amplified instead of the intended mitochondrial fragments. Numts are mtDNA-like sequences in the nuclear genome of many organisms, and they can be inadvertently amplified during PCR. In animals, Numts lose their function upon transfer into the nucleus during the repair of chromosomal breaks through non-homologous recombination. However, Numts are not necessarily uninformative. On the contrary, they are ideal loci that can be used to understand molecular ecology and evolution of species (Bensasson et al., 2001; Keller et al., 2007; Lafontaine and Dujon, 2010; Zhang and Hewitt, 1996). Effective phylogenetic signals from Numts were previously observed in other marine organisms, such as crustaceans (Williams and Knowlton, 2001, Buhay, 2009), tunicates (Richly and Leister, 2004), and fishes (Antunes and Ramos, 2005). Also, a previous study showed that Numts had been inadvertently amplified instead of the targeted cyt-b genes in freshwater crayfish (Nguyen et al., 2002). The presence or absence of Numts at specific loci can also be used to determine the phylogenetic branching order of different species (Bensasson et al., 2001). As in the case of *Merlangius merlangus*, results based on the CO1 gene (mitochondrial) and cyt-b genes (Numts) were parallel to each other. Although Numts were detected in the cyt-b gene, with a higher numbers of the mutations between two *Merlangius merlangus* subspecies, its discriminatory power was better, when compared to CO1.

Considering molecular genetic diversity indices, results of the mtDNA analyses revealed that haplotype and nucleotide diversities ($h=0.149$; $\pi=0.00035$) of the CO1 gene in the *M. m. euxinus* (Black Sea, TSS and Aegean Sea) populations were lower than those in *M. m. merlangus* (Atlantic) ($h= 0.820$, $\pi=0.00373$). Low level of the haplotype and nucleotide diversities were also observed in other studies, as well. For instance, low genetic diversities were reported by Debes et al. (2008) and Limborg et al. (2012) in the Mediterrenaen and the Black Sea, for the European sprat (*Sprattus sprattus*). Suziki et al. (2004) found two distinct lineages in the western Mediterranean and eastern Mediterranean turbot (*Scophthalmus maximus*) populations and suggested that the eastern Mediterranean lineage showed lower genetic variability than the western one, potentially due to a population bottleneck. Other marine species populations also exhibited low level of genetic diversities, such as Black Sea harbour porpoises, where the low diversities are considered to be due to recent founder events, pollution and illegal fishing (Viaud-Martínez et al., 2007; Viad-Martinez et al., 2008; Fontaine et al., 2007). Low levels of diversities were also seen bivalve Black Sea populations of lagoon cockle (*Cerastoderma glaucum*) (Nikula and Vainola, 2003) when compared

to the Atlantic-Mediterranean phylogroups, due to recent colonization and post glacial generation in the Black Sea. In this study, *M. m. euxinus* species in the eastern Mediterranean showed lower genetic variability than the *M. m. merlangus* in the Atlantic due to a potential population bottleneck in the last glacial period in the Black Sea, as well as in the eastern Mediterranean.

Regarding the origin of the extant Black Sea species, fish such as sturgeons (Acipenseridae) and crustaceans such as *Pontogammarus* are considered to be the oldest inhabitants before the formation of the Black Sea. *M. m. euxinus* is considered to be a member of the second oldest group to have dispersed into the Black Sea with origins in the Northeastern Atlantic during the period of the Neoeuxian Lake (Zaitsev and Mamaev, 1997). Mismatch distributions of the mitochondrial genes all being unimodal and Bayesian Skyline Analysis, indicate a demographic expansion for *M. m. euxinus* populations in the TSS and Black Sea, consistent with a dispersal scenario as outlined by Zaitsev and Mamaev (1997). *M. m. merlangus* populations from the Atlantic, on the other hand, with the patterns of the haplotype networks, relatively high genetic diversity levels, and multi-modal mismatch distributions indicate a long population history and presence of stable populations for long enough for the formation of new haplotypes.

Considering origin of Gadine fishes, previous studies in the literature (Carr et al., 1999; Pogson and Mesa, 2004; Coulson et al., 2006) revealed that North Eastern Atlantic was the most likely origin of these species. The role of glacial refugia in the North East Atlantic (Provan, 2013) and closing of the Strait of Gibraltar and subsequent Messinian Salinity Crisis (MSC) (between 6.0 and 5.3 Mya) (Gonzalez et al., 2012), during which the Mediterranean Sea experienced intense desiccation, may have driven the speciation of Gadine fishes. Previous studies revealed that the genus Gadus began to diversify during the Pleistocene, when fluctuating levels of sea ice may have led to episodic ice-free passages between the Atlantic and Pacific (O'Regan et al., 2011). These results suggest that glaciation cycles were the main reasons behind the diversification of Gadine species. Considering *Merlangius merlangus*, past glaciation cycles seems to have affected its diversification and distribution in the Atlantic, Mediterranean and the Black Sea. In this perspective, I propose the following scenario for diversification of *Merlangius merlangus*: ancestral *Merlangius merlangus* was differentiated from *Melanogrammus aeglefinus* approximately 7-8.5 millon years ago (Bakke and Johensen, 2005) in the eastern Atlantic. After the MSC, ancestral *Merlangius merlangus* (Boreal Atlantic relicts) entered the Mediterranean in the Pleistocene glacial, since the Mediterranean may have been a refugium for moderately cold temperate and euryhaline Atlantic species, which would have found suitable environmental conditions for their survival as observed for other fish species in the Atlantic, e.g.

Sprattus sprattus, *Sygnathus tenuirostris*, *Platichthys flesus luscus, Aphanius spp*. and *Gobius minutus* (Ekman, 1967). It is possible that, during the interglacial periods, this ancestral stock expanded to the eastern Mediterranean, as well. The estimated divergence times among *M. m. merlangus* from the Atlantic and *M. m. euxinus* from the Black Sea indicate that they began to diverge during the late Pleistocene (125 Ky BP, in the Riss-Würm interglacial (Karangatian)) based on the CO1 gene, in the Atlantic/western Mediterranean and the eastern Mediterranean, respectively. Transgression of the Black Sea occurred during the Middle Wirm interstade (40- 25 Ky BP) or Upper Wirm Glacial (25 Ky BP), and *M. m. euxinus* might have expanded into the Black Sea in this period, as indicated in the Bayesian Skyline Analysis. The actual geographic dispersal to the Black Sea might have taken place at a later time as well, after the last opening of the Dardanelles approximately 10 Ky BP (Aksu et al., 2002), and when the Mediterrenean and the Black Sea got connected. *M. m. euxinus* might have been able to disperse into the Aegean Sea first, and subsequently into the Black Sea.

In terms of population differentiation, pairwise Φ_{ST} values showed that significant and high levels of genetic differentiation were found between *M. m. merlangus* and *M. m. euxinus* populations for both CO1 and cyt-b genes (Φ_{ST}=0.95 and Φ_{ST}=0.77), respectively. Significant genetic differentiation was also observed among subpopulations of *M. m. euxinus* based on the cyt-b analysis. High and significant Φ_{ST} values were observed between Çanakkale and four populations from the Black Sea. Moreover, pairwise Φ_{ST} values between Greece and all other locations were significant, and had moderate to high values (0.10 to 0.28). For different marine species, TSS can be both a corridor and a barrier to gene flow (Öztürk and Öztürk, 1996). High values of mtDNA molecular diversity indices and significant genetic structure between subpopulations suggest that the TSS is a barrier, rather than a corridor to gene flow, for this species.

Another interesting set of results from the CO1 gene was that a total of 11 specimens from Black Sea and the TSS were clustered with *M. m. merlangus* samples in the Atlantic. Eight of these sequences were from the Black Sea (retrieved from GenBank), two of them were collected from Rize, one was collected from Çanakkale, in this study. It is likely that their presence could be due to anthropogenic disturbances such as ballast water discharge. Although *M. merlangus* individuals were not detected previously in ballast tanks, a considerable number of marine organisms such as, invertebrates, copepods (Bowman, 1978), zooplanktonic and phytoplanktonic samples (Turner, 1966, Turner and Johnson, 1971) and even fish (e.g. blennies and gobies) (Bertelsen and Ussing, 1936; Wonham et al., 2000), and fish eggs and larvae were found in the ballast tanks (Carlton, 1985).

According to literature, fish survived an average of 21 days in 16 vessels (Wonham et al., 2000). Of all the reported 28 fish collections in ballast waters (Wonham et al., 2000), eight were larval specimens. Unidentified fish larvae were reported in ballast waters from Israel to USA (Wonham et al., 2000), eggs and larvae from Florida to Corpus Christi in the US (Carlton et al., 1982), and from Italy to the US (Smith et al., 1999). Physiological characteristics of fish such as high-density schooling behavior might be another reason for their survival in ballast tanks (e.g. Clupeid and Gadoid species from Northeast Atlantic) (Wonham et al., 2000). Moreover, it should be noted that the larval stages of *Merlangius merlangus* is long enough (greater than one month) (Fischer et al., 1987), for transoceanic and interoceanic dispersal from the Atlantic to the Black Sea. Thus, these 11 *M. m. merlangus* individuals that we detected in the Black Sea and the TSS could have been transported as larvae or fish eggs, or even potentially as adults in the ballast tanks.

Nuclear DNA analysis results of *M. merlangus* were indicative of recent population structuring of the species. Nuclear DNA results also somewhat supported the results from mtDNA, even though the patterns were not as obvious. As opposed to mtDNA results, *M. m. euxinus* and *M. m. merlangus* samples from the Atlantic, Black Sea, and TSS populations could not be distinguished in the haplotype network and phylogenetic trees. This could stem from the slower rate of evolution of the RAG1 compared to that of mtDNA (Martin, 1999). On the other hand, the F_{ST} values among *M. m. euxinus* subpopulations indicated differentiation for this nuclear DNA marker. Pairwise F_{ST} values between Greece and all other locations, including those of *M. m. euxinus* from the Black Sea and the TSS were found to be significant, ranging between 0.11 and 0.25, indicating restriction of gene flow due to the Dardanelles Strait. Moreover, significant F_{ST} values were observed between İstanbul and Rize (F_{ST}=0.13), Bandırma and Rize (F_{ST}=0.12) and Çanakkale and İnebolu (F_{ST}=0.07) populations. Genetic distinctiveness between Greece and the Black Sea localities might be due to various, mutually not exclusive, factors. The genetic structure could be due to different spawning grounds and larval circulation among the whiting populations from North Aegean and Black Sea. In a similar vein, a previous study revealed that different genetic subpopulations of *Dicentrachus labrax* (Bahri-Sfar et al., 2000) in the eastern Mediterranean region such as, Adriatic, Ionian and Aegean Sea was due to different spawning grounds and hydrological conditions.

Philopatry could be another reason for the observed genetic structure. Considering stock structure of the whiting in the North Sea, *M. merlangus* populations were seen to have different stocks based on the conventional tagging experiments (Pilcher et al., 1989; Hislop and MacKenzie, 1976), and these studies reflected limited mixing between adult stocks in the North Sea. In general, whiting

populations in the northern European waters seem to be either sedentary residents or accurate homers (Robichaud and Rose, 2004) and philopatric individuals. In a similar vein, the differences of *M. m. euxinus* populations between the Aegean and the Black Sea could be due to the respective philopatry of these populations, as well.

Finally, the TSS, specifically Dardanelles Strait, could also have been instrumental in the observed differentiation. Focusing on the *M. merlangus*, Dogger Bank in the North Sea has been suggested to act as a barrier that prevents mixing between southern and northern populations of whiting (Pilcher et al., 1989). More recent genetics studies on *Merlangius merlangus* using microsatellites showed significant genetic differences due to Dogger Bank, as well (Rico et al., 1997; Charrier et al., 2007). Significant hydrographical features especially physical barriers (Zheng et al., 2001) may limit the passive dispersal of pelagic eggs and larvae, and there are many examples of this phenomenon around the TSS, such as in red mullet (*Mullus barbatus*) (Keskin and Can, 2009), Atlantic horse mackerel (*Trachurus trachurus*) (Turan et al., 2009a), Mediterranean horse mackerel (*Trachurus mediterraneus*) (Turan et al., 2009b), flathead grey mullet (*Mugil cephalus)* (Durand et al., 2013), and European anchovy (*Engraulis encrasicolus*) (Magoulas et al., 1996 and 2006; Erdoğan et al., 2009 and Keskin and Atar, 2012). TSS, especially Dardenelles Strait was reported to act as a barrier to gene flow from spawning and feeding areas between the Black Sea and the Mediterranean *Sarda sarda* populations based on the mtDNA D-loop analyses (Turan et al., 2015b), and between the Sea of the Marmara and the Aegean Sea *Sarda sarda* samples (Roberti et al., 1993) based on the cyt-b gene. Two potential *Diplodus annularis* stocks were also reported based on the cyt-b gene analyses in the Turkish coasts (Bektaş et al., 2016) as a result of the Dardanelles Strait acting as a barrier to gene flow. TSS was found to act as a barrier to gene flow for the populations of some other marine organisms as well (Öztürk and Öztürk, 1996), such as the mussel (*Mytilus galloprovincialis*) (Kalkan, 2013), sea grass (*Posidonia oceanica*) (Meinesz et al., 2009) and Cetacean harbour porpoise (*Phocena phocena*) (Tonay et al., 2017) and bivalve, lagoon cockle (*Cerastoderma glaucum*), (Nikula and Vainola, 2003). Here, we see another example of a barrier role of the TSS for *M. m. euxinus.*

Whiting in the Turkish coastal waters, especially Black Sea region is considered to be a single stock, based on morphometric and meristic characters (Akşiray, 1954; İşmen, 1995; 2001), and molecular analyses (Bektaş and Beldüz, 2007). Fisheries experts from the Black Sea Commission reported that whiting should be considered as shared stock for the Black Sea (mixing of the eggs and larvae by currents) although the species does not perform long migrations during the spawning

seasons (Radu, 2003). Delimitation of a single stock of whiting in the Black Sea results in a management problem, when the stock is shared by all the countries with coasts along the Black Sea.

In the present study, genetic differentiation of whiting populations suggest that its populations from Black Sea and TSS should be considered as different stocks (though not completely isolated), when compared to those in the Aegean. However, additional analyses are needed, such as identification of spawning areas, feeding behavior, age, size and maturity stages of species for a better understanding of the stock structures of the species, and hence for better management of whiting in these regions.

M. m. merlangus and *M. m. euxinus* species are of high commercial importance around the North East Atlantic and the Black Sea coasts, respectively. Ilegal and unregulated fishing are the main threats that affect their abundance in the Black Sea (Öztürk, 2013; Özdemir et al., 2018). In the Baltic Sea, *M. m. merlangus* is considered as vulnerable, due to a 30 % decline of stocks over the last 12 years (HELCOM, 2013). In the Mediterranean and Black Sea, however, *M. m. euxinus* has been reported in the Least Concern category by the IUCN (2011). However, according to FAO Working Group on the Black Sea (WGBS) report (FAO, 2017), whiting has been overexploited in recently (1994-2015) in the Black Sea. In this study, the results of the genetic analyses showed that whiting populations in the Black Sea, TSS, Aegean (*M. m. euxinus)* were different than those in the Atlantic (*M. m. merlangus*) populations. Moreover, Black Sea and TSS populations were considered as different stocks when compared to those in the Aegean. Therefore, separate conservation measures and monitoring system are necessary for the protection of current stocks of whiting (*M. m. euxinus*), to combat illegal and unregulated fishing in the Turkish coastal waters. Also in future studies, investigating genetic stocks of whiting by high resolution genomics approaches like RAD-seq (restriction site associated DNA sequencing) in the Turkish coastal waters is recommended for more precise stock identification.

4. CONCLUSIONS AND RECOMMENDATIONS

In this study, the effect of the Turkish Straits System (TSS) on the evolutionary history of two marine fish species with wide geographic distributions around the Turkish coasts, namely, *Spicara spp*. and *Merlangius merlangus* was investigated. Based on the results of this study, the main conclusions for each species are as follows:

4.1. *Spicara spp.*

The taxonomy of the genus *Spicara* has been problematic around the Mediterranean region. Multiple studies suggest that *S. maena* and *S. flexuosa* should be considered conspecific, whereas others support the distinction of the two species. In this dissertation, the distinction of three taxa under *Spicara* was made with the help of three different mtDNA markers, namely 16S rRNA, CO1 and cyt-b. The phylogenetic analyses and the haplotype networks using data from these three genes show three units with different evolutionary histories, corresponding to *S. maena, S. flexuosa* and *S. smaris*. Nuclear DNA (nuDNA) analysis results revealed that reproductive isolation of the two *Spicara* (*S. maena* and *S. flexuosa*) species was complete. Considering, *S. flexuosa* populations, low values of mtDNA molecular diversity indices, an absence of genetic structure between subpopulations, and star-shaped haplotype networks indicate the connectivity of the *S. flexuosa* populations from Turkey, suggesting that the TSS is a corridor, rather than a barrier to gene flow, for this species. Moreover, star-shaped haplotype networks and neutrality test results (significantly negative Fu's F_S and Tajima's D values) and unimodal mismatch distributions, support the scenario of sudden population growth of *S. flexuosa* populations after a potential bottleneck. Based on the Bayesian skyline method, this population expansion (ca. 15 Kya) was relatively recent, happening after the end of the Last Glacial Maximum (LGM), in the eastern Mediterranean.

Two morphometric measurements (head length, body depth) were used, along with genetic data, for the identification of the *Spicara maena* and *S. flexuosa*. Morphological measurements indicated that, *S. maena* samples are smaller than *S. flexuosa*, based on the HL and BD values. Specifically, based on the HL values, *S. maena* samples were found to be smaller than 3.76 cm (range 2.95-3.76 cm), and *S. flexuosa* samples were bigger than 4.00 cm (range 4.00-5.25 cm). Considering BD values, *S. maena* samples were smaller than 3.66 cm (range 3.09-3.66 cm), and *S. flexuosa* were larger than 3.76 cm (range 3.76-4.70 cm).

Spicara flexuosa is currently considered as a synonym of *Spicara maena*, and therefore, data on its population statistics have not been reported in the literature. This is a significant problem, as it can result in inaccurate determination and assessment of current stock levels for both *Spicara maena* and *S. flexuosa*. Overall picarel populations declined in the Mediterranean as well as Eastern Atlantic in the last 10 years, and therefore separate statistics and population studies are necessary to help make informed conservation decisions for both species (Pollard et al., 2014).

4.2. *Merlangius merlangus*

Whiting, *Merlangius merlangus* was investigated in the Turkish coastal waters, the Aegean Sea and the Atlantic region. *M. merlangus* comprises two subspecies: *M. m. euxinus* in the Adriatic, Mediterranean and Black Sea, and *M. m. merlangus* in the Atlantic (Quero and Vayne, 1997). The results of the mtDNA (CO1 and cyt-b) analyses indicated that *M. m. euxinus* in the Turkish coastal waters and the Aegean Sea were differentiated from *M. m. merlangus* in the Atlantic region. The genetic variation among different populations of *M. m. euxinus* based on the cyt-b gene indicated significant genetic structure in Turkish coastal waters. High values of mtDNA molecular diversity indices and significant genetic structure between its populations suggested that the TSS is a barrier, rather than a corridor to gene flow, for *M. m. euxinus*. Neutrality tests and mismatch distributions of the mitochondrial genes indicated that *M. m. euxinus* populations from Turkish coastal waters were unimodal and the subspecies might have undergone a population expansion. Based on the Bayesian Skyline Plot, the population expansion of *M. m. euxinus* started around 30 Kya in the eastern Mediterranean. Considering *M. m. merlangus* populations from the Atlantic, on the other hand, the pattern of the haplotype network, relatively high genetic diversities and multi-modal mismatch distributions indicate a long population history and a stable, constant-sized population.

As opposed to mtDNA results, *M. m. merlangus* samples from Atlantic and *M. m. euxinus* from Black Sea, TSS populations could not be distinguished in the haplotype network and phylogenetic trees of the nuclear DNA. However, genetic structure between populations of *M. m. euxinus* from Greece and Turkish coastal waters was observed, probably due to restriction of gene flow due to the Dardanelles, suggesting that the Black Sea and Aegean whiting stocks should be conserved as separate management units. In addition, Black Sea whiting populations should not be considered as a single unit stock due to having different parasite compositions, as previously reported in the literature (Volodin, 1995; Özer et al., 2015). Hence, separate conservation actions and monitoring system are necessary for the protection of the current stocks of whiting (*M. m. euxinus*). Limitation

of annual catch sizes and fishing effort, and establishment of local management systems are also important for sustainable whiting fishery and stock management in the Turkish coastal waters. Furthermore, the realization of effective regulatory measures for all countries in the Black Sea will be critical for the utilization of fish resources, including whiting, in the Black Sea. In future studies, identification of the genetic stocks of whiting using highly variable nuclear markers (e.g. microsatellites) or RAD-seq (restriction site associated DNA sequencing), the identification of spawning areas especially in the northern Aegean, and investigation of the feeding behavior of the species can be useful for a better understanding of the population structure of the *M. m. euxinus* in the Turkish coastal waters, as well as the effects of the TSS on the gene flow of its populations.

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APPENDIX A: MORPHOMETRIC MEASUREMENTS OF *Spicara spp.* INDIVIDUALS

Table A 1. Morphometric measurements of *Spicara spp.* samples collected from İstanbul (cm).

İstanbul samples	HL	BD
1	3.76	3.365
2	2.97	3.09
3	2.34	2.64
4	5.25	4.15
5	4.58	4.7
6	4.09	4.215
7	4.24	4.085
8	4.47	3.86
9	4.5	4.24
10	4.5	4.155
11	4	4.35
12	4.42	4.115
13	4.41	4.095
14	4.35	4.45
15	4.01	3.765
16	4.75	4.465
17	4.3	4.205
18	4.03	4.18
19	4.55	4.325
20	4.01	3.905
21	4.35	4.34
22	4.24	4.4
23	4	3.305

APPENDIX B: CODON USAGE ANALYSIS

Table B. 1. Relative synonymous codon usage analysis for CO1 (454 bp.) sequences of all *Spicara* spp. individuals (Stop codon is represented as *).

Codon	AA	ObsFreq	RSCU
UAG	*	0	0
UAA	*	0	0
AGA	*	0	0
AGG	*	0	0
GCU	A	108	1.245
GCG	A	1	0.012
GCC	A	149	1.718
GCA	A	89	1.026
UGU	C	0	0
UGC	C	0	0
GAU	D	32	0.853
GAC	D	43	1.147
GAG	E	0	0
GAA	E	24	2
UUU	F	121	1.186
UUC	F	83	0.814
GGU	G	71	0.956
GGG	G	48	0.646
GGC	G	36	0.485
GGA	G	142	1.912
CAC	H	44	1.833
CAU	H	4	0.167
AUU	I	244	1.632
AUC	I	55	0.368
AAA	K	21	2
AAG	K	0	0
CUA	L	51	0.463
CUC	L	174	1.578
CUG	L	21	0.19
CUU	L	195	1.769
UUA	L	117	2
UUG	L	0	0
AUG	M	88	0.903
AUA	M	107	1.097

Table B. 1. Relative synonymous codon usage analysis for COI (454 bp.) sequences of all *Spicara* spp. individuals (Stop codon is represented as *) (cont.).

Codon	AA	ObsFreq	RSCU
AAC	N	111	1.298
AAU	N	60	0.702
CCU	P	41	0.626
CCG	P	19	0.29
CCC	P	97	1.481
CCA	P	105	1.603
CAA	Q	25	0.98
CAG	Q	26	1.02
CGA	R	45	4
CGC	R	0	0
CGG	R	0	0
CGU	R	0	0
AGC	S	27	2
AGU	S	0	0
UCA	S	106	2.109
UCC	S	36	0.716
UCG	S	6	0.119
UCU	S	53	1.055
ACA	T	90	1.644
ACU	T	39	0.712
ACC	T	84	1.534
ACG	T	6	0.11
GUU	V	127	2.807
GUG	V	2	0.044
GUC	V	5	0.11
GUA	V	47	1.039
UGA	W	59	1.372
UGG	W	27	0.628
UAC	Y	36	1.469
UAU	Y	13	0.531

Table B. 2. Relative synonymous codon usage analysis for cyt-b (355 bp.) sequences of all *Spicara* spp. individuals (Stop codon is represented as *).

Codon	AA	ObsFreq	RSCU
UAG	*	0	0
UAA	*	0	0
AGA	*	0	0
AGG	*	0	0
GCU	A	30	0.706
GCG	A	10	0.235
GCC	A	42	0.988
GCA	A	88	2.071
UGU	C	9	2
UGC	C	0	0
GAU	D	7	0.233
GAC	D	53	1.767
GAG	E	5	0.286
GAA	E	30	1.714
UUU	F	57	0.708
UUC	F	104	1.292
GGU	G	29	0.823
GGG	G	25	0.709
GGC	G	35	0.993
GGA	G	52	1.475
CAC	H	38	1.357
CAU	H	18	0.643
AUU	I	134	1.295
AUC	I	73	0.705
AAA	K	47	1.649
AAG	K	10	0.351
CUA	L	110	1.452
CUC	L	75	0.99
CUG	L	49	0.647
CUU	L	69	0.911
UUA	L	57	1.781
UUG	L	7	0.219
AUG	M	37	1.276
AUA	M	21	0.724

Table B. 2. Relative synonymous codon usage analysis for cyt-b (355 bp.) sequences of all *Spicara* spp. individuals (Stop codon is represented as *) (cont.).

Codon	AA	ObsFreq	RSCU
AAC	N	46	0.979
AAU	N	48	1.021
CCU	P	43	0.994
CCG	P	7	0.162
CCC	P	99	2.289
CCA	P	24	0.555
CAA	Q	46	1.917
CAG	Q	2	0.083
CGA	R	40	2.807
CGC	R	2	0.14
CGG	R	14	0.982
CGU	R	1	0.07
AGC	S	12	1.5
AGU	S	4	0.5
UCA	S	28	0.966
UCC	S	35	1.207
UCG	S	11	0.379
UCU	S	42	1.448
ACA	T	43	1.11
ACU	T	19	0.49
ACC	T	85	2.194
ACG	T	8	0.206
GUU	V	68	1.52
GUG	V	16	0.358
GUC	V	56	1.251
GUA	V	39	0.872
UGA	W	62	1.879
UGG	W	4	0.121
UAC	Y	62	1.459
UAU	Y	23	0.541

Table B. 3. Relative synonymous codon usage analysis for CO1 (481 bp.) sequences of all *Merlangius merlangus* individuals (Stop codon is represented as *).

Codon	AA	ObsFreq	RSCU
UAG	*	0	0
UAA	*	0	0
AGA	*	0	0
AGG	*	0	0
GCU	A	120	1.463
GCG	A	0	0
GCC	A	56	0.683
GCA	A	152	1.854
UGU	C	0	0
UGC	C	0	0
GAU	D	53	1.493
GAC	D	18	0.507
GAG	E	1	0.111
GAA	E	17	1.889
UUU	F	70	0.805
UUC	F	104	1.195
GGU	G	88	1.437
GGG	G	50	0.816
GGC	G	35	0.571
GGA	G	72	1.176
CAC	H	17	0.654
CAU	H	35	1.346
AUU	I	209	1.62
AUC	I	49	0.38
AAA	K	17	2
AAG	K	0	0
CUA	L	107	1.237
CUC	L	85	0.983
CUG	L	18	0.208
CUU	L	136	1.572
UUA	L	88	2
UUG	L	0	0
AUG	M	17	0.219
AUA	M	138	1.781

Table B. 3. Relative synonymous codon usage analysis for CO1 (481 bp.) sequences of all *Merlangius merlangus* individuals (Stop codon is represented as *) (cont.).

Codon	AA	ObsFreq	RSCU
AAC	N	67	1.107
AAU	N	54	0.893
CCU	P	53	1.024
CCG	P	0	0
CCC	P	69	1.333
CCA	P	85	1.643
CAA	Q	52	1.962
CAG	Q	1	0.038
CGA	R	18	3.789
CGC	R	0	0
CGG	R	0	0
CGU	R	1	0.211
AGC	S	18	1.895
AGU	S	1	0.105
UCA	S	66	1.714
UCC	S	8	0.208
UCG	S	2	0.052
UCU	S	78	2.026
ACA	T	70	2
ACU	T	53	1.514
ACC	T	17	0.486
ACG	T	0	0
GUU	V	51	1.085
GUG	V	21	0.447
GUC	V	47	1
GUA	V	69	1.468
UGA	W	69	2
UGG	W	0	0
UAC	Y	0	0
UAU	Y	52	2

Table B. 4. Relative synonymous codon usage analysis for cyt-b (612 bp.) sequences of all *Merlangius merlangus* individuals (Stop codon is represented as*).

Codon	AA	ObsFreq	RSCU
UAG	*	173	1.602
UAA	*	204	1.889
AGA	*	55	0.509
AGG	*	0	0
GCU	A	231	2.444
GCG	A	1	0.011
GCC	A	88	0.931
GCA	A	58	0.614
UGU	C	29	1.16
UGC	C	21	0.84
GAU	D	58	0.571
GAC	D	145	1.429
GAG	E	173	1.326
GAA	E	88	0.674
UUU	F	407	1.22
UUC	F	260	0.78
GGU	G	62	1.097
GGG	G	3	0.053
GGC	G	82	1.451
GGA	G	79	1.398
CAC	H	86	0.847
CAU	H	117	1.153
AUU	I	88	1.011
AUC	I	86	0.989
AAA	K	90	2
AAG	K	0	0
CUA	L	176	1.275
CUC	L	28	0.203
CUG	L	87	0.63
CUU	L	261	1.891
UUA	L	145	1.429
UUG	L	58	0.571
AUG	M	147	0.916
AUA	M	174	1.084

Table B. 4. Relative synonymous codon usage analysis for cyt-b (612 bp.) sequences of all *Merlangius merlangus* individuals (Stop codon is represented as *) (cont.).

Codon	AA	ObsFreq	RSCU
AAC	N	67	1.107
AAU	N	54	0.893
CCU	P	53	1.024
CCG	P	0	0
CCC	P	69	1.333
CCA	P	85	1.643
CAA	Q	52	1.962
CAG	Q	1	0.038
CGA	R	18	3.789
CGC	R	0	0
CGG	R	0	0
CGU	R	1	0.211
AGC	S	18	1.895
AGU	S	1	0.105
UCA	S	66	1.714
UCC	S	8	0.208
UCG	S	2	0.052
UCU	S	78	2.026
ACA	T	70	2
ACU	T	53	1.514
ACC	T	17	0.486
ACG	T	0	0
GUU	V	51	1.085
GUG	V	21	0.447
GUC	V	47	1
GUA	V	69	1.468
UGA	W	69	2
UGG	W	0	0
UAC	Y	0	0
UAU	Y	52	2

Table B. 5. Relative synonymous codon usage analysis for cyt-b (611 bp.) sequences of all *Merlangius merlangus* individuals (1 bp. was deleted in the original sequences) (Stop codon is represented as *).

Codon	AA	ObsFreq	RSCU
UAG	*	88	0.934
UAA	*	87	0.923
AGA	*	57	0.605
AGG	*	145	1.538
GCU	A	82	3.184
GCG	A	0	0
GCC	A	0	0
GCA	A	21	0.816
UGU	C	147	1.262
UGC	C	86	0.738
GAU	D	32	0.744
GAC	D	54	1.256
GAG	E	84	1.541
GAA	E	25	0.459
UUU	F	203	1.08
UUC	F	173	0.92
GGU	G	0	0
GGG	G	3	4
GGC	G	0	0
GGA	G	0	0
CAC	H	0	0
CAU	H	120	2
AUU	I	118	0.894
AUC	I	146	1.106
AAA	K	175	1.716
AAG	K	29	0.284
CUA	L	196	1.054
CUC	L	174	0.935
CUG	L	145	0.78
CUU	L	229	1.231
UUA	L	176	0.804
UUG	L	262	1.196
AUG	M	145	1.45
AUA	M	55	0.55

Table B. 5. Relative synonymous codon usage analysis for cyt-b (611 bp.) sequences of all *Merlangius merlangus* individuals (1 bp. was deleted in the original sequences) (Stop codon is represented as *) (cont.).

Codon	AA	ObsFreq	RSCU
AAC	N	32	0.309
AAU	N	175	1.691
CCU	P	112	1.383
CCG	P	57	0.704
CCC	P	116	1.432
CCA	P	39	0.481
CAA	Q	114	0.88
CAG	Q	145	1.12
CGA	R	29	0.446
CGC	R	59	0.908
CGG	R	57	0.877
CGU	R	115	1.769
AGC	S	58	0.674
AGU	S	114	1.326
UCA	S	56	0.486
UCC	S	88	0.764
UCG	S	115	0.998
UCU	S	202	1.753
ACA	T	184	2.062
ACU	T	58	0.65
ACC	T	115	1.289
ACG	T	0	0
GUU	V	33	1.451
GUG	V	0	0
GUC	V	29	1.275
GUA	V	29	1.275
UGA	W	29	0.5
UGG	W	87	1.5
UAC	Y	203	1.077
UAU	Y	174	0.923

Table B. 6. Relative synonymous codon usage analysis for cyt-b (610 bp.) sequences of all *Merlangius merlangus* individuals (2 bp. was deleted in the original sequences) (Stop codon is represented as *).

Codon	AA	ObsFreq	RSCU
UAG	*	0	0
UAA	*	107	1.012
AGA	*	142	1.343
AGG	*	174	1.645
GCU	A	58	1.143
GCG	A	2	0.039
GCC	A	143	2.818
GCA	A	0	0
UGU	C	116	0.665
UGC	C	233	1.335
GAU	D	28	0.651
GAC	D	58	1.349
GAG	E	27	1.862
GAA	E	2	0.138
UUU	F	235	1.009
UUC	F	231	0.991
GGU	G	57	0.781
GGG	G	0	0
GGC	G	116	1.589
GGA	G	119	1.63
CAC	H	58	0.991
CAU	H	59	1.009
AUU	I	268	1.644
AUC	I	58	0.356
AAA	K	58	2
AAG	K	0	0
CUA	L	60	0.529
CUC	L	86	0.758
CUG	L	28	0.247
CUU	L	280	2.467
UUA	L	116	1.983
UUG	L	1	0.017
AUG	M	49	0.56
AUA	M	126	1.44

Table B. 6. Relative synonymous codon usage analysis for cyt-b (610 bp.) sequences of all *Merlangius merlangus* individuals (2 bp. was deleted in the original sequences) (Stop codon is represented as *) (cont.).

Codon	AA	ObsFreq	RSCU
AAC	N	145	0.831
AAU	N	204	1.169
CCU	P	144	1.806
CCG	P	0	0
CCC	P	146	1.831
CCA	P	29	0.364
CAA	Q	183	2
CAG	Q	0	0
CGA	R	58	1.349
CGC	R	28	0.651
CGG	R	0	0
CGU	R	86	2
AGC	S	1	0.067
AGU	S	29	1.933
UCA	S	145	1.111
UCC	S	143	1.096
UCG	S	29	0.222
UCU	S	205	1.571
ACA	T	86	1.19
ACU	T	145	2.007
ACC	T	58	0.803
ACG	T	0	0
GUU	V	87	0.926
GUG	V	2	0.021
GUC	V	58	0.617
GUA	V	229	2.436
UGA	W	145	1.472
UGG	W	52	0.528
UAC	Y	145	0.831
UAU	Y	204	1.169

APPENDIX C: A LIST OF SEQUENCES WITH AMBIGUITY CODES FOR THE IRBP GENE

>161

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TTTGCTCCTAGACCTGGTCTGGAATAAAATCCTGCCAACCTCAGCTCTCATCTTTGACTT
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>163

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>172

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AGGCCGAGCCTCAGCTTCACATTGATAGCGTGTATGACCGACCCTCCAACACCACCAC
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>221
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AGGCCGAGCCTCAGCTTCACATTGATAGCGTGTATGACCGACCCTCCAACACCACCAC
TAAGTTGCTGTCTATGGACACACTGCTGGGGGAAAGATATGGCGTCACCAAACCCCTC
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>331

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APPENDIX D: PHOTOGRAPHS OF *Spicara spp.* SAMPLES COLLECTED FROM ISTANBUL



Figure D.1. *Spicara maena,* İstanbul 1.



Figure D.2. *Spicara maena,* İstanbul 2.



Figure D.3. *Spicara flexuosa,* İstanbul 3.



Figure D.4. *Spicara flexuosa,* İstanbul 4.



Figure D.5. *Spicara flexuosa,* İstanbul 5.



Figure D.6. *Spicara flexuosa*, İstanbul 6.



Figure D.7. *Spicara flexuosa*, İstanbul 7.



Figure D.8. *Spicara flexuosa*, İstanbul 8.



Figure D.9. *Spicara flexuosa*, İstanbul 9.



Figure D.10. *Spicara flexuosa*, İstanbul 10.



Figure D.11. *Spicara flexuosa*, İstanbul 11.



Figure D.12. *Spicara spp.* İstanbul 12.



Figure D.13. *Spicara flexuosa*, İstanbul 13.



Figure D.14. *Spicara flexuosa*, İstanbul 14.



Figure D.15. *Spicara flexuosa*, İstanbul 15.

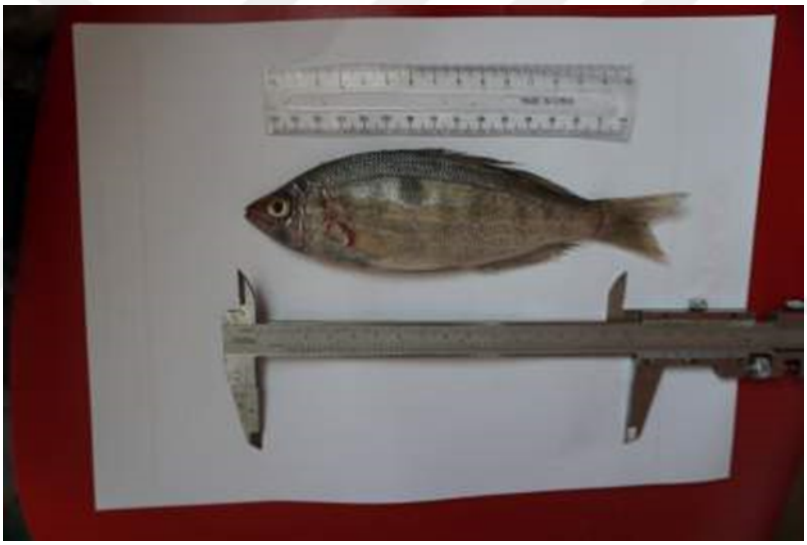


Figure D.16. *Spicara spp.,* İstanbul 16.



Figure D.17. *Spicara spp.,* İstanbul 17.



Figure D.18. *Spicara spp.*, İstanbul 18.



Figure D.19. *Spicara flexuosa*, İstanbul 19.



Figure D.20. *Spicara flexuosa*, İstanbul 20.



Figure D.21. *Spicara flexuosa,* İstanbul 21.



Figure D.22. *Spicara flexuosa*, İstanbul 22.



Figure D.23. *Spicara flexuosa*, İstanbul 23.

APPENDIX E: SAMPLING PERIODS AND LOCATION OF FISH SAMPLES

Table E.1. Sampling periods and location of fish samples.

Species	Stations	Locations	Dates	Sample size
S. flexuosa	İstanbul	40°52’ 12" N, 29° 06' 04" E	28.08.2013	25
S. flexuosa	Çanakkale	40° 9′ 0″ N, 26° 24′ 0″ E 40.15, 26.4	19.06.2014	25
S. flexuosa	Armutlu	40° 31′ 10″ N, 28° 49′ 41″ E 40.519444, 28.82805	05.09.2015	25
S. flexuosa	Rize	41° 06’ 44" N, 40° 43’ 18"	23.12.2013	25
S. flexuosa	Sinop	42° 03' 45" N, 34° 50' 15" E 42° 05' 30" N; 34° 51' 00" E	09.12.2013	25
S. flexuosa	İzmir	38° 18′ 0″ N, 26° 45′ 0″ E 38.3, 26.75	14.11.2014	25
S. flexuosa	Muğla	37° 3′ 0″ N, 28° 19′ 0″ E 37.05, 28.316667	05.11.2013	25
S. flexuosa	Antalya	36° 54′ 0″ N, 30° 41′ 0″ E 36.9, 30.683333	12.10.2014	25
S. flexuosa	Mersin	36° 48′ 0″ N, 34° 37′ 59.88″ E 36.8, 34.6333	23.06.2014	25
S. maena	Thessaloniki	40° 38′ 0″ N, 22° 57′ 0″ E 40.633333, 22.95	16.04.2015	25
S. flexuosa/maena	İstanbul	40°52’ 12" N, 29° 06' 04" E	23.02. 2017	23
M. merlangus	İstanbul	40°52’ 12" N, 29° 06' 04" E	10.08.2013	25
M. merlangus	Çanakkale	40° 9′ 0″ N, 26° 24′ 0″ E 40.15, 26.4	01.06.2015	25
M. merlangus	Bandırma	40.360924, 27.973957	22.09.2014	25
M. merlangus	Rize	41° 06’ 44" N, 40° 43’ 18"	23.12.2013	30
M. merlangus	Sinop	42° 03' 45" N, 34° 50' 15" E 42° 05' 30" N; 34° 51' 00" E	09.12.2013	25
M. merlangus	Thessaloniki	40° 38′ 0″ N, 22° 57′ 0″ E 40.633333, 22.95	15.11.2015	25
M. merlangus	Zonguldak	41.556015, 32.011411	09.10.2013	25
M. merlangus	İnebolu	41.973559, 33.784968	02.10.2015	25

APPENDIX F: TOTAL NUMBERS OF SAMPLES BASED ON THE SAMPLING SITES AND GENE RESULTS

Table F.1. Total numbers of the collected samples and sequences based on the sampling sites and CO1, cyt-b, CO1+cyt-b, 16S rRNA and IRBP genes for *Spicara spp*.

		Gene				
Species	**Sampling sites**	**CO1**	**Cyt-b**	**CO1+cyt-b**	**16S**	**IRBP**
Spicara flexuosa	İstanbul	17	20	15	1	22
	Çanakkale	19	17	15	2	31
	Armutlu	22	25	19	1	27
	Rize	19	20	12	2	20
	Sinop	13	5	3		25
	İzmir	24	7	5	2	27
	Muğla	17	15	8	1	19
	Antalya	23	21	16		35
	Mersin	22	9	8		27
	Total *S. flexuosa*	176	139	101	9	233
Spicara maena	Greece	23	18	15	7	22
	Total *S. maena*	23	18	15	7	22

Table F.2. Total numbers of the collected samples based on the CO1, cyt-b, CO1+cyt-b, and RAG1 genes for *Merlangius merlangus.* *Includes *M. m. merlangus* samples detected in these water bodies.

		Gene			
Species	**Sampling sites**	**CO1**	**Cyt-b**	**CO1+cyt-b**	**RAG1**
Merlangius m. euxinus	İstanbul	20	10	10	20
	Çanakkale	18 *	16	9	24
	Bandırma	22	15	11	20
	Rize	28 *	18	12	25
	Sinop	14	10	6	19
	İnebolu	6	16	5	13
	Zonguldak	18	8	5	20
	Greece	36	32	23	30
	Total *M. m. euxinus*	162	125	81	171
M. m. merlangus	France	2	1	1	2
	Total *M. m. merlangus*	5	1	1	2