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RESEARCH PAPER

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First Molecular Record and Distribution of *Padina pavonica* (Linnaeus) Thivy from the Southern Black Sea

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Abstract

Padina is a brown algal genus widely distributed in warm-temperate and tropical regions. Species-level identification of specimens is often difficult due to intraspecific and interspecific morphological plasticity. This study reveals the results of the phylogenetic relationships of specimens, by analyzing the cytochrome oxidase subunit 3 (Cox3) and RuBisCO large subunit (rbcL) gene sequences. A total of 144 new sequences were analyzed together with other *Padina* sequences obtained from GenBank. Phylogenetic inference identified one well-resolved clade. All of these analyses showed that this genus has only one species in the Black Sea. This is the first study on the molecular diagnosis of macroalgae in the Black Sea, and it contains the first molecular records of *Padina* in the Black Sea Region.

Introduction

The Black Sea has a complex geological history in terms of its biology and ecology. It has been isolated from and reconnected to other seas and oceans many times throughout history (Zaitsev, 1998). Continuous changes in salinity along these periods caused it to have a complex biota. The Modern Black Sea has had five main groups: Pontian relics, Boreal-Atlantic relics, Freshwater species, Alien species and Mediterranean species.

Mediterranean species have formed the basic components of the Black Sea biota (Zaitsev et al., 2002). However, there are significant physical differences between the two seas like salinity and productivity. Different environmental factors may influence species composition and genetic differentiation (LaJeunesse et al., 2010; Kostamo, 2008; Yan et al., 2020). Additionally, different environmental conditions may cause intraspecific and interspecific variations and a high rate of polymorphism (Hwang et al., 2005). Many algal species are known to exhibit substantial intraspecific morphological variation (Leliaert et al., 2014). This situation causes problems in macroalgal classification in which morphological characteristics are predominantly used. Due to these high polymorphism rates, researchers around the world have developed various molecular approaches to determine the species boundaries of algae. In light of these developments, some macroalgal taxa have been revised (Leliaert et al., 2014). The genus *Padina* (Dictyotales, Phaeophyceae) is one of the taxa revised worldwide. Win et al. (2020), denoted that the *Padina* taxonomy has a lack of information on reliable morphological characteristics for

species delineation, coupled with insufficient/inadequate DNA sequences with the convergent evolution of morphological traits. As a result of recent studies, there has been an increase in regional diversity and species numbers (Win et al., 2008, 2010, 2011a, b, 2012, 2013, 2018, 2020). According to the Algaebase database, the number of recognized *Padina* species in 2008 was 32, whereas this number is recently 53 (Win et al., 2008, Guiry and Guiry, 2021).

In the Black Sea, the taxonomy of the genus *Padina* has been based on morphological characteristics only. Therefore, molecular data are not available, and the phylogenetic relationships of the Black Sea lineage remain unsolved. It is important to find an answer to the question of whether the genus *Padina* is actually represented by a single species in the Black Sea. Based on plastid rbcL and mitochondrial cox3 partial gene sequencing data, Silberfield et al. (2013), and Win et al. (2020), performed studies on large-scale global species diversity. However, since there are no molecular data on the genus *Padina* in the Black Sea, this region was excluded from both studies. In this study, to fill this gap in the literature, using molecular data, the diversity of *Padina* in the Black Sea was investigated, and the results were combined with morphological data.

Materials and Methods

Taxon Sampling and Collection

Selection of the sampling stations was achieved based on the literature (Aysel et al., 2000, 2004, 2005b, 2005c, 2008a, 2008b; Karaçuha and Gönülol, 2007; Karaçuha and Ersoy Karaçuha, 2013). Figure 1 shows the 24 stations where algae specimens were explored. Sampling was conducted in two seasons (July - September 2015 and July - September 2016) when thallus size and coverage percentage were the highest. Rocky, stony and relatively wave-closed areas were selected for sampling algae. Depending on the depth of the stations, by utilizing snorkeling at 0-5 m of depth and scuba diving at 5-20 m of depth, algae samples were collected.

The collected specimens were cleaned with sterile sea water, and each one was divided into three aliquot parts. The first aliquot was dried in silica gel for DNA extraction (Chase & Hills, 1991). The second was preserved with 4% formalin/seawater (Voucher). The third was preserved with absolute ethanol; it was kept as a backup plan for both morphological examination and DNA extraction. Each silica gel specimen was linked by a call number with voucher specimens.

Morphological and Anatomical Examination

Both the morphological and anatomical characteristics of the collected specimens were inspected using hand-selected sections (basal, middle and margins) of the voucher specimen. The examined characteristics were respectively (1) number of cell layers and thickness of the thallus (apical, mid- and basal), (2) degree of calcification on superior and inferior blade surfaces, (3) presence of hairlines and reproductive cells and their position against each other, and (4) presence or absence of an indusium (Lawson & John, 1987; Womersley, 1987; Lee & Kamura, 1991; Abbott & Huisman, 2004). The species were identified according to studies in the current literature such as those by Win et al., (2008, 2010, 2011, 2012, 2013).

DNA Extraction

The total DNA was extracted from the silica gel-dried samples according to the protocol described by Snirc et al. (2010). Finally, to the extracted DNA, 50 µl of 0.1X TE buffer (10 mM Tris at pH 8, 0.1 mM EDTA) was added, and the sample was frozen (−20°C) for further analyses.

PCR Amplification

According to the literature reviews, two protein-coding regions were selected as the targets for PCR amplification. The first target region was the plastid *rbc*L gene region. This region was amplified with overlapping primer pairs in the order of *rbc*L-68F /*rbc*L-708R and

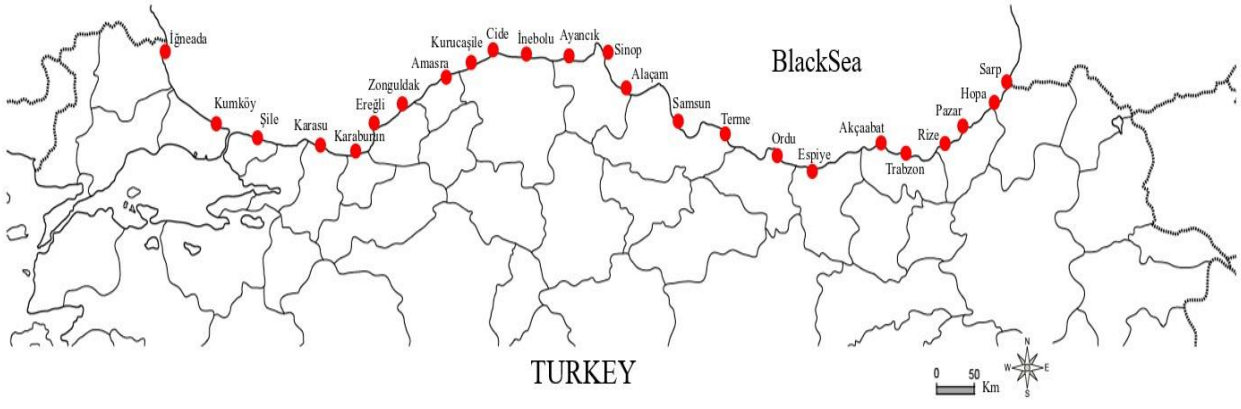

Figure 1. Sampling stations (Anonymous, 2017)

*rbc*L-543F/*rbc*L-1381R. The second target region was amplified with mitochondrial *cox*3 and sequenced using the primer pairs *cox*3-44F/*cox*3-739R. The primer pairs that were used are shown in Table 1. 50 µl of the PCR mix for the PCR amplification of the gene regions consisted of 0.8 mM dNTP mixture (0.2 mM of each base), 1.5 mM $MgCl_2$, 0.6 pmole of each forward and reverse primer, 1X Green GoTaq Flexi PCR Buffer, 1.25 units of GoTaq DNA polymerase (Promega), 0.5 mg template DNA and double distillated H_2O (ddH_2O). The PCR conditions are presented in Table 2.

The purification and sequencing of the PCR products were performed commercially by the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea).

Data Analysis

In order to create a dataset for phylogenetic analysis, the *rbc*L and *cox*3 gene sequences of different *Padina* species in previous studies were downloaded from the NCBI (National Biotechnology Information Center) database (Supplementary Table). While generating datasets for the indicated genes, an outer group sequence other than *Padina* species was selected. Attention was paid to ensure that the database used for the analysis represented the geography in which the genus was distributed and included the databases used in previous studies. After the datasets were generated, Clustal X (Thompson et al., 1997) and MAFT (Katoh et al., 2002) were used to align the homologous bases in the sequences. The datasets aligned by the program were re-checked using the BioEdit (Hall, 1999) program, the required regions were corrected, and the columns containing indel regions were removed with GBLOCKS (Castresana, 2000). Prior to the phylogenetic analysis, jModelTest (Posada, 2008), AIC (Akaike Information Criteria) (Akaike, 1974) and BIC (Bayesian Information Criteria) analyses were carried out. For the phylogenetic analysis, the maximum-likelihood (ML) and Bayesian (BI) methods were performed using the MEGA7 (Kumar et al., 2015) and MrBayes 3.2 (Ronquist et al., 2012) software, respectively. The maximum-likelihood analysis was performed separately for each of the base change models determined using the AIC and BIC test, and bootstrap analyses were performed with 1000 replications. The bootstrap values are shown on trees for over 50% of the supported nodes. For the BI analyses, MCMC (Markov chain Monte Carlo) tests were performed, 10.000.000 generation runs were carried out, and sampling was made in every 1000 generations. The BI analysis results were assessed using Tracer v1.6 (Rambaut et al., 2014), and the first 1.000.000 trees were discarded as 'burn-in'.

Results

Padina specimens were found in 7 stations. The coordinates of these stations are given in Table 3.

Table 1. Primer pairs

Primer	Gene regions	Sequence (5'-3')	Reference
cox3-44F	cytochrome c oxidase subunit 3	CAACGNCAYCCWTTTCATTT	Silberfeld et al., 2010
cox3-739R	cytochrome c oxidase subunit 3	CATCNACAAAATGCCAATACCA	Silberfeld et al., 2010
rbcL-68F	Rubisco LSU DNA (*rbc*L)	GCNAAAATGGGNWAYTGGGATGC	Draisma et al., 2001
rbcL-708R	Rubisco LSU DNA (*rbc*L)	TTAAGNTAWGAACCYTTAACTTC	Bittner et al., 2008
rbcL-543F	Rubisco LSU DNA (*rbc*L)	CCWAAATTAGGTCTTTCWGGWAAAAA	Bittner et al., 2008
rbcL-1381R	Rubisco LSU DNA (*rbc*L)	ATATCTTTCCATARRTCTAAWGC	Burrowes et al., 2003

Table 2. PCR conditions

	rbcL 68F/708R		*rbcL 543F/1381R*		*cox*3	
İnitial denaturalization	94°C/3 min.		94°C/3 min.		94°C/3 min.	
Denaturalization	94°C/45 sec.	38 cycles	94°C/45 sec.	35 cycles	94°C/1 min.	38 cycles
Annealing	52°C/1 min.		50°C/1 min.		48°C/1 min.	
Extension	72°C/90 sec.		72°C/90 sec. min.		72°C/90 sec.	
Final extension	72°C/5 min.		72°C/5 min.		72°C/5 min.	

Table 3. Coordinates of sampling stations

Sample ID	Coordinates
Dedkup0202	41° 15' 18" N, 29° 02' 20" E
Dedsil0301	41° 10' 41" N, 29° 36' 54" E
Dedkap0401	41° 11' 13" N, 30° 21' 03" E
Dedayp1203	41° 56' 54" N, 34° 33' 59" E
Dedsip1301	42° 03' 03" N, 35° 02' 36" E
Dedfap1703	41° 07' 54" N, 37° 40' 55" E
Dedesp1805	40° 58' 14" N, 38° 37' 55" E

Morphological Observation of Specimens

1.Dedkup0202 (*Padina* sp.)

The color was bright brown, thalli erect 4.7 ± 0.3 cm tall 5.2± 0.9 cm in diameter, stipe length 0.45 ± 0.1 cm that was attached by a stupose base, basal section 3-4 cells, middle portion 3 cells, involute margin 2-cell-layer-thick. Hairlines were found in both surfaces of the thallus. The superior surface of thalli moderately, inferior surface slightly calcified (Figures 2 a, b, c, d). The habitat is a rocky shore.

2. Dedsil0301 (*Padina* sp.)

The color was yellowish brown, thalli erect 6 ± 0.5 cm tall, 5 ± 0.15 in cm diameter, stipe length 0.5 ± 0.15 cm that was attached by a stupose base, basal section 3 cells, middle portion 3 cells, involute margin 2-cell-layer-thick. Hairlines were found in both surfaces of the thallus. The superior surface of thalli moderately, inferior surface slightly calcified, sori covered by indusium (Figures 3a, b, c, d). The habitat is a rocky shore.

3. Dedkap0401 (*Padina* sp.)

The color was bright brown, thalli erect 5 ± 0.7 cm tall 6 ± 0.3 cm in diameter, stipe length 0.45 ± 0.1 cm that was attached by a stupose base, basal section 3-4(5) cells, middle portion 3 cells, involute margin 2-cell-layer-thick. Hairlines were found in both surfaces of the thallus. The superior surface of thalli slightly, inferior surface slightly calcified (Figures 4 a, b, c, d). The habitat is a rocky shore.

4. Dedayp1203 (*Padina* sp.)

The color was yellowish brown, thalli erect 6.5 ± 0.4 cm tall, 7 ± 0.15 cm in diameter, stipe length 0.5 ± 0.05 cm that was attached by a stupose base, basal section 3(4) cells, middle portion 3 cells, involute margin 2-cell-layer-thick. Hairlines were found in both surfaces of the thallus. The superior surface of thalli moderately, inferior surface slightly calcified, sori covered by indusium (Figures 5 a, b, c, d). The habitat is a rocky shore.

5. Dedsip1301 (*Padina* sp.)

The color was brown, thalli erect 5.1 ± 0.07 cm tall, 7.1 ± 0.15 cm in diameter, stipe length 0.5 ± 0.05 cm that was attached by a stupose base, basal section 3(4) cells, middle portion 3 cells, involute margin 2-cell-layer-thick. Hairlines were found in both surfaces of the thallus. The superior surface of thalli heavily, inferior surface slightly calcified, sori covered by indusium (Figures 6 a, b, c, d). The habitat is a rocky shore.

6. Dedfap1703 (*Padina* sp.)

The color was bright brown, thalli erect 5.3 ± 0.19 cm tall, 7± 0.9 cm in diameter, stipe length 0.48 ± 0.05 cm that was attached by a stupose base, basal section 3 cells, middle portion 3 cells, involute margin 2-cell-layer-thick. Hairlines were found in both surfaces of the thallus. The superior surface of thalli heavily, inferior surface slightly calcified, sori covered by indusium (Figures 7 a, b, c, d). The habitat is a rocky and stony area.

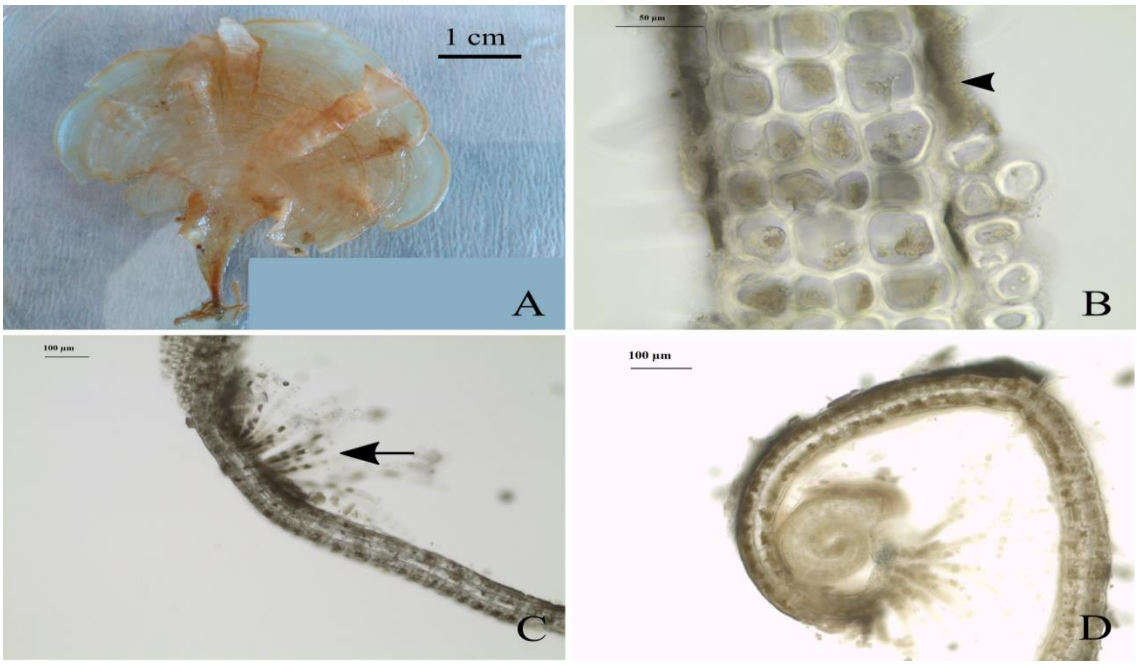

Figure 2. DEDKUP0202: **A**. Whole thallus, **B**. Dense calcification on the upper surface (Arrowhead) in the basal cross section, **C**. Cell layers and hairlines in the middle of the thallus (Arrow), **D**. Cell layers in the longitudinal section from the tip of the thallus, Scale; A: 1 cm, B: 50 µm, C, D: 100 µm.

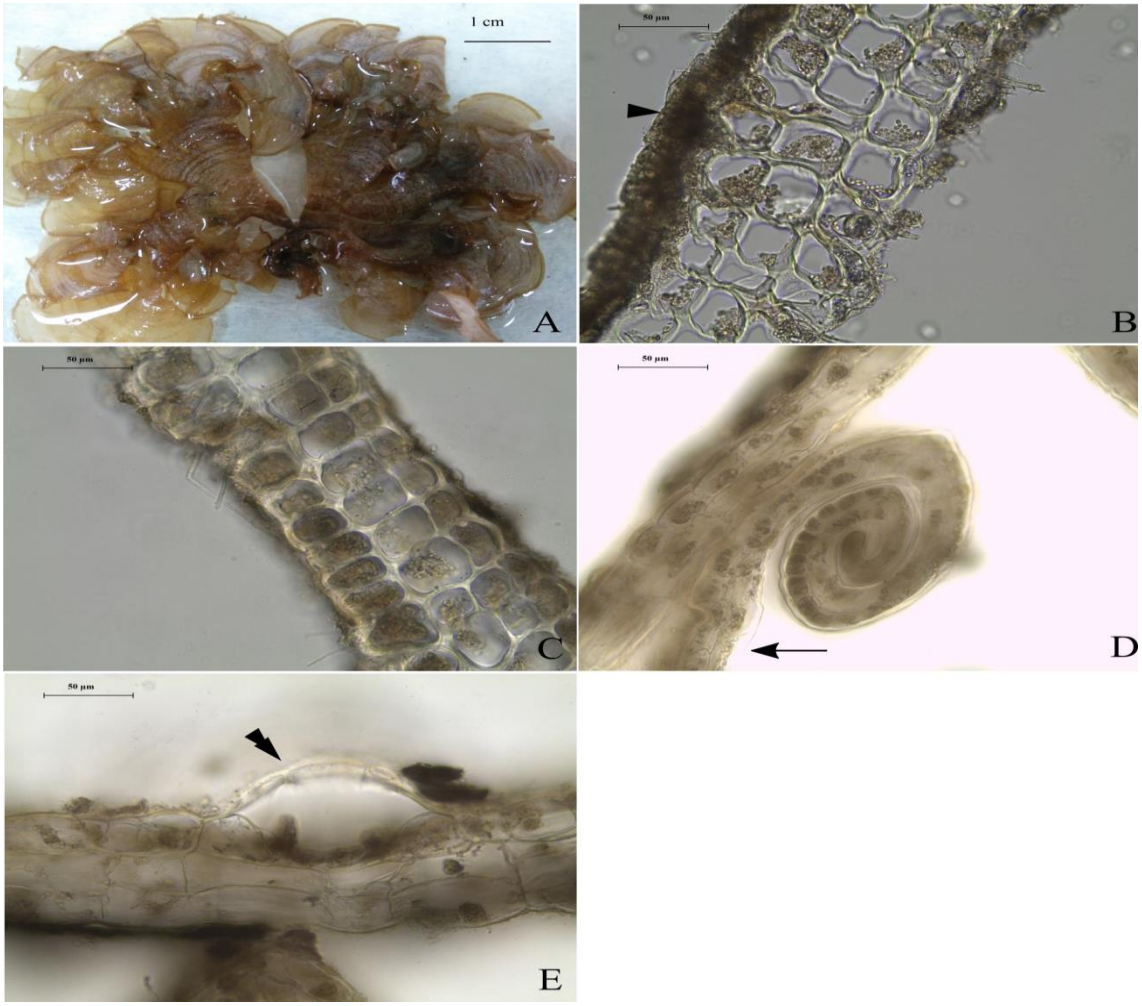

Figure 3. DEDSIL0301: **A**. Whole thallus, **B**. Dense calcification on the upper surface in the basal cross-section (Arrowhead), **C**. Cell layers in the transverse section in the middle part of the thallus, **D**. Cell layers in the longitudinal section from the enrolled margin (Arrow), **E**. indusium (double arrowhead), Scale; A: 1 cm, B, C, D, E: 50 µm.

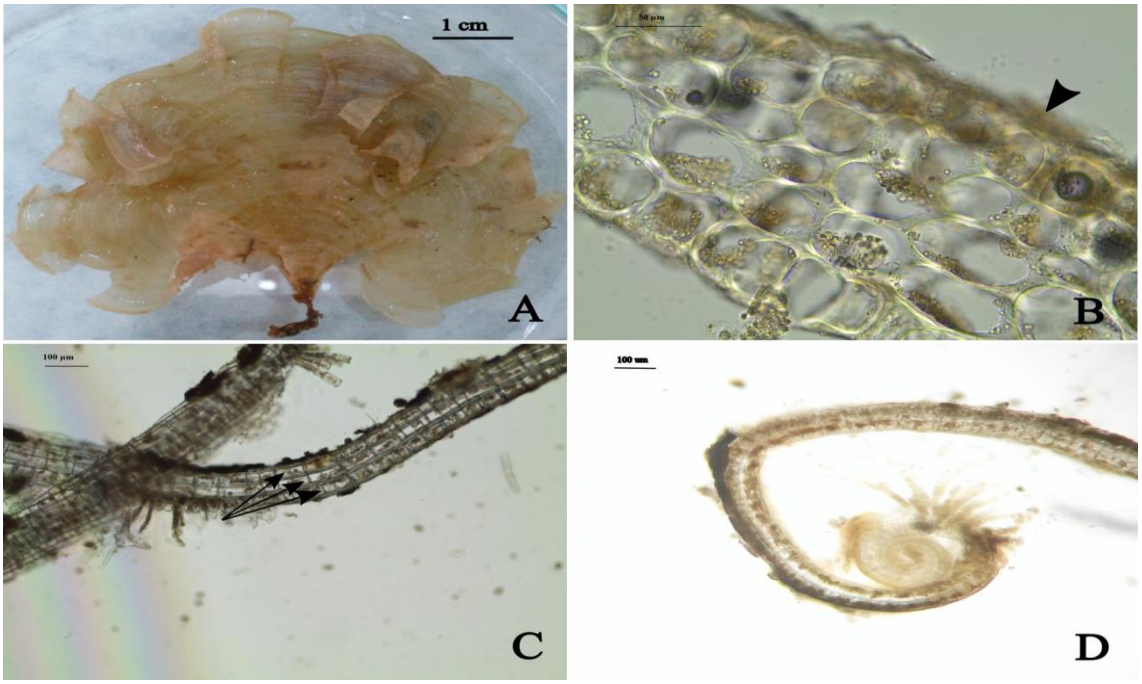

Figure 4. DEDKAP0401: **A**. Whole thallus, **B**. Basal part calcification at the upper surface (Arrowhead), **C**. Cell layers in the transverse section in the middle part of the thallus, **D**. Cell layers in the longitudinal section from the tip of the thallus, Scale; A: 1cm, B: 50 µm, C, D: 100 µm.

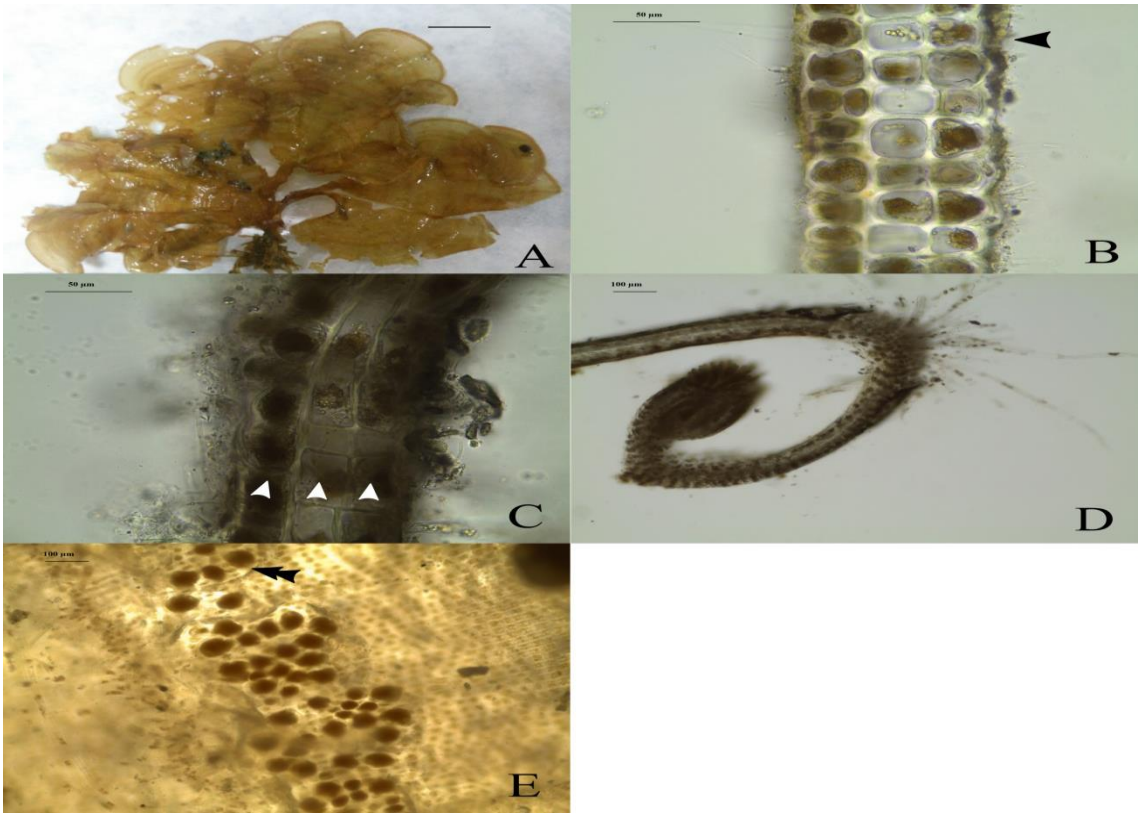

Figure 5. DEDAYP1203: **A**. Whole thallus, **B**. Moderate calcification on the upper surface in the basal cross-section (Arrowhead), **C**. Cell layers in the middle of the thallus, **D**. Cell layers in the longitudinal section from the enrolled margin, **E**. indusium (double arrowhead), Scale; A: 1 cm; B, C: 50 µm; D, E: 100 µm.

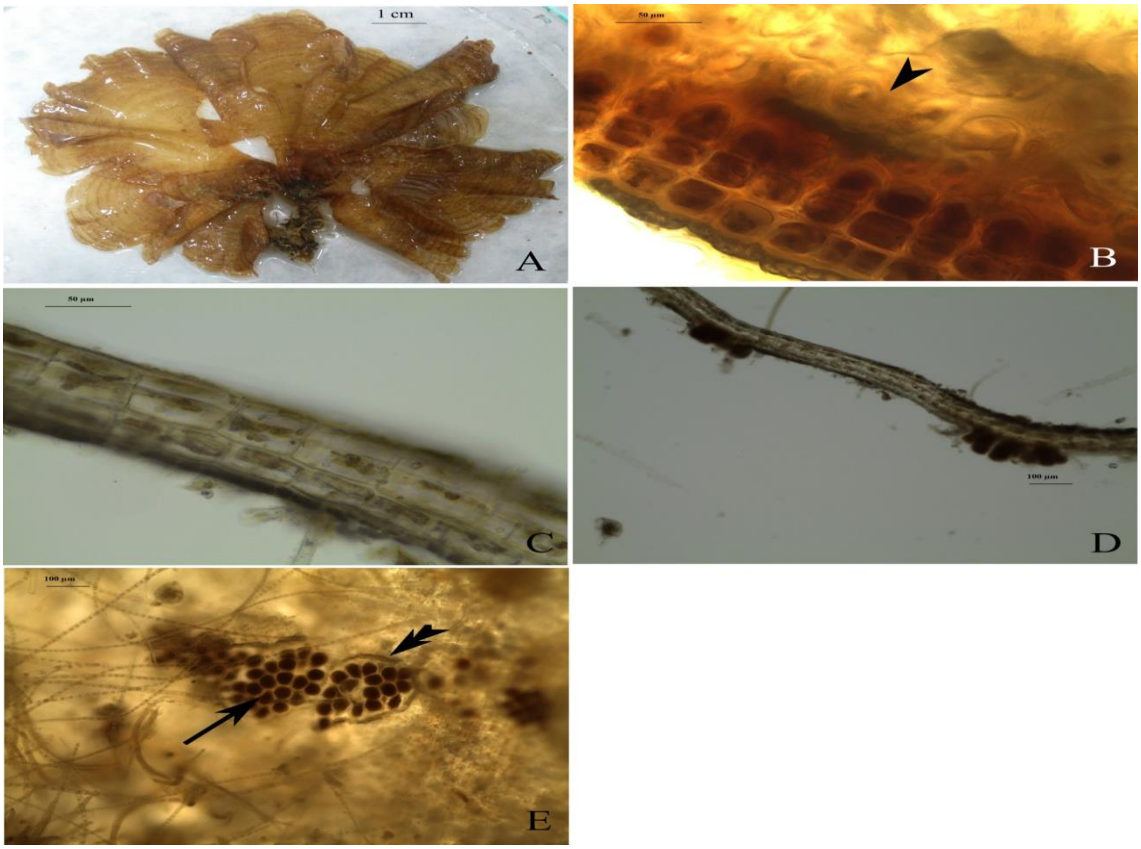

Figure 6. DEDSIP1301: **A**. Whole thallus, **B**. Intense calcification on the upper surface of the basal section in the cross-section (Arrowhead), **C**. Cell layers in the middle section of the cross-section, **D**. Cell layers in the longitudinal section from the tip of the thallus, **E**. Mature tetrasporangium (Arrow), indicum covering the tetrasporangium (Double arrowhead), Scale; A: 1 cm, B, C: 50 µm, D, E: 100 µm.

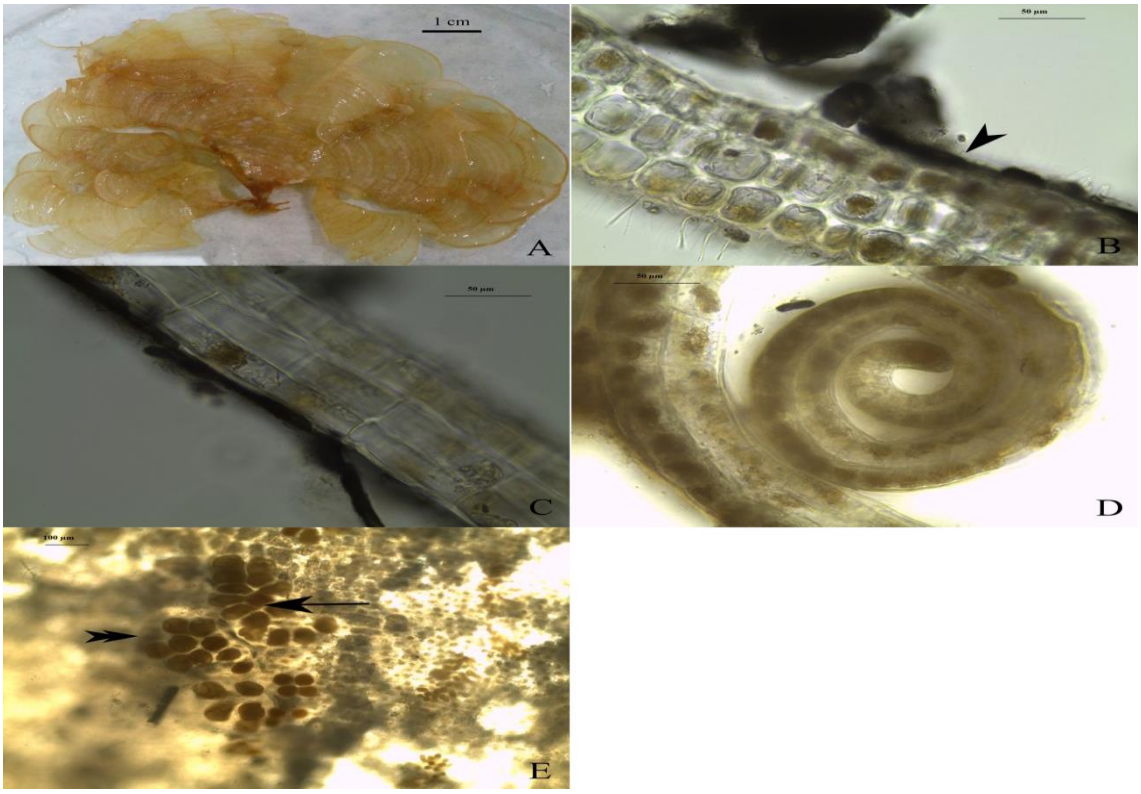

Figure 7. DEDFAP1703: **A**. Whole thallus, **B**. Calcification on the upper surface of the basal section in the cross-section (Arrowhead), **C**. Cell layers in the middle section of the cross-section, **D**. Cell layers in the longitudinal section from the tip of the thallus, **E**: Mature tetrasporangium (Arrow), indicum covering the tetrasporangium (Double arrowhead), Scale; A: 1 cm, B, C, D: 50 µm, E: 100 µm.

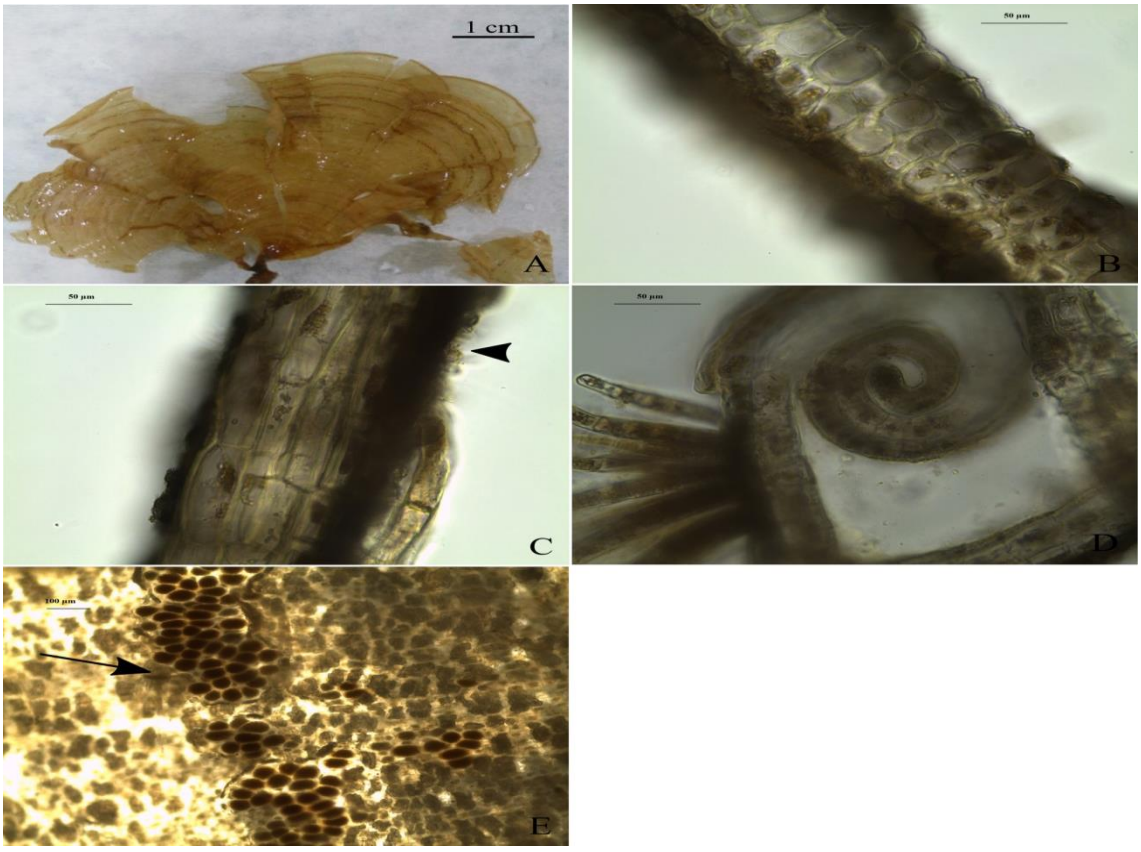

Figure 8. DEDESP1805: **A**. Whole thallus, **B**: Basal cross section, **C**: Intense calcification in the middle of the thallus transverse section cell layers and upper surface (Arrowhead), **D**: Cell layers in the longitudinal section from the tip of the thallus, **E**: Indicum covering mature tetrasporangium (Arrow) and tetrasporangium, Scale; A: 1 cm, B, C, D: 50 µm, E: 100 µm.

7. Dedesp1805 (*Padina* sp.)

The color was yellowish brown, thalli erect 4.1 ± 0.04 cm tall, 5± 0.2 cm in diameter, stipe length 0.3 ± 0.2 cm that was attached by a stupose base, basal section 3 cells, middle portion 3(4) cells, involute margin 2-cell-layer-thick. Hairlines were found in both surfaces of the thallus. The superior surface of thalli heavily, inferior surface slightly calcified, sori covered by indusium (Figures 8 a, b, c, d). The habitat is a stony area.

Molecular Phylogenetic Analysis

The final *rbc*L dataset consisted of 26 in-group (among 64 sequences, 7 newly generated) and three out-group taxa (*Dictyota dichotoma* (Hudson) J.V. Lamor, *Zonaria flabellata* (Okamura) Papenfuss, *Stypopodium australasicum* (Zanardini) Allender et Kraft), and the *cox*3 dataset consisted of 27 in-group (61 sequences, 7 newly generated) and one out-group (*Stypopodium* sp. Kützing) taxa (Supplementary Table). The alignments were *rbc*L and *cox*3, 1051 bp and 485 bp, respectively. In order to determine the most suitable base change model for the created datasets, analyses were carried out according to the AICc and BIC criteria. According to both sets of selection criteria, the highest bootstrap values were obtained by the Tamura-Nei + G + I (G = 0.86802, I = 0.57622) model for rbcL and (G = 0.82569, I = 0.50722) for *cox*3. The Maximum Likelihood and Bayesian analyses for both datasets produced the same tree topology (Figures. 9-10). When the ML trees of the *cox*3 and *rbc*L datasets were examined, it was observed that the Mediterranean and Black Sea sequences were branched together with 99% and 100% bootstrap values, respectively. Within these groups, the Mediterranean and the Black Sea samples were completely diverged, and they formed two subgroups. It was observed that the Black Sea subgroups were supported with bootstrap values of 64% and 96%, respectively, for the two ML trees.

Discussion

In the morphological view, our specimens were seen to be compatible with *P. pavonica* (Nizamuddin, 1981, Win et al., 2011a, Win et al., 2013). The number of cell layers in the transverse and longitudinal sections taken from all *Padina* samples diagnosed in this study was determined as 3-4 cell layers on the basis, 3 cell layers in the middle and 2 cell layers in the involute margins. Traditionally, *Padina* species have been grouped by their numbers of cell layers as 2, 2-3, 2-4 and multi-layered. Recent morphological data on the genus *Padina* revealed the taxonomic significance of the number of cell layers for species identification (Win, 2011a). The phylogenetic inference of the *P. pavonica* sequences of the Mediterranean and the Black Sea formed a strongly supported two sister clades, rather than a single one (Figures. 9-10). Although this divergence was not seen in morphology, it is suggested that it had started to differentiate genotypically.

Silberfeld et al. (2013), reported that speciation events more recent than ~4 Ma are not captured well by the rbcL gene. From this suggestion, it may be concluded that the *P. pavonica* species has settled in the Black Sea, not only through the straits, which have a history of a few thousand years, but in the geological times through the Sea of Tethys. Similar inferences have been proposed for *Halimeda* J.V. Lamour (Verbruggen et al., 2009) and *Dictyota* J.V. Lamour (Tronholm et al., 2012). As a result of the study, it was observed that the genus *Padina* was represented by only one species (*P. pavonica*) in the Black Sea, and there was one genetic population apart from the Mediterranean lineage.

The specimens in this study were collected from an area of the entirety of the Turkish coast of the Black Sea (Western, Central and Eastern Black Sea Regions), and *Padina* samples were found only on Turkey's Western and Central Black Sea coasts (7 stations). In comparison to previous studies on macro algae in the Black Sea (Aysel et al., 2000, 2004, 2005b, 2005c, 2008a, 2008b; Karaçuha and Gönülol, 2007; Karaçuha and Ersoy Karaçuha, 2013; Taşkın, 2014), it was seen that the *Padina* populations had been decreasing in the last 20 years in the Turkish Black Sea coasts. In fact, in previous studies, it is seen that the *P. pavonica* species is distributed in 11 provinces, namely Kocaeli, Sakarya, Bolu, Zonguldak, Bartın, Kastamonu, Sinop, Samsun, Trabzon, Rize and Artvin (Aysel et al., 2000). However, in this study, the *P. pavonica* species was not found in the areas previously reported in the provinces of Samsun, Trabzon, Rize and Artvin. This situation in Turkey was an indication of the habitat loss, and thus, the slow extinction of the *P. pavonica* species, and it pointed to the urgent need to develop the necessary conservation policies. Unlike the shores of Turkey, *Padina pavonica*, which is distributed only on the shores of Sozopol in Bulgaria, is in conservation status as an "endangered species" (Anonymous, 2020).

It was observed that the *P. pavonica* species was spread in local patches rather than large areas on the Black Sea coast of Turkey. The common characteristic of these stations was being located away from anthropogenic impact and in a partially clean zone. Previously, Aysel et al. (2000), reported *Padina* samples from the Eastern Black Sea region. It is thought that the road project and filling works carried out on the Eastern Black Sea coasts of Turkey today have been effective in the habitat loss of these populations.

This study showed that, if the necessary conservation policies are not developed in the near future, the *P. pavonica* species in the regions identified on the Black Sea coast of Turkey will also be endangered.

Ethical Statement

Specific permission was not required to conduct sampling for this research. No experiments have been

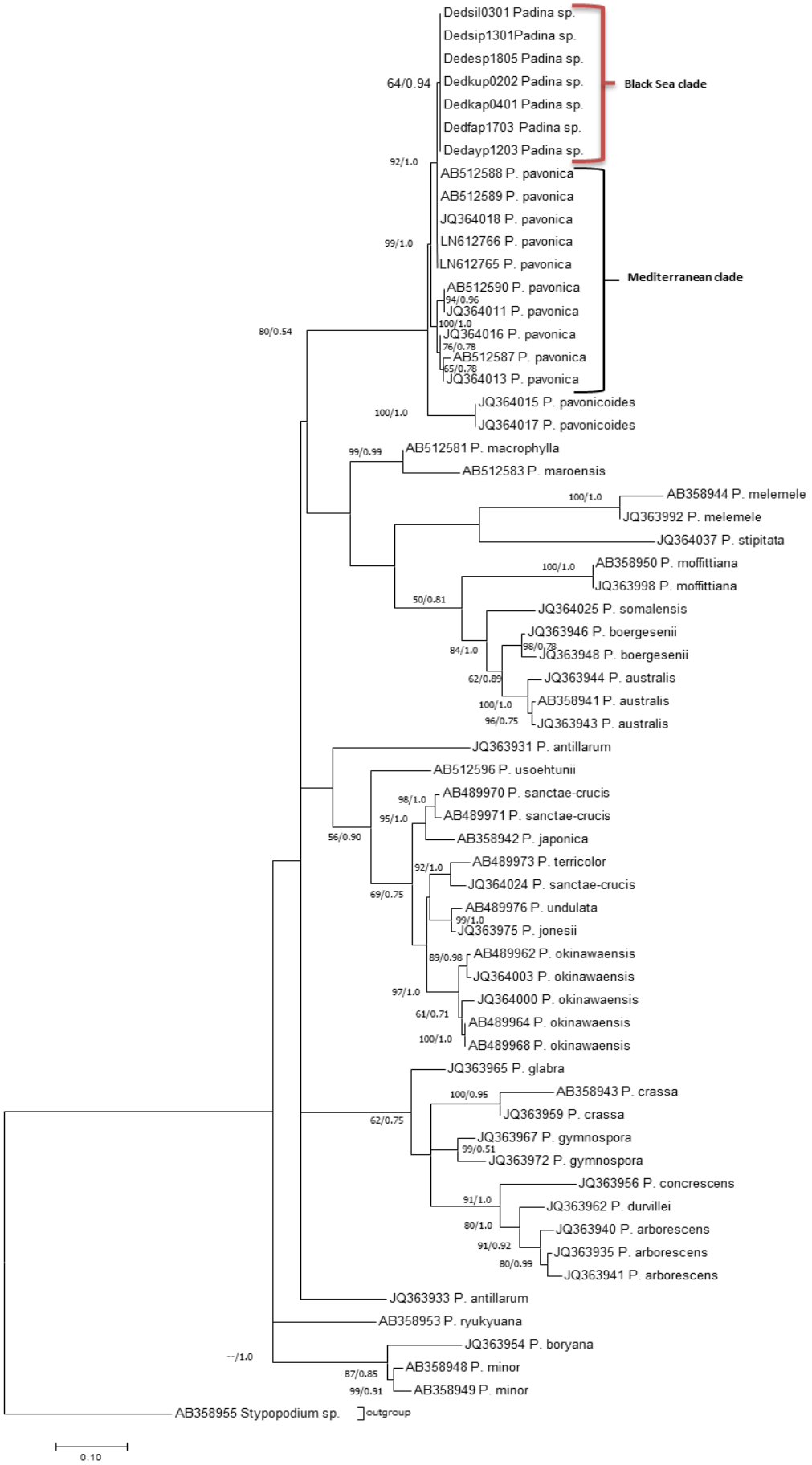

Figure 9. ML tree cox3 gene sequences. The first numbers at each node indicate bootstrap values for ML analysis, and the second (posterior probabilities) are for BI.

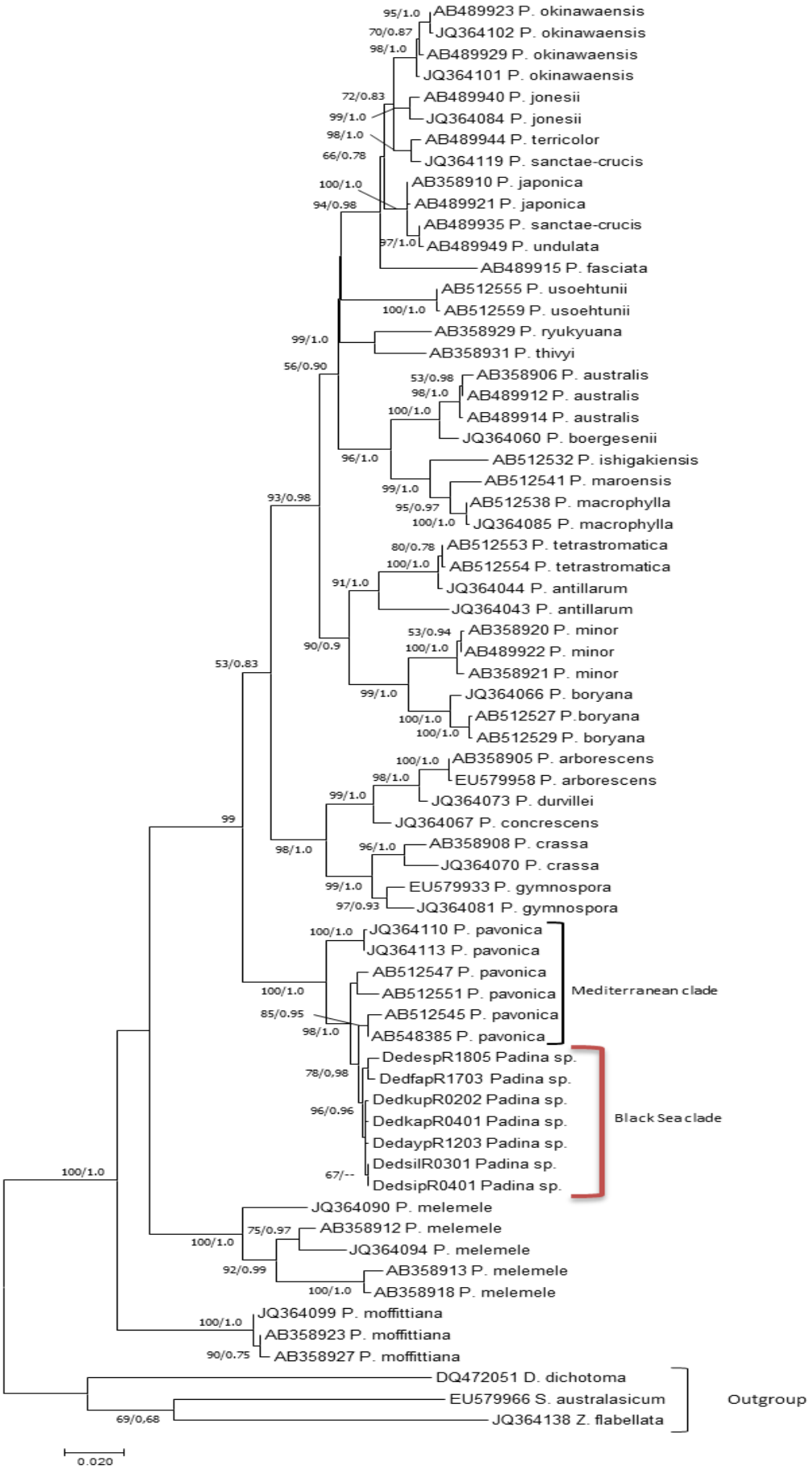

Figure 10. ML tree rbcL gene sequences. The first numbers at each node indicate bootstrap values for ML analysis, and the second (posterior probabilities) are for BI.

carried out using living organisms. The authors confirm that the field studies did not involve any endangered or protected species.

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Author Contribution

The research was designed by Gümüş and Gönülol. Laboratory and field studies were carried out by Gümüş. Morphological identification of samples was made by Gönülol and molecular characterization was made by Gümüş. All authors contributed to the writing of the article.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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