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RESEARCH ARTICLE

Evaluation of Siamese Twins of *Testudo graeca* Linnaeus, 1758

Ayfer Şirin[1], Serdar Düşen[1], Eyüp Başkale[1]

Abstract

Objective: In the present study, the morphological features of common tortoise (*Testudo graeca* Linnaeus, 1758) found in the Pamukkale ruins and determined to be two-headed Siamese twins, were investigated using computed tomography.

Material and Methods: The size and weight of the twin tortoise were measured and placed under care in a terrarium similar to their natural habitat. The anatomical features of the two headed Siamese twins were evaluated by three-dimensional volumetric computed tomography.

Results: The Siamese twins combined from the end of the shell have 4 anterior extremities and 2 posterior extremities. Even though tortoises were conjoined, they were reacting independently, but had to move together due to their abnormal shell structures. The Siamese twins weighed 13.2 grams when they were found. They survived 47 days in the terrarium. The tomography scanning showed that that one of the twins was fused from the 7th vertebrae and the other from the 8th vertebrae. Although the internal organ structure is not clear, it has been observed that they use a shared cloaca.

Conclusion: The anomalies rarely seen in reptiles can be caused by many factors, including environmental and genetic factors.

Keywords: Anatomy, Computed tomography, Common tortoise, Pamukkale ruins, Siamese twins

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Introduction

Developmental anomalies in living organisms have always aroused interest. Conjoined twins or congenital duplications (congenital anomalies) were first described for the family Chelonidae in the 17th and 18th centuries (Edwards, 1751). Although the description of the species cannot be performed properly in those records, it is thought that it is a hawksbill sea turtle and it is stated that it has a single body with two separate heads. The heads connected to the body with the fusion of two necks and moved independently. Since the late 20th century, conjoined twins or congenital duplications have been recorded in some domestic animals (Hiraga & Dennis, 1993; Mostafa *et al*., 2005), salamanders (Pereira & Rocha, 2004), reptiles (Harkewicz, 2002) and marine mammals (Dabin *et al*., 2004). Likewise, examples of congenital twinning have been observed in some species of the family Testudinidae (Dimitropoulos, 1985; Eckert, 1990; Hildebrand, 1938; Molina *et al*., 1996; de Silva *et al*., 2020; Tucker & Funk; 1976; Tucker, 1996; Tucker & Janzen 1997; Yntema, 1970). The only record from Türkiye belongs to the Green Turtle

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Chelonia mydas species detected on Samandağ beach, in Hatay (Sönmez *et al*., 2017).

The aim of this study is to investigate the morphological and anatomical characteristics of the Siamese twin common tortoise (*Testudo graeca*) specimen found in the Pamukkale ruins in Denizli using computed tomography (CT).

Materials and Methods

The Siamese twins were delivered to Pamukkale University, Animal Ecology Research Laboratory via the General Directorate of Nature Conservation and National Parks on November 10, 2021. There is no information about the hatching date of the tortoises. Straight carapace length (SCL) and straight carapace width (SCW) of the Siamese twins were measured (Başoğlu & Baran,1977; Tiar Saadi *et al*., 2022) with a digital calliper with a precision of 0.02 mm, and curved carapace length (CCL) and curved carapace width (CCW) were measured with a tape measure with a precision of 0.1 mm. The weight of the Siamese twins was weighed with a precision balance with a sensitivity of 0.01g (Fig. 1).

After a general health examination and external parasite control (Fig. 2) by a veterinarian, the tortoises were placed in a 25×40×30 cm terrarium prepared similarly to their natural habitat and placed under care. The terrarium was covered with bryophytes, natural grasses and leaves to create an optimal environment for the tortoises, all daily activities were observed every 24 hours, and changes were recorded. Since the tortoises are herbivores, they were fed with natural grasses, lettuce, cucumbers, etc. while they were in care. Drinking water and nutrients were provided without limitation and food was reachable at any time.

After the tortoises died, the specimen was preserved in alcohol, and three-dimensional images were taken using computed tomography (Vimago brand, high-resolution three-dimensional volumetric computed tomography) in a veterinary clinic.

Figure 1. Weight and carapace measurement of tortoises.

Figure 2. Cleaning and care of tortoises.

Results

Morphological observations: The individuals have 2 heads, 4 anterior extremities and 2 posterior extremities. The tortoises are referred to as right and left individuals from the dorsal view for ongoing definitions in this study (Fig. 3).

The right and left individuals conjoined were from the 8th marginal and supracaudal scutes of the right individual and from the 10th marginal and supracaudal scutes of the left individual. The supracaudal was unique and formed separately in each individual. It was calculated that the outer sites of the carapace of both individuals had 10 marginal plates, while we counted different numbers of marginal of the inner (connection) sites of the carapace (the right individual had 10 marginal plates and the left individual had 8). It was also observed that the right and left individuals had 4 costal plates on both sides and 5 vertebral plates, and these plates were not abnormal. On the other hand, both individuals had their own pair of gular, humeral, pectoral and abdominal scutes on the plastron. In detail, the posterior portion of the abdominal scute was deformed, and the conjoining started from this part. At the site of connection, the femoral and anal scutes fused in

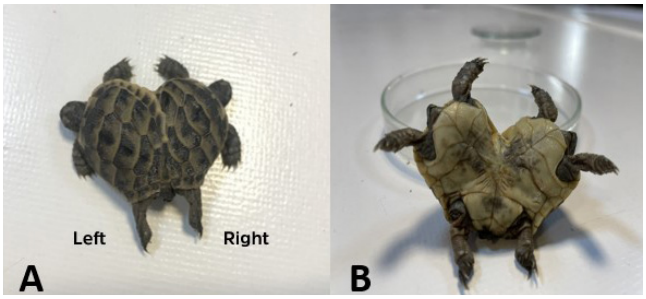

Figure 3. Dorsal (A) and ventral (B) views of tortoises.

both individuals, each with only one pair of these scutes. In addition, three ant heads with their mandibles belonging to the genus *Formica* were found attached to the skin of the tortoises. Two of them were located on the hind leg of the right individual, and the third one was located under the neck of the left individual.

Body measurements: The SCLs of the right and left individuals were 29.42 mm and 32.96 mm, while the SCWs were 25.75 mm and 28.55 mm, respectively. CCLs were 33.00 mm and 37.00 mm, while CCWs were 35.00 mm and 37.00 mm, respectively. The total body weight of both individuals was 13.2 g.

Observation on the thirtieth day at under care: Both individuals showed an enlargement in the width of the flat carapace due to growth, while their weights decreased by 0.1 g. The SCW measurements of the right individual increased from 25.75 mm to 30.16mm, and the left individual increased from 28.55 mm to 30.66 mm during this period. Therefore, the individual on the right grew by 4.41 mm and the individual on the left grew by 2.11 mm. The total weight of the two individuals decreased to 12.3 g until the first 15th day in terrarium conditions due to adaptation stress, and then it was measured as 13.1 g at the end of the first month.

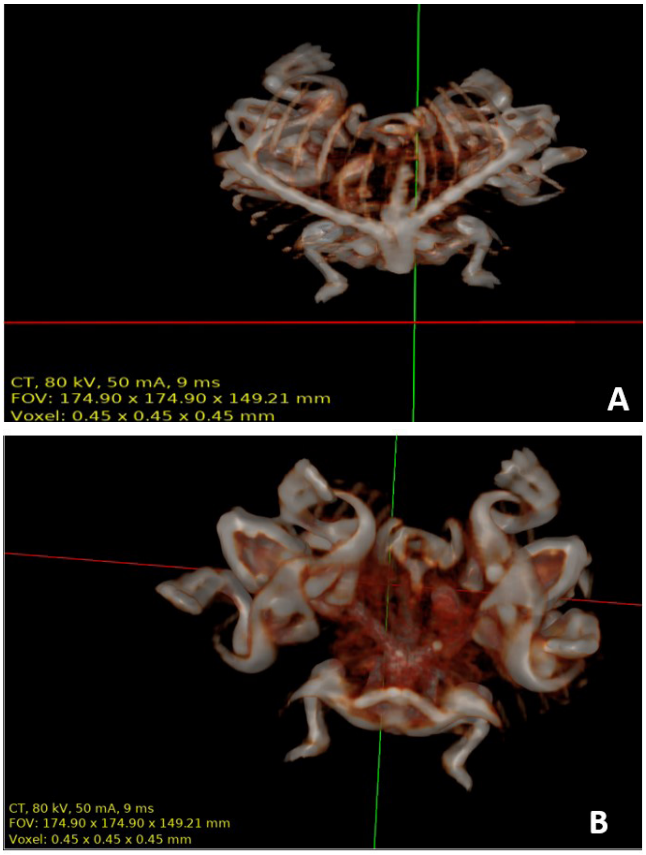

Figure 4. Dorsal CT image (A) and ventral CT image (B).

The results of computed tomography: It was observed that the skeleton in the anterior part of the two individuals was formed separately. In the posterior part of the individuals, it was determined that the right individual was connected from the 7th thoracic vertebra, and the left individual was connected from the 8th thoracic vertebra. They had common sacral and caudal vertebrae (Fig. 4). The tibia lengths of the individuals were measured as 6.9 mm and 5.16 mm, and the femur lengths were measured as 7.29 mm and 6.52 mm, respectively. The right individual was found to be longer than the left one.

Observation of some behaviour: Under care, the twin tortoises survived a total of 47 days in the terrarium, even though they showed self-feeding behaviour. Although the twins were able to respond independently, they were found to move together in one direction due to the conjoined and abnormal shell structure. The direction of movement is usually directed by the right individual that was stronger than the left individual. It was also recorded that both individuals moved towards the food separately.

Discussion

Anomalies in living organisms are not often observed due to the breeding and nesting behaviour of animals. General morphological descriptions of the two-headed tortoise specimens are available in the literature (Barbour, 1888; Cooper, 2009). However, there is no detailed information on internal anatomy. Due to their abnormal body structure, they are unlikely to survive in the wild, as they would have difficulty feeding in the natural environment and could be easily hunted. For instance, a twinning case was observed in *Geochelone elegans* in Sri Lanka. This tortoise in the national zoo did not eat any food given under observation and lived for only 12 days (de Silva *et al*., 2020), whereas the Siamese twin common tortoises in this study were capable of self-feeding and lived for 47 days in the terrarium.

Stockard (1921), Newman (1917, 1923) and others have noted that anomalies can occur and that temporary developmental arrest or regression at a critical stage of the twins' egg development can cause such differences. Interference with normal development or various natural factors can cause these anomalies. Stockard (1921), in his experiments cooling fish eggs during the cell division stages and limiting the oxygen demand, found that malformed embryos were formed as a result of irregular division.

Thus, it was concluded that environmental conditions such as sudden and sharp changes in temperature or oxygen deficiency in the habitat where the organisms spend their embryological period may cause these anomalies to occur (Stockard, 1921; Newman, 1923). It has also been suggested that various factors such as genetic defects and biotic and abiotic factors may play a role in the occurrence of these anomalies and malformations (Velo-Antón *et al*., 2011). It is very difficult to say anything about the cause of Siamese twinning because we have only one case. However, considering the factors mentioned above, the changes in the ecological environment during the incubation period and the effects of these changes on the embryonic development of the common tortoise, genetic factors and the combined effects of these factors may affect the formation of the Siamese twin. It would be beneficial to increase the number of studies on malformation or anomaly for more precise results and evaluations.

In conclusion, the general morphological characteristics of the Siamese twin common tortoises found in the Pamukkale ruins were determined in this study for the first time in the world. Especially up to the 21st century, the small number of individuals with anomalies in nature made it difficult to understand such anomalies. The study of developmental abnormalities in more reptile specimens will facilitate the understanding of such anomalies or malformations that may occur in the wild. Furthermore, when considering species conservation or area conservation strategies, an increase in the number of individuals with anomalies and malformations in a population will inform us about population or ecosystem health and enable early action to be taken.

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RESEARCH ARTICLE

Genetic Structure and Phylogenetic Analysis of *Liquidambar orientalis* Mill. (Altingiaceae) Populations Based on Non-Coded *psaA/ycf3* Intergenic Region in The Chloroplast Genome in Türkiye

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Abstract

Objective: The genus *Liquidambar*, is one of many woody genera with morphologically similar species in America, Southeast Europe, and Asia and is thought to have existed on Earth for about 65 million years. *Liquidambar orientalis* (Anatolian sweetgum tree) is distributed in southwestern Türkiye. With this study, it was aimed to reveal the sequence differences of the *psaA/ycf3* inter-gene space region of chloroplast DNA (cpDNA) in *L. orientalis* populations for the first time in the scientific literature.

Materials and Methods: In addition, the polymorphism levels, and the molecular evolution of the studied gene region of the *L. orientalis* populations were investigated. 154 samples were collected from 12 different populations belonging to four provinces, Muğla, Burdur, Antalya, and Aydın, and were studied and the *psaA/ycf3* gene encoded by chloroplast DNA was analysed by partial base sequence analysis.

Results: Haplotype diversity (h) and the average nucleotide diversity (π) values for all populations were found to be 0.52933 ± 0.011, and 0.00086 ± 0.0001, respectively. According to the results of our research, the gene flow level (N_m) among the populations was 1.40. F_{ST} values revealed statistically significant genetic differences between the Fethiye-Yanıklar population of Muğla province and other studied populations.

Conclusion: According to the results of the study, Fethiye-Yanıklar, Marmaris-National Park, and Fethiye-İnlice locations, where the highest genetic diversities detected among the studied populations, were found to be important in terms of conservation studies.

Keywords: *Liquidambar*, Sweetgum tree, cpDNA, Conservation genetics

Introduction

Global climate change can have significant impacts on populations in multiple ways. Climate change can lead to habitat loss, fragmentation, and alteration, which can reduce the size and connectivity of populations. This can lead to a reduction in genetic diversity and an increased risk of inbreeding and genetic drift. As temperatures and precipitation patterns change, plant, and animal species may shift their ranges to track suitable habitats. This can result in the loss of genetic diversity in populations that are left behind and the establishment of new populations with reduced genetic diversity. Climate change can also affect the ability of species to adapt to changing environmental

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conditions. Genetic diversity is essential for adaptation, and reductions in genetic diversity can limit the ability of species to respond to changing environmental conditions. As species shift their ranges, they may encounter other species and hybridize. This can result in the loss of genetic diversity in the parent species and the creation of new genetic combinations (Pauls *et al.,* 2013).

Conservation genetics is a field of study that uses genetic data to inform conservation efforts for threatened and endangered species. It involves the application of molecular genetic techniques to understand the genetic diversity, population structure, and evolutionary history of species, as well as to develop strategies for their conservation and management (Frankham, 2019). Conservation genetics is important because genetic diversity is essential for the long-term survival of populations and ecosystems. Genetic diversity provides the raw material for adaptation and evolution, and loss of genetic diversity can reduce the ability of populations to adapt to changing environmental conditions and increase the risk of extinction (Doğaç, 2008). Genetic diversity decreases from generation to generation due to factors such as genetic drift, migration, and selection. The effect of genetic drift is dependent on population size, and as genetic drift becomes dominant in small populations, genetic diversity is lost over time and the survival of a population is compromised (Beaumont & Wang, 2019). Wright (1931) proposed a parameter called effective population size (*Ne*) to measure the effect of many factors, such as population size, number of breeding individuals, sex ratio, variation in reproductive success, and non-random mating, on genetic drift. Low *Ne* means high loss of genetic variation, high inbreeding, low fitness, and low adaptation potential. Therefore, the conservation of populations with low *Ne* should be considered. Although demographic data can be used in *Ne* calculations, it is nearly impossible to obtain parameters describing the pedigree and demographics of wild populations. However, genetic drift and inbreeding effects can be determined using genetic marker data and converted into *Ne* estimates (Beaumont & Wang, 2019).

Undoubtedly, the first step in developing appropriate population conservation strategies is to determine the amount and structure of genetic diversity in populations. In the last decades, our knowledge of the genetic diversity of forest trees has improved through the use of molecular markers as alternatives and supplements to classical methods (Tong *et al.,* 2020). It has been noted that DNA sequence data can provide a more precise prediction of separation from allozymes, and studies have reported that both nuclear and chloroplast DNA sequence data are informative in analysing the phylogenetic relationships of discrete taxa (Crawford *et al.,* 1992). The *psaA/ycf3* gene region is a useful marker for studying the genetic diversity of plants because it exhibits a high degree of variation between species, which can be used to distinguish between closely related taxa. This region has been used in molecular phylogenetic studies to infer the evolutionary relationships among plant species and to reconstruct the Tree of Life (Shi *et al.,* 2001; Ickert-Bond & Wen, 2006).

Conservation genetics involves a variety of techniques, including DNA sequencing, microsatellite analysis, and population genetics modelling. These techniques can be used to identify genetically distinct populations, estimate effective population size, and assess the genetic health of populations (Hedrick, 2001). Conservation genetics has many applications, including the development of genetic management plans for endangered species, the identification of priority areas for conservation, and the monitoring of populations over time. It can also be used to identify threats to genetic diversity, such as habitat loss, fragmentation, and climate change, and to develop strategies to mitigate these threats (Willi *et al.,* 2022). Overall, conservation genetics is a critical tool for preserving biodiversity and ensuring the long-term survival of endangered species and ecosystems.

Conservation genetics can be used to protect endangered plant species in several ways. Conservation genetics can help identify genetically distinct populations of endangered plant species. These populations may have unique adaptations or genetic diversity that should be conserved. By identifying these populations, conservationists can develop targeted conservation strategies that focus on protecting these unique populations (Hedrick & Hurt, 2012). Conservation genetics can be used to assess the genetic diversity of populations of endangered plant species. This information can be used to determine the genetic health of populations and to identify populations that are at risk of extinction due to low genetic diversity. By identifying populations with low genetic diversity, conservationists can develop strategies to increase genetic diversity, such as habitat restoration or crossbreeding with genetically diverse populations (Doğaç, 2008). Conservation genetics can inform the development of breeding programs for endangered plant species. For example, genetic analysis can identify populations or individuals that are genetically diverse or have traits that are important for survival in a changing environment. This information can be used to maximize genetic diversity and adaptive potential in captive breeding programs (Witzenberger & Hochkirch, 2011).

Conservation genetics can help understand the structure of populations of endangered plant species, including their relationships to other populations. This information can be used to determine the best locations for translocations, which involve moving individuals to new locations to establish or augment populations (Moritz, 1999). Conservation genetics can be used to monitor changes in genetic diversity over time in endangered plant species. This information can be used to assess the success of conservation actions and to modify management strategies as needed (Willi *et al.,* 2022). Overall, conservation genetics can provide valuable information for developing effective conservation strategies for endangered plant species. By identifying key populations, assessing genetic health, and guiding conservation actions, conservation genetics can help ensure the long-term survival of endangered plant species and the ecosystems they inhabit.

The genus *Liquidambar* belongs to the family Altingiaceae, although previously species of this genus were generally considered within the *Hamamelidaceae*. The *Liquidambar* genus is the only genus in the Altingiaceae family with discrete distributions seen in Turkey, East Asia, and North America (Li *et al.,* 1997a). The distribution of closely related plant species across different geographies has long attracted the attention of both plant systematists and biogeographers (Wen & Zimmer, 1996). The origin and development of the Asian and North American divides have been extensively discussed by various researchers (Wen, 1999; Milne, 2004; Beatty & Pro van, 2010; Schmickl *et al.,* 2010).

The genus *Liquidambar* is distributed at approximately the same latitudes on Earth. They spread across America, Southeast Europe, and Asia. The species has four different species; *L. acalycina* (Chang sweetgum tree) located in Central and Southern China, *L. formosana* (Chinese frankincense) located in South China, North Korea, South Korea, Taiwan, Laos, and North Vietnam, *L. styraciflua* (American sweetgum tree) located at Southeast and Central America and Mexico and *L. orientalis* (Anatolian sweetgum tree) located at southwestern Turkey (Ickert-Bond *et al.,* 2005). Genetic divergence studies conducted on *Liquidambar* species using isozymes (Hoey & Parks, 1991, 1994) and molecular techniques (Li *et al.,* 1997a, b; Li & Donoghue, 1999; Shi *et al.,* 2001; Ichert-Bond & Wen, 2006; Özdilek *et al.,* 2012) showed that *L. orientalis* and *L. styraciflua* are phylogenetically closer than the others.

The genus *Liquidambar* is one of many woody genera with morphologically similar species on different continents and is thought to have existed on Earth for about 65 million years. Therefore, species of the genus *Liquidambar* are called relict species (Hoey, 1990; Hoey & Parks, 1991; Akman *et al.,* 1992). The fragmentation and spread of species in the genus *Liquidambar* can be attributed to a combination of geological events, climate change, seed dispersal mechanisms, and human influence. These factors have caused the isolation and divergence of sweetgum species, ultimately leading to the current distribution observed today (Sun *et al.,* 2019; Hoey & Parks, 1991; Özdilek *et al.,* 2012; Joannin *et al.,* 2007; Wang *et al.,* 2020; Ďurkovič & Lux, 2010).

Although some researchers studying *Liquidambar* argue that *L. orientalis* cannot be endemic to Turkey as *L. orientalis* is found outside the country, in northern Syria, the 12 Islands, and Rhodes Island, some researchers argue that this is not true and that *L. orientalis* species have been transported to distribution areas outside the country through culture. Despite these different views, it is generally accepted that *L. orientalis* species is an endemic species to Turkey (Acatay, 1963; Atay, 1985; Efe, 1987; Günal, 1994; İstek & Hafızoğlu, 1998; Alan & Kaya, 2003; Velioğlu *et al.,* 2008). *Liquidambar orientalis* species has two different varieties in Turkey. These varieties are *L. orientalis* var. *orientalis* and *L. orientalis* var. *integriloba* Fiori (Doğaç, 2008). Relict endemic *L. orientalis* species spreads in Western Anatolia. Although it mainly spreads in Köyceğiz, Marmaris, Fethiye, Ula, and Dalaman districts in Muğla province, there are *Liquidambar* trees in certain regions in Aydın, Denizli, Antalya, Burdur and Isparta provinces (Acatay, 1963; Atay, 1985; Efe, 1987; Günal, 1994; İstek & Hafızoğlu, 1998; Alan & Kaya, 2003; Velioğlu *et al.,* 2008; Aydıngöz & Bulut, 2014).

Liquidambar orientalis is a long-lasting tree and can live for about 200-300 years. It has a shallow root structure. The tree has a thin and long body structure when it is young. A thicker trunk structure is observed in older trees (Günal, 2006; Doğaç, 2008). In addition to its ecological value, *Liquidambar* is an important species with its everyday use and economic value. There are many different areas where *Liquidambar* trees have been used from past to present.

Liquidambar is used in the construction of furniture, houses, and ornaments, and as a landscape material. As a wood structure, it is highly resistant to rotting that may be caused by water (Acatay, 1963; Atay, 1985; Bozkurt *et al.,* 1989). Today, sweetgum oil is still used in diseases such as asthma, bronchitis, lung disease, ulcer, and gastritis. In addition to these, it is widely used among people to relieve rheumatic pain due to its analgesic properties. Besides, it is

believed to have an antibacterial effect and allows wounds to heal quickly without leaving scars (Huş, 1949; Örtel, 1988; Acar, 1989; Bozkurt *et al.,* 1989; İstek & Hafızoğlu, 1998; Aydıngöz & Akbulut, 2014). Sweetgum oil is used in many natural fragrance perfumes and is known to effectively remove the smell of sweat. Sweetgum oil is used as a fixative in perfumery and prevents volatile fragrances from flying for a long time. For this reason, it is a highly preferred raw material in the perfume industry (Hus, 1949; Örtel, 1988; Acar, 1989; Bozkurt *et al.,* 1989; İstek & Hafızoğlu, 1998; Aydıngöz & Akbulut, 2014).

In its native range, *L. orientalis* is an important component of many forest ecosystems, providing habitat and food for a variety of wildlife species. However, like many tree species, it is threatened by habitat loss and overexploitation, and conservation efforts are needed to ensure its survival. The area of *Liquidambar* forests has been greatly reduced due to fires in recent years, the cutting down of trees for agricultural and tourism purposes, and the unconscious destruction of trees to produce sweetgum oil (Ürker *et al.,* 2014). While the range of distribution of *Liquidambar* forests was 7,000 hectares in 1947, these areas have shrunk over the years. These areas were recorded as 6,312 hectares in 1949, 4,316 hectares in 1955, 1,337 hectares in 1980, 1,215 hectares in 1988, and 3,200 hectares in 2002. Today, the distribution area of *Liquidambar* is thought to be in the range of 1,500-2,000 hectares (Ürker *et al*., 2014; Arslan & Şahin, 2016). In recent years, many decisions have been made to protect the sweetgum forest lands, and steps have been taken to increase the distribution areas of sweetgum forests (Ürker *et al.,* 2014).

The general purpose of this study is to determine the level and structure of genetic diversity in 12 populations of *L. orientalis* by using the polymorphic *psaA/ycf3* region of chloroplast DNA (cpDNA) for the first time in Turkey. Since the *L. orientalis* populations are a good example of populations that have been fragmented recently and very quickly, the findings to be obtained are increasing rapidly in nature as a result of intense human activities and are important in terms of their contribution to the literature in the fields of population and conservation genetics regarding the genetics of such populations. Given the suggestions presented following the findings, it is believed that the development of conservation programs suitable for the characteristics of the region for the species and their immediate implementation are important in terms of the last goal for implementation. We also investigate the molecular diversity pattern of *L. orientalis* populations by cultivar and geographic region to provide additional data to address cultivar and species-level taxonomy problems of *L. orientalis*.

Materials and methods

Plant material

To obtain a complete representation of the natural distribution of Eastern Sweetgum, 12 natural populations were identified from different regions in southwestern Turkey in collaboration with the Turkish Ministry of Environment and Forestry, Forest Tree Seeds, and Tree Growth Research Directorate (Fig. 1, Table 1). Transects

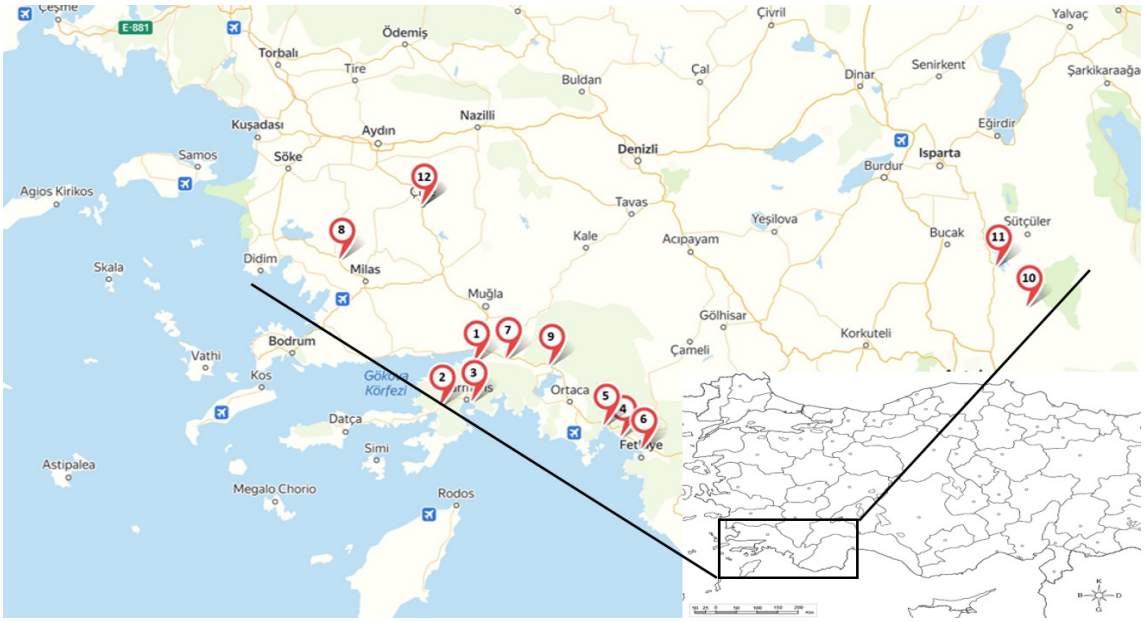

Figure 1. *Liquidambar orientalis* populations that were studied in the field.

(sampling units extending between two different points in the study area) were taken at different points so that the most homogeneous sampling within the determined population would be achieved. These transects were collected from young leaves, preferably on older trees, at intervals of 50-80 meters depending on population size. The samples were placed in ice boxes with ice molds, and the samples were brought to Muğla Sıtkı Koçman University, Faculty of Science, Plant Biotechnology laboratory and stored at -80 °C until DNA extraction.

Table 1. Information on the samples collected and the locations where the samples were collected.

LOCATION NUMBERS	LOCATION INFORMATION	Variety information	Number of Sequenced Individuals
1	Marmaris - Çetibeli	*L. orientalis* var. *integriloba* Fiori	10
2	Marmaris - Değirmenyanı	*L. orientalis* var. *integriloba* Fiori	21
3	Marmaris - Milli Park	*L. orientalis* var. *integriloba* Fiori	10
4	Fethiye - Günlüklü	*L. orientalis* var. *orientalis*	10
5	Fethiye - İnlice	*L. orientalis* var. *orientalis*	10
6	Fethiye - Yanıklar	*L. orientalis* var. *orientalis*	9
7	Ula - Kızılyaka	*L. orientalis* var. *integriloba* Fiori	22
8	Milas - Selimiye	*L. orientalis* var. *orientalis*	10
9	Köyceğiz - Toparlar	*L. orientalis* var. *integriloba* Fiori	22
10	Antalya- Gebiz	*L. orientalis* var. *integriloba* Fiori	10
11	Burdur- Bucak	*L. orientalis* var. *integriloba* Fiori	11
12	Aydın- Çine	*L. orientalis* var. *orientalis*	9
Total			**154**

DNA Extraction, PCR amplification, and DNA sequencing

Total genomic DNA was isolated from leaf tissues by using a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle, 1987). Within the scope of the study, a total of 154 sequences were obtained from 12 locations, and the *psaA/ycf3* gene encoded by chloroplast DNA was analysed by partial base sequence analysis. The amplification of the relevant region and sequencing was performed by amplifying with PG1 (5'-CATTCCTCGAACGAAGTTTTTACGGGATCC-3') and PG2R (5'-TCCCGGTAATTATATTGAAGCGCATAATTG -3') primers (Ickert-Bond & Wen, 2006). PCR reactions were performed by the PCR conditions (Shi *et al.,* 2001; Ickert-Bond & Wen, 2006) for the relevant region; the first denaturation step was at 94°C for 5 min, then at 94°C for 1 min, at 54°C for 1 min, at 72°C for 1 min and the last step was carried out at 72°C for 10 minutes, with a total of 35 cycles. Sequencing was conducted on an ABI 310 genetic analyser automatic sequencer (Applied Biosystems) by BM Labosis.

Data Analyses

The 720-bp portion of *psaA/ycf3* cpDNA sequences from 154 *L. orientalis* samples were acquired in the present study and previously published chloroplast *psaA/ycf3* gene sequences (GenBank accession numbers DQ352230-DQ352257) were aligned using CLUSTALW in MEGA 7 software (Kumar *et al.,* 2016). The sequences were analyzed by grouping them into two datasets. The first dataset contained only the sequences of *L. orientalis* from the current study. The second dataset included both studied *L. orientalis psaA/ycf3 s*equences and sequences from *Liquidambar* species available from the GenBank. The basic molecular diversity statistics (number of variable sites, average number of nucleotide differences between haplotypes, number of haplotypes, number of parsimony-informative sites, haplotype diversity) were performed with Dnasp (ver. 5.0) (Librado & Rozas, 2009). Descriptive statistics, namely divergence within species, genetic distance, transition, and transversion, were performed for *Liquidambar* species as well as among varieties and geographical regions for *L. orientalis* using MEGA 7 software (Kumar *et al.,* 2016). The Analysis of Molecular Variance (AMOVA) test was carried out with Arlequin version 3.5 (Excoffier *et al.,* 2007). Median-joining networks of haplotypes, with the inclusion of previously defined haplotypes, were constituted by using NETWORK (ver. 4.6) (Bandelt *et al.,* 1999; Polzin & Daneschmand, 2003).

Results

The *psaA/ycf3* sequences from natural *L. orientalis* populations from Turkey and other *Liquidambar* species

find out that the number of variable and conserved regions differentiated between the species, meanwhile the GC contents did not change significantly (32.1% to 32.8%). Among the analyzed species, *L. orientalis* has the most variable and least conserved sites. The parsimony-informative sites were found as 4 in *Altingia*, 2 in *L. orientalis,* and 0 in *L. macrophylla*, *L. styraciflua*, *L. acalycina*, *L. formosana,* and *Semiliquidambar*. The transition to transversion ratio was highest in *Semiliquidambar* and lowest in *L. orientalis* (Table 2a). The highest number of parsimony-informative sites was found in *Altingia* and *L. orientalis* (4 and 2 respectively). Within the varieties *L. orientalis* var. *orientalis* had the least conserved sites and highest variable sites, parsimony-informative sites, and transition rates, while *L. orientalis* var. *integriloba* had the lowest variable and parsimony-informative sites and highest transversions (Table 2b).

Six haplotypes were obtained in a variable sequence form in a total of 154 individuals studied. New haplotypes were deposited in GenBank under accession numbers OR365068- OR365073 (available after August 01, 2023). It was found that 4 of these haplotypes were unique to Turkey, and 1 of them was common with the haplotype in the previous study (Ickert-Bond & Wen, 2006). A 720 bp sequence analysis of the *psaA/ycf3* intergenic spacer region of 12 populations of *L. orientalis* (a total of 154 individuals) was performed. 6 haplotypes were found in a total of 154 samples belonging to the 12 populations studied (Fig. 2).

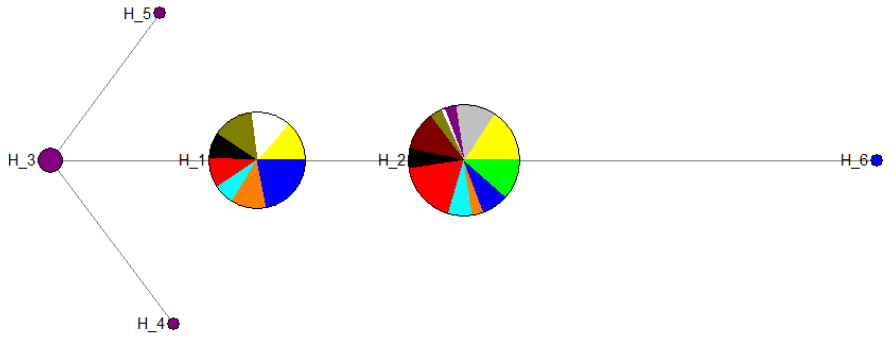

Figure 2. Network analysis result of the haplotypes of the *psaA/ycf3* inter-gene space region. (Toparlar: Yellow, Kızılyaka: Red, Çetibeli: Green, National Park: Black, Değirmenyanı: Blue, Daily: Orange, İnlice: Ice blue, Selimiye: Grey, Burns: Purple, Gebiz: Brown, District: Olive green and Çine: Shown in white).

Table 2. cpDNA *psaA/ycf3* gene molecular diversity values; a) *Liquidambar*, *Semiliquidambar*, and *Altingia* species (data obtained from this work and Genbank), b) *Liquidambar orientalis* (data contained only in this study)

(a) Characteristics of cp DNA *Psa-Ycf* gene region	***L. orientalis***	***L. macrophylla***	***L. styraciflua***	***L. acalycina***	***L. formosana***	***Semiliquidambar* sp.**	***Altingia* sp.**	**Overall**
Sequence length	720	720	720	720	720	720	720	720
Number of singleton sites	4	0	0	0	0	1	1	5
Number of parsimony-informative sites	2	0	0	0	0	0	4	12
Number of conserved sites	714	720	720	720	720	719	715	703
Number of variable sites	6	0	0	0	0	1	5	17
GC content range (%)	32.1	32.6	32.6	32.6	32.8	32.7	32.6	32.3
Transitions (%)	16.95	33.33	33.33	33.33	33.33	99.58	49.99	26.02
Transversions (%)	83.05	66.67	66.67	66.67	66.67	0.42	50.01	73.98
Transition/transversion bias (R)	0.193	0.45	0.45	0.45	0.45	187.95	0.96	0.33

(b) Characteristics of cp DNA *Psa-Ycf* gene region	***L. orientalis* var. *orientalis***	***L. orientalis* var. *integriloba***	**Overall**
Sequence length	720	720	720
Number of singleton sites	2	2	4
Number of parsimony-informative sites	2	1	2
Number of conserved sites	716	717	714
Number of variable sites	4	3	6
GC content range (%)	32.1	32.1	32.1
Transitions (%)	27.23	4.65	17.13
Transversions (%)	72.77	95.35	82.87
Transition/transversion bias (R)	0.35	0.05	0.20

Table 3. Distribution and frequencies of haplotypes of the *psaA/ycf*3 intergene space region of *L. orientalis* populations.

Haplotype/ Populations	Köyceğiz Toparlar	Ula Kızılyaka	Marmaris Çetibeli	Marmaris Millipark	Marmaris Değirmenyanı	Fethiye Günlüklü	Fethiye İnlice	Fethiye Yanıklar	Milas Selimiye	Antalya Gebiz	Burdur Bucak	Aydın Çine
H1	8 (0.364)	6 (0.273)		5 (0.5)	13 (0.619)	7 (0.7)	4 (0.4)				8 (0.727)	8 (0.889)
H2	14 (0.636)	16 (0.727)	10 (1)	5 (0.5)	7 (0.333)	3 (0.3)	6 (0.6)	3 (0.333)	10 (1)	10 (1)	3 (0.273)	1 (0.111)
H3								4 (0.444)				
H4								1 (0.111)				
H5								1 (0.111)				
H6					1 (0.0476)							

Haplotype 2, which contains 57.14% of the total 720 base sequences, was found to be common to all populations and is considered the inherited haplotype for our country. It was observed in all populations except 4 populations in the other high-frequency Haplotype 1 (38.31%). The other haplotypes had a low frequency. The frequency of Haplotype 3 was 2.59%, and it was observed to be specific to the Muğla Fethiye Yanıklar population. The frequencies of Haplotype 4 and Haplotype 5 are 0.65 and are specific to the Muğla Fethiye Yanıklar population. The frequency of haplotype 6 was also 0.65, and it was observed to be specific to the Muğla Marmaris Değirmenyanı population (Table 3).

Haplotypes are similar to each other, with base changes ranging from 1 to 6 nucleotides (no deletion or insertion observed). Genetic diversity data for all populations are presented in Table 4. Haplotype diversity (*h*) ranges from 0.0000 (Selimiye, Çetibeli, and Çine) to 0.75000 (Yanıklar). The haplotype diversity (*h*) value for all populations is 0.52933 ± 0.011. The nucleotide diversity (π) between haplotypes ranges from 0.0000 (Selimiye, Çetibeli, and Cine) to 0.0021 (Yanıklar) and the average nucleotide diversity (π) for all populations is 0.00086 0.0001. Although more samples were taken from Toparlar, Değirmenyanı, and Kızılyaka populations, the haplotype numbers in the populations ranged from 2 (Toparlar and Kızılyaka) to 3 (Değirmenyanı). All populations except Selimiye, Çetibeli, and Cine populations showed relatively high levels of genetic variation. There are 6 polymorphic regions in 720 base pairs (bp) (0.83% of the total length); 4 of these 6 regions are singleton, that is, the mutation occurs in a single haplotype base sequence, and 2 of them are determined as parsimony-informative regions. The G+C content was 0.321 and Tajima's D value was -0.8877.

Genetic relationships between populations were determined by comparing F_{ST} (genetic differentiation coefficient between species) values. F_{ST} values showed that the Yanıklar population differed significantly from other populations genetically. While among the *L. orientalis* populations, the Cine population, and the populations with the highest genetic differentiation were Gebiz, Çetibeli, and Selimiye populations (0.88166), the populations with the lowest genetic differentiation, although not statistically significant, were observed between the Bucak and Günlüklü populations with a value of -0.10351 (Table 5). The gene flow level *L. populations* (*Nm*) was observed as 1.40. The main group was formed based on the presence of two varieties of the *L. orientalis* species. Accordingly, Group 1 (*integriloba* variety) was formed as Muğla Toparlar, Muğla Değirmenyanı, Burdur Bucak, Antalya Gebiz, Muğla National Park, Muğla Kızılyaka and Muğla Çetibeli, Group 2 (*orientalis* variety) was formed as Muğla Selimiye, Muğla Yanıklar, Muğla İnlice, Muğla Günlüklü and Aydın Çine. As a result of the AMOVA analysis of the species, it was concluded that the main contribution to the genetic variation was caused by the variation among individuals with 71.52%. The genetic variation between the groups was observed to be low with -0.46% (Table 6).

Table 4. Haplotype diversity in *L. orientalis* populations. N: Sample number, Hp: Haplotype number, *h*: Haplotype diversity, π: nucleotide diversity.

Populations	N	Hp	*h*	π
Muğla Toparlar	22	2	0.48485	0.00067
Muğla Selimiye	10	1	0.00000	0.00000
Muğla Yanıklar	9	4	0.75000	0.00201
Muğla Değirmenyanı	21	3	0.52857	0.00095
Muğla Mill Park	10	2	0.55556	0.00077
Muğla Kızılyaka	22	2	0.41558	0.00058
Muğla İnlice	10	2	0.53333	0.00074
Muğla Günlüklü	10	2	0.46667	0.00065
Muğla Çetibeli	10	1	0.00000	0.00000
Antalya Gebiz	9	2	0.22222	0.00031
Burdur Bucak	11	2	0.43636	0.00061
Aydın Çine	10	1	0.00000	0.00000
Average	**12.83**	**2**	**0.52933**	**0.00086**

The relationships between the previously published base sequences and the haplotypes obtained from the study (GenBank access numbers DQ352230-DQ352257) were determined using the Network program (Fig. 3). Network analysis based on haplotype provides a better understanding

Table 5. The F_{ST} values of the *psaA/ycf3* intergenic gap region of *L. orientalis* populations and the significance of differentiation according to these values (*P < 0.05)

Populations	Toparlar	Değirmenyanı	Bucak	Gebiz	Millipark	Kızılyaka	Çetibeli	Selimiye	Yanıklar	Çine	İnlice	Günlüklü
Toparlar	****											
Değirmenyanı	0.06818	****										
Bucak	0.17603	-0.04417	****									
Gebiz	0.24063*	0.42731*	0.68824*	****								
Millipark	-0.03733	-0.04692	0.00927	0.44444*	****							
Kızılyaka	-0.02785	0.15317*	0.29631*	0.15466	0.03861	****						
Çetibeli	0.24063	0.42731*	0.68824*	0.00000	0.44444*	0.15466	****					
Selimiye	0.24063	0.42731*	0.68824*	0.00000	0.44444*	0.15466	0.00000	****				
Yanıklar	0.40123*	0.30939*	0.30246*	0.55326*	0.28810*	0.44884*	0.55326*	0.55326*	****			
Çine	0.37135*	0.05699	-0.02507	0.88166*	0.21493	0.49233*	0.88166*	0.88166*	0.33824*	****		
İnlice	-0.07536	0.00838	0.11239	0.33333	-0.08889	-0.03821	0.33333	0.33333*	0.31318*	0.33870*	****	
Günlüklü	0.14036	-0.05916	-0.10351	0.66667*	-0.02222	0.25977	0.66667*	0.66667*	0.28665*	-0.00411	0.07407	****

Table 6. Molecular analysis of variance (AMOVA) of the *psaA/ycf3* intergene gap region.

Structure	Source of Variation	% of total variance	Fixation index
Two Variety	Between groups	-0.46	*FCT*= -0.00458
	Between populations / within groups	28.94	*FSC* = 0.28808
	Within individuals	71.52	*FST*=0.28482

of the phylogenetic relationships between *Liquidambar*, *Semiliquidambar*, and *Altingia* species in Turkey and the world, and the status of the genetic structure. As a result of the network analysis, *Semiliquidambar* and *Altingia* samples exhibited a strong phylogeographic structure by showing a very good structure and differed quite well from *L. orientalis* haplotypes. However, while *L. acalycina* haplotypes were clustered with *Semiliquidambar* and *Altingia* haplotypes, and *L. formosana* haplotypes were clustered with *Altingia* haplotypes. While *L. orientalis* samples were clustered closely with *L. macrophylla* and *L. styraciflua* samples, both the gene bank and the haplotypes obtained from the study showed a strong structure.

Discussion

While there is not much information available specifically on the genetic diversity of this species, studies of related species in the family Altingiaceae have shown that there can be significant genetic variation within and between populations. This genetic diversity is important for maintaining the long-term viability of populations and ecosystems, and efforts to conserve *L. orientalis* through habitat protection and restoration are essential for ensuring its survival. As a result of literature reviews, it has been determined that polymorphism determination studies for *L. orientalis* were carried out with various methods. However, a limited number of samples (Ickert-Bond & Wen, 2006) has been detected among these methods investigating the polymorphic *psaA/ycf3* region of the chloroplast genome. Since the chloroplast genome has been studied for the first time in *L. orientalis* populations in terms of this region, it is essential to compare the results obtained from these different methods with the results of our study in terms of revealing the genetic variation and polymorphism within and between populations. According to the accepted mutation rate and base substitution in plant chloroplasts, the evolutionary divergence time of the species of the *Liquidambar* genus was predicted to be 8.6 MYA. This divergence time corresponds to the Late Miocene of the Tertiary period. The divergence time of *L. styraciflua* and *L. orientalis* was estimated about a million years later. The results support the probability of a more extended linkage

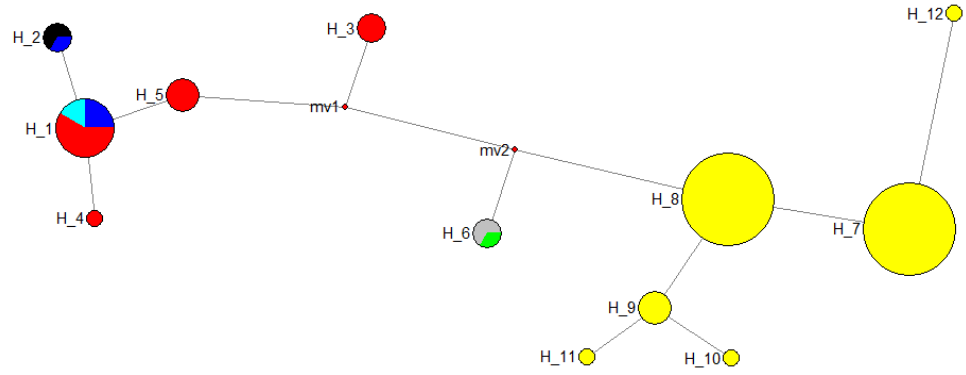

Figure 3. Network graph obtained from analysis of the cpDNA *psaA/ycf3* intergene gap region. (*Semiliquidambar* sp.: Blue, *Altingia* sp.: Red, *L. orientalis*: Yellow, *L. acalycina*: Ice blue, *L. formosana*: Black, *L. macrophylla*: Grey, *L. styraciflua*: Green).

between *L. styraciflua* and *L. orientalis* by way of the North Atlantic Land Bridge (Hoey & Parks, 1991; Özdilek *et al.,* 2012). For floristic swaps between eastern Asia and eastern North America through the North Atlantic Land Bridge, two ways were recommended. One of them is between Europe and east Asia, and the other one is between eastern North America and Europe (Tiffney, 1985, 2000; Wen, 1999). In the early Tertiary, a land connection between Europe and eastern North America took place with Greenland, and this connection seems like a more acceptable North Atlantic Land Bridge connection. This situation may have enabled the continuousness of *Liquidambar* species (Wen, 1999; Jiao & Li, 2009). The pollen and fossil studies showed that the *L. orientalis* was widespread in Europe (Joannin *et al.,* 2007; Sadori *et al.,* 2010; Worobic & Gedl, 2010; Hristova & Ivanov, 2009; Sakala & Prive-Gill, 2004) and Turkey (Kasapligil, 1976) before the Pleistocene. The Miocene cooling may have hindered the continuousness of North American and Turkish species of the *Liquidambar* genus.

The results obtained from the studies support the sister group relationship between the western Asian *L. orientalis* and the eastern North American *L. styraciflua*, in parallel with the results previously obtained with allozyme (Hoey & Parks, 1991, 1994) and DNA sequence data (Li *et al.,* 1997a, Li *et al.,* 1997b; Li & Donoghue, 1999, Shi *et al.,* 1998; Shi *et al.,* 2001; Ickert-Bond & Wen, 2006; Özdilek *et al.,* 2012). *Liquidambar macrophylla* Oersted from Central America is generally considered congener with *L. styraciflua*. Results from sequence analyzes of cpDNA show that North American sweetgum (*L. styraciflua*) and East Asian sweetgum (*L. formosana* and *L. acalycina*) are the most distant intercontinental species. In the obtained network graph, it was observed that *L. orientalis* samples were closely related to *L. styraciflua* and *L. macrophylla* samples, and *L. acalycina* and *L. formosana* were closely related to *Semiliquidambar* and *Altingia* species (Fig. 3). Hoey & Parks (1991) estimated divergence times between *Liquidambar* species based on isozyme data using the methods of Nei (1987) and Thorpe (1982) and *L. styraciflua* differentiated from *L. orientalis* approximately 7 mya (Nei's) or 13 mya (Thorpe's) and 10 mya (Nei's) or 17 mya (Thorpe's) ago from east Asian *L. formosana*. Our results are consistent with the studies carried out with sweetgum species (Hoey & Parks, 1991, Li *et al.,* 1997a; Li & Donoghue, 1999; Shi *et al.,* 2001; Ickert-Bond & Wen, 2006; Özdilek *et al.,* 2012), and this result should be a consequence of longer connection between *L. orientalis* and *L. styraciflua* through the North Atlantic Land Bridge.

Plant genetic sources are one of the basic components of biodiversity. Plant conservation genetics ensures instruments to lead restoration and conservation endeavours, monitor and measure achievement, and eventually reduce extinction hazards by preserving species as living creatures capable of evolving in spite of changing conditions (Kramer & Havens, 2009). Throughout the years, conservation genetics has voluminously developed our understanding of processes that are associated with small population size and habitat fragmentation. Inbreeding, which is admitted a significant phenomenon in conservation genetics, is thought to be the intermediary between the loss of variants owing to genetic drift and the impacts of this loss on the possibility of extinction (Ouborg *et al.,* 2010). Even though conservation genetics has concentrated on sequence variation, much less attention has been paid to variation in gene expression. It is largely unclear how gene expression is altered by changes in regulatory mechanisms as a function of genetic drift and inbreeding. In addition to this, it has been suggested that gene expression may be closer to phenotypic variation compared to the gene sequences. Environmental stress, such as increased temperature, drought, and lack of nutrients, can cause changes in genomic pathways, both in animals and plants (Ouborg *et al.,* 2010).

In previous studies conducted to determine polymorphism in *L. orientalis* populations, different markers were used (Öztürk, 2008, Doğaç, 2008, Velioğlu *et al.,* 2008, Yüzer, 2019). Eighteen polymorphic loci were found in 320 individuals belonging to 14 populations where polymorphism with 7 different isozymes was investigated (Öztürk, 2008). In another study, a total of 453 loci were determined as a result of the screening performed using 30 RAPD primers on 320 individuals belonging to 14 populations, and an average of 15 polymorphic loci were determined for each primer (Doğaç, 2008). As a result of another study using ISSR primers, an average of 27 polymorphic loci were found (Yüzer, 2019). The high level of genetic diversity determined according to the results of our research indicates that chloroplast DNA is more suitable in polymorphism determination studies for this species.

While the *Nm* value was given as 0.265 for the species that can spread their seeds and pollen over short distances and for the self-pollinated species, the *Nm* value was determined as 4,750 for the species that can spread their seeds and pollen over long distances (Hamrick *et al*., 1990). In addition, while the gene flow level (*Nm*) of 0.50 is considered a critical value, it has been reported that

higher values indicate the opposite results of genetic drift. According to the results of our research, the gene flow level (*Nm*) was determined as 1.40. The fact that this value is above the value of 0.265 indicates that the individuals of the population have pollinated with trees at not very close distances. If the gene flow level (*Nm*) value is less than 4,750, it is an indication that fertilization originating from long distances is not at high levels or does not occur at all.

In Turkey, there are two varieties of *L. orientalis*, namely *L. orientalis* var. *orientalis* and *L. orientalis* var. *integriloba.* Divergence at the intra-species level and relatively low genetic diversity propose that *L. orientalis* populations share ancestral polymorphism from which two varieties may have evolved. Additionally, anthropogenic factors, more recent climatic alterations, and breeding systems may also have conducted to divergence and low genetic diversity. In this study, the three populations with the highest level of genetic diversity were identified as Yanıklar (0.7500), National Park (0.5556), and İnlice (0.53333), respectively. Species must be protected in order to preserve the genetic diversity within the species and to transfer these variations to the next generations. This could be locations evaluated as the important genetic diversity centre and refugiums for the species. Therefore, it is recommended that priority should be given to these three populations in a conservation program to be initiated for the *L. orientalis* species.

In the studies conducted by Doğaç (2008) and Öztürk (2008) using RAPD and isoenzymes, the populations with the highest genetic diversity were reported as Marmaris Çetibeli, Köyceğiz Toparlar and Ula Kızılyaka and Toparlar, Kızılyaka, and İnlice, respectively. However, in the study conducted by Yüzer (2019) using the ISSR marker, it was reported that the three populations with the highest genetic diversity were Fethiye Günlüklü, Fethiye İnlice, and Ula Kızılyaka populations. The studies performed by Özdilek (2007) and Or (2007) using matK and trn regions are the most similar studies in terms of a method to our research using chloroplast DNA regions. Özdilek (2007) reported that the populations with the highest polymorphism are Pamucak, Günlükbaşı, and Toparlar. Yatağan, Serik, and Çetibeli populations were given by Or (2007) as the populations with the highest polymorphism. Considering that some locations and marker systems used in the studies are different and the results obtained from the studies are different, it shows that studying many gene regions by taking samples from all locations in the conservation programs to be planned will yield more successful results.

As a result of the AMOVA analysis of the species in this study, it was concluded that the main contribution to genetic variation was the variation between individuals by 71.52%. This result was similar to the studies conducted by (Özdilek, 2007) and (Or, 2007), using *matK* and *trn* regions; (Özdilek, 2007) reported the variation among individuals as 86.10%, and (Or, 2007) as 91.97%. In addition, no variation was observed between the varieties in AMOVA results, which is the same as the result we obtained in our study.

Currently, *psaA /ycf3* gene sequences from databases for *Liquidambar* species are limited. The more *psaA /ycf3* gene sequences for *Liquidambar* species that become available in databases, will help to better understand the phylogenetic relationship among *Liquidambar* species. As a result, in this study, the *psaA /ycf3* region was used for the first time to determine the genetic diversity of *L. orientalis* species. Since *L. orientalis* species is a relict endemic species in Turkey, it is very important for the species to continue its existence in the future. Therefore, it is recommended to start research on the conservation of *L. orientalis* species as earliest as possible and strengthen the measures taken. Considering the previous studies on the *L. orientalis* species, it is clear that the forest areas of the species continue to decrease, and it is not known exactly how far it will spread. So, first of all, the exact distribution area of *L. orientalis* species, which is one of the important riches of our country, should be determined by authorized persons and institutions, and then these forests should be quickly taken into *in-situ* conservation. After the study, it was recommended that Fethiye-Yanıklar, Marmaris-National Park, and Fethiye-İnlice locations, where the highest genetic diversity was detected in the studied populations, should be taken under protection as a priority. Though some conservation programs are being implemented in the Fethiye Günlüklü and Marmaris National Park locations today, it is recommended to strengthen the measures taken, and it is believed that the population in the Fethiye-Yanıklar area should be taken under protection as soon as possible. It is very important to develop *ex-situ* conservation strategies with or simultaneously with the provision of *in-situ* conservation. Otherwise, we will continue to follow the story of the tragic disappearance of sweetgum forests and sweetgum oil that have lost their value in the last 50-60 years.

Conservation genetics can play an important role in mitigating the impacts of climate change on biodiversity. By identifying genetically distinct populations, assessing genetic diversity and health, and developing breeding

programs or other management strategies, conservation genetics can help ensure the long-term survival of species in a changing environment. However, conservation genetics alone is not sufficient to address the complex and multifaceted challenges of climate change, and broader conservation efforts, including habitat protection and restoration, are also necessary.

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RESEARCH ARTICLE/ARAŞTIRMA MAKALESİ

Biyoçeşitlilik Koleksiyonlarının Dijitalleştirilmesi ve Aydınlatma Kabini Tasarımı

Hakan Çalışkan [1], Hatice Kübra Yalçın[1]

Öz

Amaç: Biyoçeşitlilik koleksiyonlarında ve müzelerinde milyonlarca numune bulunmaktadır. Koleksiyonlardaki bu numunelerin dijital veriye dönüştürülmesi son çeyrek yüzyılda başlamış ve hızla artmıştır. Bu süreç müzelerin dijital numunelerini oluşturmasına neden olmuştur. Bu çalışmada ülkemizde var olan biyoçeşitlilik koleksiyonlarının dijitalleştirilmesi ve farklı koleksiyonlar ile birleştirilerek açık ulaşıma sunulmasında kullanılabilecek dijital fotoğraf çekimini basit yöntemlere kavuşturmak ve veri setlerinin oluşturulmasında standartlaşmayı sağlamak amaçlanmıştır.

Materyal ve Yöntem: Çalışmada ESOGÜ Zooloji Müzesi koleksiyonunda yer alan omurgalı ve omurgasız hayvanlara ait numunelerin dijital görsellerinin alınması ve veri setleri oluşturulması sürecinde kullanılan yöntemler paylaşılmaktadır. Numunelerin dijital görsellerinin alınması için 60 × 60 cm ölçülerinde 40 W LED panellerden oluşan bir aydınlatma kabini tasarımı yapılmış ve kullanılmıştır.

Bulgular: Çalışmada biyoçeşitliliğe ait numunelerinin envanterlerinin oluşturulması için kullanılacak standart altı harf ve altı sayıdan oluşan bir kayıt kodu, sergilenen numuneler için üç farklı numune veri etiketi tasarlanmıştır. Tüm dijital görsellerin sayısal ortamda arşivlenebilmesinde kullanılacak adlandırma kuralı oluşturulmuştur.

Sonuç: Tasarımı yapılan ışıklandırma kabininin farklı hayvansal müze numunelerinde kullanımı ile ilgili bilgiler verilmiş ve sonuçlar paylaşılmıştır.

Anahtar Kelimeler: Anadolu, biyofotografi, doğa tarihi, veri kaydı

Digitization of Biodiversity Collections and a Lighting Cabinet Design

Abstract

Objective: There are millions of specimens in biodiversity collections and museums. The conversion of these specimens in collections to digital data started in the last quarter century and has increased rapidly. This process has caused museums to create digital specimens. In this study, it is aimed to simplify digital photography and to standardize the creation of data sets in digitizing the existing biodiversity collections in our country and to present them to open access by combining them with different collections.

Materials and Methods: In the study, the methods used for obtaining digital images of vertebrate and invertebrate animals in the collection of the ESOGÜ Zoology Museum and creating data sets are shared. A lighting cabinet consisting of 60 × 60 cm 40 W LED panels was designed and used to take the digital images of the samples.

Results: A registration code of six letters and six numbers, which will be used to create inventories of biodiversity samples, and three different sample data labels for the exhibited samples were designed in the study. A naming rule has been established for archiving all digital images in a digital environment.

Conclusion: Information on using the designed lighting cabinet in different animal museum specimens is provided and the results are shared.

Keywords: Anatolia, biophotography, data record, natural history

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Giriş

Biyoçeşitlilik müzelerinde dijital verilerin toplanması ve kullanılması giderek önem kazanmaktadır. Bireysel koleksiyonlardan doğa tarihi müzelerinde stoklanan örneklere kadar her dijitalleştirilen numune geniş kitlelere ulaşma fırsatı bulmaktadır. 1999 yılında kurulan Küresel Biyoçeşitlilik Bilişim Tesisi (GBIF: the Global Biodiversity Informatics Facility) 2018 yılı ortasına kadar yaklaşık 150 milyonu doğa tarihi koleksiyonlarında bulunan bir milyar numuneden fazla biyoçeşitlilik verisini kaydetmiştir. Paris herbaryumu 5,4 milyon, Londra Doğa Tarihi Müzesi 8,4 milyon numuneyi sayısallaştırmıştır. Berlin Doğa Tarihi Müzesi tüm böcek koleksiyonunu dijitalleştirmeyi amaçlamaktadır (Nelson & Ellis, 2018).

Koleksiyonlarda ve müzelerde görsel verilerin kaydedilmesinde analog sistemlerle elde edilen fotoğrafların yerini günümüzde dijital teknolojiler almıştır. Meehan (2022) dijital müze nesnesini, fiziksel bir müze nesnesinin gerçek ve aslına sadık sayısallaştırılmış görüntüsü olarak tanımlar. Uluslararası Müzeler Konseyi Uluslararası Belgeleme Komitesi (CIDOC) dijital nesnelerin oluşturduğu sınıfı, Doerr *vd.* (2016)'e göre, metin setleri, resimler, ses ve video öğeleri ve yazılımlar olarak değerlendirmektedir. Dijital nesneler yaygınlık ya da her yerde bulunabilme özellikleri ile benzersiz bir kategoriye yerleşmektedir (Hui 2012, 2016).

Burada biyolojik numunenin dijitalleştirilmesi, onun numune etiketinden, bilgi metinlerine, fiziksel numuneyi temsil eden iki boyutlu görsellerinin sayısal verilere dönüştürülmesine kadar birçok konuyu içermektedir. İster fiziksel olsun, ister dijital veriye dönüşmüş nesne olsun tüm numuneler üzerlerinde kendilerini tanımlayan verileri taşırlar. Bunlar birer bilgi kategorisi içinde değerlendirilir. Bir "tip", bir bilgi kategorisi, disiplin gelenekleri tarafından oluşturulmuş ayrı bir analiz birimidir. Antropologlar için bir tip, kültürel bir davranış veya insan yapımı olabilir. Biyologlar için tür, onu temsil eden numune ile bir tip olarak değerlendirilir. Koleksiyonlarda türün tanımlanmasında kullanılan ilk numune türün holotipi ve aynı türden olan diğer numuneler de paratipleri olarak yer alır. Tipoloji uygulamaları, ilgili özellikleri taşıyan nesnelerin birlikte gruplandırılmasına olanak tanır (Crick, 2005; Speers, 2005; Nichols, 2021). Biyoçeşitlilik müzelerinde oluşturulan dijital numune, bir türü temsil eden koleksiyon numunesinin nesnel durumunda yer alan tipin özelliklerini eksiksiz yansıtması gerekir. Bu hem taksonomik açıdan hem de müze dijital numunesi olması durumunda büyük önem taşımaktadır. Biyolojik bir numunenin dijital görselinde aynı zamanda metrik doğruluk ve geometrik doğruluk da aranan bir zorunluluktur. Doğru ölçüm ihtiyacı, mevcut taksonomik uygulamalardan kaynaklanmaktadır. Vücut bölümlerinden alınan ölçümler veya taksonomik karakterler genellikle türlerin açıklamalarında kullanılır. Bu nedenle görselin elde edilmesi aşamasında, temsili ve doğru bir ölçüm örneği (ölçek) kullanılarak metrik ve geometrik doğruluk sağlanabilir. Ölçek kullanımında oluşacak yanlışlıkları önlemek için ölçek, numune ile aynı odak düzlemine ve aynı görsel açıya yerleştirilir. Bu sayede optik bozulmalar ve alan derinliği nedeniyle oluşan metrik ve geometrik bozulmalardan meydana gelen ölçüm hataları azaltılmış olur. İyi çözümlenmiş bir görüntü, numunenin taksonomik özelliklerini, morfolojisini ve morfometrisini doğru bir şekilde yansıtmalıdır. Bu özelliklerde görselin elde edilmesi birçok teknik detay ve teknolojinin kullanılmasıyla gerçekleşebilmektedir. Dijitalleşmede beklenti, görselin güzelliğinden çok doğruluğu yönündedir. İyi odaklanmış, iyi kontrastlı bir görüntü daha iyi doğruluk sağlar. Numunenin iyi aydınlatılması, iyi kontrast ve görsel doğruluk için en önemli kriterlerin başında gelir. Homojen bir aydınlatma biyolojik numunenin tüm özelliklerini göstermesini sağlar. Geniş aydınlatıcı yüzeyler, numune üzerindeki istenmeyen gölgelerin solmasına, spot ışıklara göre daha iyi etki eder. Bu konuda fotoğraf ışık kutuları kullanılmaktadır. Bu tür aydınlatma kutularında numunelerin dijital görüntülerinin arka planı isteğe göre değiştirilebilir ve özne ile kontrastı en üst düzeye çıkaracak şekilde seçilebilir. Renk taksonomik bir karakter oluşturabilir. "Gerçek" renkler yalnızca belirli bir standart açısından tanımlanabilir. Beyaz ışık, yani belirli bir renk sıcaklığında (güneş ışığı için tipik olarak 5500°K) yayılan siyah bir cismin spektrumu olarak standardize edilir. Algılanan renkler, tayfın numuneden yansıtılan (yani soğurulmayan) veya girişim süreçleriyle oluşturulan kısımlarıdır. Dijital fotoğraf makinesinin renkleri doğru algılamasında beyaz denge önemli rol oynar. Özel bir beyaz test kartı kullanılarak, çekim ortamında kullanılan aydınlatmaların ışığı altında bir nötr fotoğraf çekilerek beyaz denge ayarı yapılması önerilen yöntemlerin başında gelmektedir. Dijital görüntüler piksel cinsinden ölçülür, piksel sayısı ayrıntıyı sınırlar. Görselde pikselden daha küçük bir ayrıntı yer alamaz. Bir kameranın sensörü her piksel için üç değer kaydeder, bu değerler kırmızı, yeşil ve mavidir (red, green, blue: RGB). Görünen her renk tonu, bu üç değerin belirli bir oranda birleşimidir. Görüntünün sayısallaşmasında görüntünün boyutu, renk değerleri toplam veriyi oluşturur. Görüntü dosyası iki temel türe ayrılır. Birincisi, üç renk kanalının ayrı ayrı kaldığı dosyalardır.

Bu dosya türünün en bilineni TIF dir. İkincisi her piksel için üç rengin bir değer üzerinde karıştırıldığı dosyalardır. Bu dosya türü JPEG olarak bilinir ancak sıkıştırılmış dosya olması nedeniyle boyutu küçültülmüştür. Görüntü boyutu piksel olarak ölçüldüğü için baskı boyutundan bağımsızdır. Basılı bir fotoğrafta nokta/inç (DPI) sayısı kullanılır. DPI değeri yüksek dosya oluşturmak baskı ölçüsünü büyütmek açısından önemlidir. 6 Mpx bir DSLR fotoğraf makinesi, 3000 piksel genişliğinde bir görüntü üretir ve standart ofset kağıdına, tek tek piksellerin görünmediği yaklaşık 28 cm çapında görsel olarak basılabilir. Görseli dijitalleştirilen numunelerin yapısal özellikleri de fotoğraflamada önem taşır. Refleks dijital fotoğraf makineleri kullanılarak görece düz numuneler ile üç boyutlu derinliğe sahip numunelerin görsellerinin alınması sırasında, çekim teknikleri değişmektedir. Fotoğraf makinesinde yer alan diyafram ışık miktarının sensöre giriş açıklığını belirler. Kural olarak, herbaryum numuneleri gibi düz nesneler her zaman büyük diyafram açıklıklarından yararlanılarak fotoğraflanırlarken, hacme sahip numuneler (örneğin bir kafatası), tüm nesnenin odakta olmasına izin veren düz nesnelere göre daha dar bir diyafram açıklığından yararlanılarak görseli alınır. Diyafram açıklığının kısılması alan derinliğin artıracaktır. Alan derinliğindeki artış, üç boyutlu nesnelerin, derinlik sınırlarında net görüntüsünün elde edilmesini sağlar (Arino & Galicia, 2005; Hauser *vd.,* 2005; Riedel, 2005).

Çok sayıda numunenin dijitalleştirilmesi çalışmalarında elde edilen görsellerin dijital dosyaları için benzersiz adlar oluşturulması gerekir. Dosya adında görüntü içeriği hakkında da bilgilerin yer alacağı bir kod düzeni kullanılır. Bu sayede belirli bir isimlendirme düzeniyle binlerce görselin sıralanması ve arşivlenmesi mümkün olacaktır (Hauser *vd*., 2005).

ESOGÜ Zooloji Müzesi 2007 yılından günümüze Eskişehir'de bir üniversite müzesi olarak hizmet vermektedir. Koleksiyonunda yer alan omurgalı ve omurgasız hayvanlara ait 24.241 örnek yer almaktadır. TÜBİTAK 2209-A kapsamında "Alternatif bir müze deneyimi için "ONLINE ESOGÜ ZOOLOJİ MÜZESİ tasarımı" konulu 1919B012220285 numaralı proje ile numunelerin dijital görsellerinin alınmasına başlanmıştır. Bu proje kapsamında müzelerde müze numunelerinin görsellerinin alınması için kullanılabilecek bir "fotoğraf ışıklandırma kabini" tasarımı geliştirilmiş olup bu çalışmada özellikleri ve kullanımı ile ilgili bilgiler sunulmaktadır.

Yapılan çalışmada verilerin standartlaştırılması hedeflenmiştir. Örneklerin dijitalleştirilebilmesi sürecinde isimlendirme etiketlerinin standart olması önem taşımaktadır. Çalışmada müze için tasarlanmış isimlendirme ve kayıt etiketleri için önerilere de yer verilmiştir. Elde edilen dijital görseller için bir dosya isimlendirme sistemi tasarlanmış ve sunulmuştur. Böylece hem müze dijital koleksiyonunun düzenli arşivlenmesi hem de gelecekte farklı koleksiyonlarda yer alan fiziki ve dijital numunelerin bir veri tabanında bir araya getirilmesini sağlayabilecek örnek bir model oluşturulması amaçlanmıştır.

Materyal ve Yöntem

ESOGÜ Zooloji Müzesi envanterinde yer alan taksonların sınıflandırılmasında Küresel Biyoçeşitlilik Bilgi Aracı, GBIF (Global Biodiversity Information Facility)'de belirlenen nomenklatur kullanılmıştır. Envanterdeki her numune altı harf ve altı sayısal değerle kodlanmıştır. Altı harfin ilk üçü, taksonun ait olduğu şube, alt şube, üst sınıf ya da sınıfı, son üç harf ise numunenin müzedeki durumunu, altı rakamlı sayısal değer ise numunenin müzeye kayıt sırasını belirtmektedir (Tablo 1).

Sadece fosil numuneler için üçlü kodlama kullanılmamış, "FOSSIL" ifadesi tercih edilmiştir. Envanter kayıt kodu, müzemiz ilk kaydı *Felis silvestris* subsp. *catus* Linnaeus, 1758 türüne ait olan iskelet numunesi için hazırlanarak

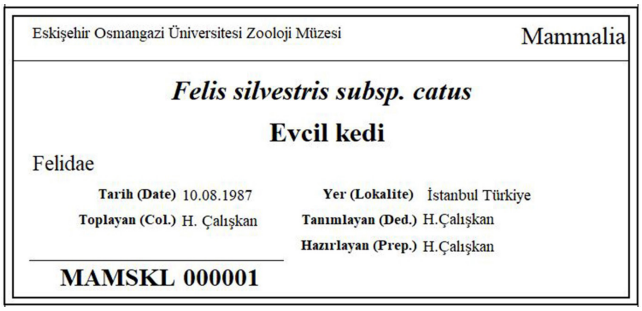
Tablo 1. Envanter kodu oluşturulurken takson ve numune durumu için kullanılan kısaltmalar.

Kodlamada ilk üç harfe göre taksonlar		**Son üç harfe göre numunelerin durumu**	
MAM:	Mammalia	SKL:	İskelet
AVS:	Aves	TAX:	Taksidermi
REP:	Reptilia	DRY:	Kuru
AMP:	Amphibia	LIC:	Sıvı
PSC:	Pisces	PLY:	Polyester
ECH:	Echinodermata		
CRS:	Crustacea		
INS:	Insecta		
MYR:	Myriapoda		
CHL:	Chelicerata		
MOL:	Mollusca		
POR:	Porifera		
CND:	Cnidaria		

kullanılmıştır. Numune kayıt kodu **"**MAMSKL000001"dir. Burada "MAM" memelileri, "SKL" numunenin iskelet olduğunu, "000001" bir numaralı kayıtlı müze numunesi olduğunu ifade etmektedir.

Sergilenen taksonlar için üç ayrı biçimde etiket tasarlanmıştır. Numune veri etiketlerinde müze adı, numunenin şube, alt şube, üst sınıf ya da sınıfı, bilimsel adı, Türkçe adı, türün familyası, toplandığı tarih, örneği toplayan, materyalin toplandığı yer, tanımlayan, eğer hazırlanması gerekiyorsa numuneyi hazırlayan kişi ve numunenin envanter kayıt numarası yer almaktadır. Büyük boy etiket (Şek. 1), çoğu omurgalı hayvan numuneleri olan, özellikle sergilenmesi için tahnit ya da iskelet oluşturma benzeri özel işlemler gerektiren görece büyük boyutlu numuneler için kullanılmaktadır. Orta boy etiketler (Şek. 2) omurgasız hayvanlar için, küçük boy etiketler (Şek. 3) ise numunenin kaybolmasını, karıştırılmasını önlemek amacıyla numunelerin üzerlerinde bulundukları platform ya da numunenin kendi üzerine yerleştirilmesi için tasarlanmıştır.

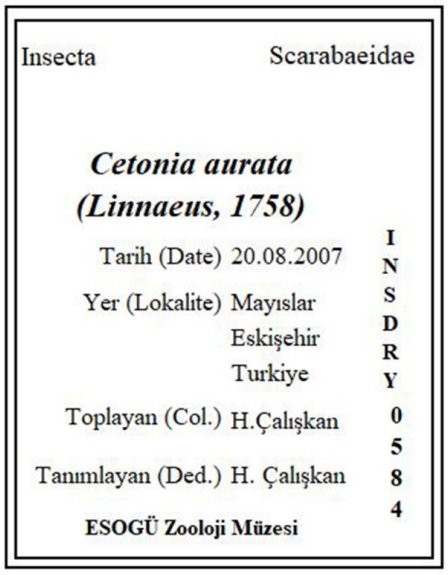
Tüm numunelerin dijital fotoğraflarının çekimi sırasında numune ile aynı düzlem ve fotoğraf makinesi objektifinin görüş açısıyla aynı açıda konumlandırılan metrik bir ölçek (Şek. 4) yerleştirerek görseli alınmıştır.



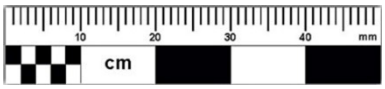
Şekil 1. Büyük boy numune veri etiketi (7,5×3,5 cm).

Şekil 2. Orta boy numune veri etiketi (2,5×8 cm).

Numunenin dijital fotoğrafının çekilmesinde fotoğraf karesi hayali dört bölüme ayrılmıştır. Sağ alt bölüm veri etiketi, sağ orta üst bölüm ölçek, sol orta üst bölüm numune ve sol alt bölüm ise oluşan görsele eklenebilecek karekod ve benzeri uygulamalar için boş bırakılmıştır.



Şekil 3. Küçük boy numune veri etiketi (3×2,8 cm).



Şekil 4. Dijital görsellerde yer alan (50 mm)'lik ölçek siyah ve beyaz referans değeri ile birlikte verilmiştir.

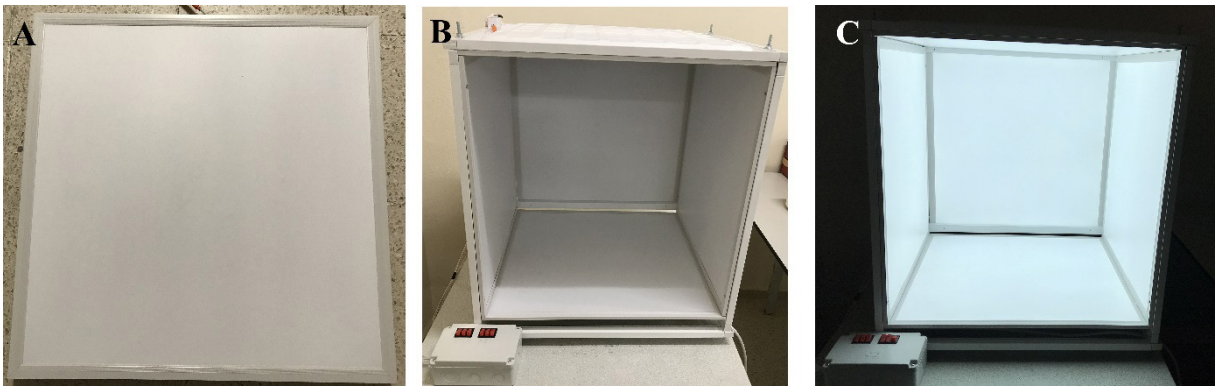
Elde edilen tüm görsel dosyalar RGB modunda işlenmiştir. Dijital görseller TIF formatında 300 dpi'da kaydedilmişlerdir. Dosyaların isimlendirilmesinde numune ve görselle ilgili olabildiğince fazla bilgiye yer verilmesi dikkate alınmıştır. Buna göre dijital dosyalar aşağıdaki şekilde isimlendirilerek kaydedilmektedirler:

"Numune kodu – tür adı – fotoğrafçı adı – müze adı – fotoğraf yönü – fotoğraflama sayısı"

Fotoğrafı çekenin adının ilk harfi kullanılır ve büyük, soyadının ilk harfi büyük diğer harfleri küçük olarak yazılır. Müze adı olarak Eskişehir Osmangazi Üniversitesi Zooloji Müzesi'için "ESOGÜ-ZM" kısaltması kullanılmıştır. Fotoğraf yönü için, sırt (D), karın (V), sağ yan (RL), sol yan (LL), baş (H), bacak (Lg), erkek genitalya (Hyp), dişi genitalya (Ov), göğüs (Th) kısaltmaları kullanılır. Buna göre, ESOGÜ Zooloji müzesindeki bir numaralı numunenin sırtından çekilen iki numaralı dijital görselin dosyası aşağıdaki şekilde isimlendirilmiştir:

"MAMSKL000001_*Felis_silvestris*_subsp_*catus*_HCaliskan_ESOGU_ZM_d_2.tif"

Numunelerin dijital görsellerinin elde edilmesinde 2004 yılı üretimi 8 MP, Olympus E300 DSLR (Digital single-lens reflex) fotoğraf makinesi kullanılmıştır. Objektifi Zuiko Digital 50 mm, 1:2 makro objektiflerden yararlanılmıştır. Çalışmada çekilen fotoğraflarda ISO 100 olarak ayarlanmıştır. Fotoğraf makinesi üç ayak tripota yerleştirilmiştir.



Şekil 5. A) Işıklandırma kabininde kullanılan NAOS marka LED panel (60×60 cm); B) Işıklandırma kabini (ışıklar kapalı); C) Işıklandırma kabini (ışıklar açık).

Işıklandırma kabini için 2,5×1,8 cm kutu alüminyum profilden 60×60×60 cm ölçülerinde iskelet hazırlanmış ve bunun beş yüzeyinin her birine, NOAS marka 60×60 cm ölçülerinde 40 W LED paneller yerleştirilmiştir (Şek. 5). Panellerin CRI değeri (Renksel Geriverim Indeksi) >80 dir. Renk sıcaklığı 6400 K dir.

Işıklandırma kabini aydınlatma led panellerinin spektrofotometrik ölçümleri Ocenaoptics USB 2000 Tak-Çalıştır spektrofotometre ile ölçülmüştür. Panel aydınlatmalar Leica MZ 16 doğal görünüm görüntüleme sisteminde kullanılan Leica marka Fiber Optik Cool Light ve Leica Ring Light ile karşılaştırılmıştır. Değerler Şekil 6'da verilmiştir.

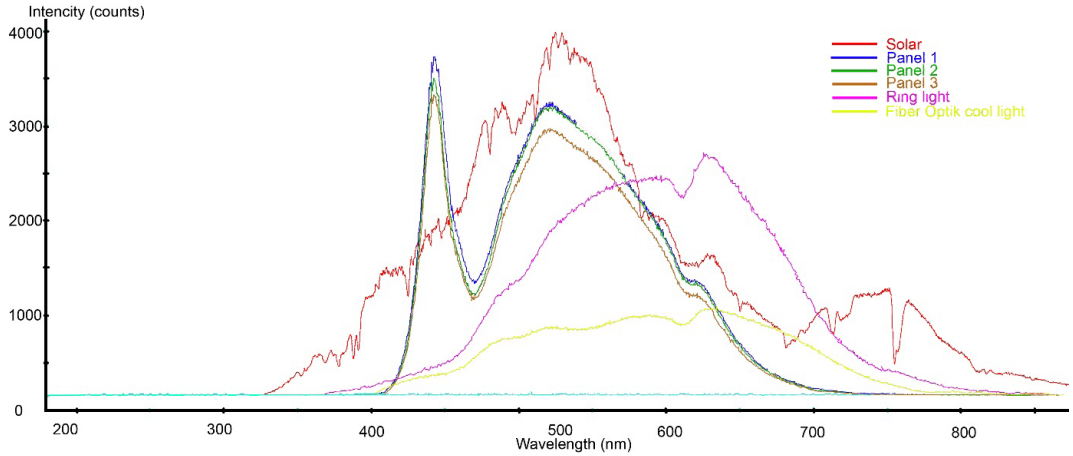
Panellerin yüzeyi Opal polikarbon difüzörle örtülüdür. Çekimlerde ek difüzör olarak görev yapabilecek kağıt ya da kumaş kullanılarak ışık şiddeti düşürülebilmektedir. Her panel kontrol kutusundaki bir anahtar ile kontrol edilmektedir. Bu sayede fotoğraf çekiminde, yön ve arka plan seçimine göre paneller ayrı ayrı kullanılabilmektedir. Numunelerin arka planı için mat siyah ve beyaz kağıt fon kullanılmıştır.

Dijital görselin alınmasından önce fotoğraf makinesi beyaz denge (WB) ayarı seçilen arka plan ve kullanılmasına karar verilen aydınlatma panellerin açık olduğu ışık şiddetinde göre ayarlanmıştır. Objektifin görsel alanına yerleştirilen beyaz bir kağıt kullanılarak One–Touch WB ayarıyla optimum beyaz denge fotoğraf makinesi tarafından belirlenmiştir.

Müzedeki biyoçeşitlilik numunelerinden elde edilen tüm dijital görsel dosyaları ESOGÜ Zooloji Müzesi Dijital görüntü arşivinde uygun bir ara yüzde sunulana kadar saklanmaktadır.

Bulgular

ESOGÜ Zooloji Müzesi envanterinde yer alan 24.241 adet biyoçeşitlilik numunelerinin arasından seçilen 915 örnek sergi salonunda ziyaretçilere sunulmaktadır. Bu çalışmada, omurgasızlar içinde Mollusca filumundan Gastropoda sınıfına ait [MOLDRY000563 *Galeodea echinophora* (Linnaeus, 1758)], Arthropoda filumunda Crustacea alt subesinde yer alan Malacostraca'ya ait [CRSDRY000068



Şekil 6. Işıklandırma kabininde kullanılan NAOS marka LED panel (60×60 cm); B) Işıklandırma kabini (ışıklar kapalı); C) Işıklandırma kabini (ışıklar açık).

Astacus leptodactylus Eschscholtz, 1823; CRSDY000113 *Calappa granulata* (Linnaeus, 1758); CRSDRY000079 *Eriphia verrucosa* (Forskål, 1775)], ile omurgalılarda Aves sınıfına ait [AVSTAX000623 *Anas crecca* Linnaeus, 1758] olmak üzere toplam beş türün görsellerine yer verilmiştir. Elde edilen dijital fotoğraflar bir bilgisayar programı ile renksel bir düzenleme yapılmaksızın bu çalışmada kullanılmıştır.

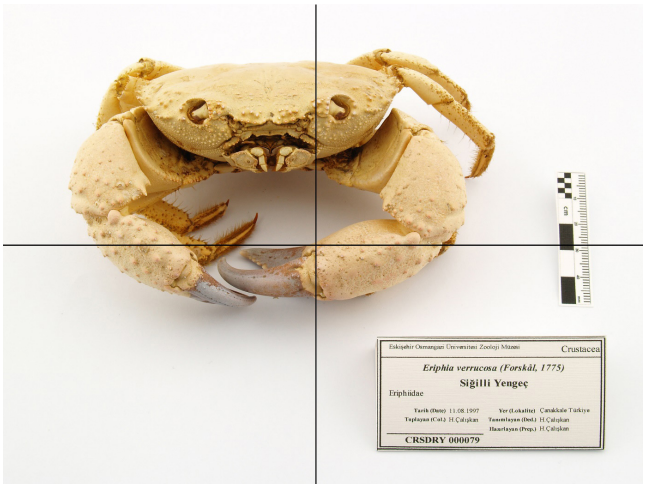
Işıklandırma kabini

Işıklandırma kabini aydınlatma LED panellerinin spektrofotometrik ölçümleri Şekil 6'da görülmektedir. Ölçümlere göre gün ışığı spektrumu çeşitli dalga boylarında atmosferin sahip olduğu gaz, nem ve aeroseller nedeniyle eksiktir. Gün ışığı modeli için yapılan kabinin duvarlarından kabinin merkezine gelen ışımanın spektrumu ise güneş spektrumuna benzer olmakla birlikte, temel de çeşitli eksiklikler vardır. LED kullanılarak yapılan aydınlatma sisteminde Şekil 6'da görüldüğü gibi 420 nm'nin altındaki dalga boyları eksiktir ve 470 nm'de de bir eksiklik görülmektedir. Üst dalga boylarda solar spektrumla da bir miktar farklılık ortaya çıkmaktadır. Kırmızı ötesi bölgede eksiklikler mevcuttur. Panel aydınlatmalar Leica MZ 16 doğal görünüm görüntüleme sisteminde kullanılan Leica marka Fiber Optik Cool Light ve Leica Ring Light ile karşılaştırılmıştır. Her iki lambanın da ışık şiddetleri düşük olmakla birlikte yine mavi ve UV bölgede eksik dalga boyları mevcuttur. Buna ilave olarak kırmızı ötesi bölgede ise ışıma şiddetinde düşüklük mevcuttur. Her iki lambanın ışığı beyaz değil aksine sarı ışık vermektedir.

Görsel Alana Yerleştirme

Numune, etiket ve ölçeğin görsel alana yerleştirilmesinde standart bir düzene ihtiyaç duyulmaktadır. Bu çalışmada fotoğrafta yer alacak görsel alan birbirleriyle kesişen dikey ve yatay hayali iki doğruyla dört bölüme ayrılmıştır (Şek. 7). Sağ alt köşeye veri etiketi yerleştirilmiştir. Sağ üst bölümün sağ kenarı ölçek için ayrılmıştır. Ölçek, materyalin konumuna bağlı olarak metrik doğruluğu yakalayabilmek için numunenin odak noktasına, alan derinliği doğrultusunda yerleştirilmiştir, sol üst ve sağ üst bölüme büyük oranda numune yerleşmiştir. Sol alt köşeye görsel üzerine ilerde yerleştirilebilecek karekod ya da benzeri bir bilgi kutusu için yer bırakılmıştır. Şekil 7'de, yer alan numune kontrast açısından görece zayıf ancak vücut örtüsü açısından ışığı yansıtmayan mat bir yüzeye sahip deniz kabuklusu (CRSDRY000079) *Eriphia verrucosa* (Forskål, 1775)'dır. Beyaz arka planı oluşturan zeminde opal polikarbon levha kullanılmıştır.

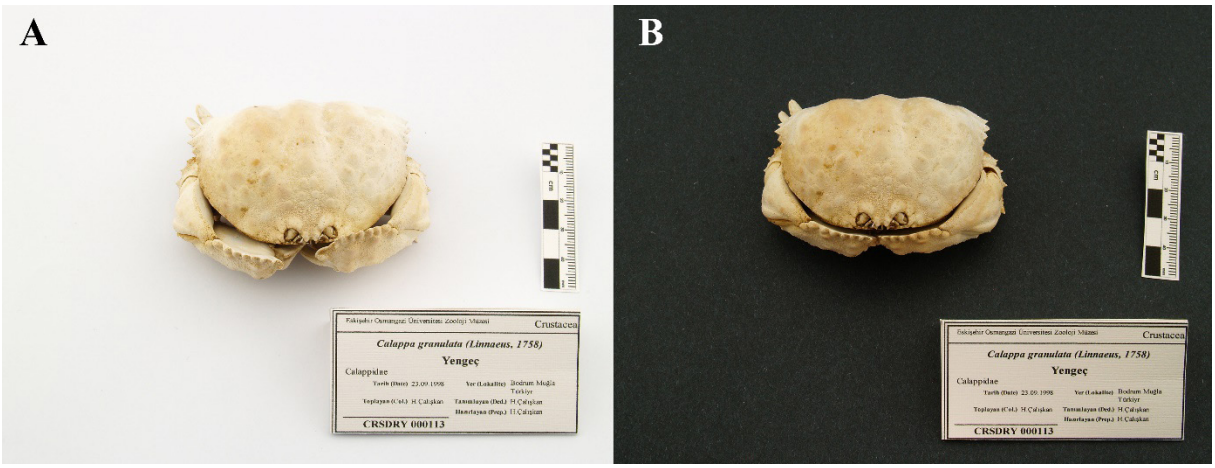
Bir platform üzerinde bulunan numunelerin etiket ve ölçeğinin görselle yerleştirilmesinde bazı zorluklarla



Şekil 7. CRSDRY000079 *Eriphia verrucosa* (Forskål, 1775), Olympus E300 body, Zuiko 50 mm 1:2 objektif, diyafram f/16, pozlama süresi 1/25 sn, odak uzunluğu 29 mm, ölçek

karşılaşılmıştır. Şekil 8'de yer alan (AVSTAX000623) *Anas crecca* Linnaeus'un sol kanat renk ve deseni sağ tarafına nazaran daha belirgindir. Bu nedenle sol yan yüzeyinden fotoğrafı çekilmek zorunda kalınmıştır. Bu da numunenin yönünün sola bakmasına neden olmaktadır. Bire bir görüntü yakalama kuralına sadık olmak amacıyla görüntünün yönü yazılım kullanılarak değiştirilmemiştir. Ölçek numunenin bulunduğu tablanın köşesine konumlandırılmıştır. Ölçek odak noktadan önde yer almış bu da metrik doğruluğu elde etmeyi zorlaştırmıştır. Bu tür numunelerde ölçeğin yeri materyalin bakış yönünde ve alan derinliğine paralel doğrultuda konumlandırılabilir. Veri etiketini, numune ve

Şekil 8. AVSTAX000623 *Anas crecca* Linnaeus, 1758; Olympus E300 body, Zuiko 50 mm, 1:2 objektif, diyafram f/11, pozlama süresi 1/30 sn, odak uzunluğu 24 mm, ölçek 50 mm.



Şekil 9. CRSDY000113 *Calappa granulata* (Linnaeus, 1758); Her iki fotoğraf Olympus E300 body, Zuiko 50 mm 1:2 objektif ile çekilmiştir. Ölçek 50 mm, odak uzunluğu 33 mm'dir. A) Beyaz arka plan, diyafram f/16, pozlama süresi 1/25 sn; B) Siyah arka plan, diyafram f/16, pozlama süresi 1/30 sn.

tablasından bağımsız yerleştirmek görselden uzaklaşmaya neden olmakta, etiketin numunenin görsel alan derinliğinin dışında kalmasına ve netliğinin bozulmasına neden olmaktadır. Numunenin karışmasını engellemek için küçük etiketler kullanılmaktadır. Söz konusu küçük etiketler tablanın üzerine değil alt köşesine yerleştirilecektir. Bu sayede görselde gereksiz yer kaplaması engellenmiş olacaktır.

Arka Plan Seçimi

Numunenin yerleştirildiği zemin için bu çalışmada üç seçenek kullanılmıştır. İlki opal polikarbon levhadır. Led aydınlatmanın üzerinde diffüzör görevi yapan levha aynı zamanda numunenin arka plan zeminini oluşturmaktadır. Daha doygun bir yüzey hissi vermiştir. İkinci seçenek olarak mat beyaz kâğıt kullanılmıştır. Alan derinliğinin dışına çıkıldıkça özellikle zemin kaybolmakta ve nesnel görselliğini yitirmektedir. Ancak Şekil 9 (B)'da olduğu gibi numunenin zemine yakın ve yüksekliğinin görece az olduğu durumlarda zeminin dokusu belirginleşebilir. Şekil 9 (A)'da kullanılan opal polikarbon zemin levha, dokusunu belli etmez iken, Şekil 9 (B)'da siyah zemin olarak kullanılan siyah mat kartonun dokusu tozlu bir görüntü şeklinde görselin zemininde belirginleşmiştir. Siyah zemin için kullanılacak malzemenin seçimi bu nedenle önemli olmaktadır. Burada diğer bir çözüm ise numuneyi zeminden uzaklaştırmak ve zemini alan derinliğinin dışına çıkartmaktır. Bu teknik eğer olanak varsa zemindeki tüm kusurları yok edecek ve numuneye zemine temas etmiyor hissi verecektir. Bunun için özel donanımlar ya da ekipmanlara ihtiyaç duyulur. Şekil 9'da seçilen numune beyaz zeminde arka planı ile arasında kontrasının düşük olması nedeniyle önerilen çözümler için iyi bir örnek niteliğindedir. Beyaz arka plana sahip görselde (Şek. 9), numunenin objektiften en uzak olan kenarı giderek zorlaşan sınır hattına sahiptir. Numunenin düşük kontrastlı dokusu arka kenarını belirginsizleştirmiştir. Diyafram ve pozlama süresiyle yapılacak müdahaleler numunenin orijinal rengini etkileyecek ve görselin orjinale benzerlik ölçüsünü bozacaktır. Mat siyah arka plan bu gibi numuneler için iyi bir çözüm olarak görülebilir. Ayrıca, arka plan rengi siyah olmak zorunda değildir. Görsel numune ile gelecekte yapılacak çalışmalara uygun olacak arka plan renkleri seçilebilir. Siyah zeminde numunenin tüm sınırları ayırt edilebilir hale gelmiştir. Ancak seçilen arka plan malzemesinin yapısı nedeniyle tozlu görünmesi tercih edilmeyen bir durumdur. Arka plan olarak kullanılabilecek farklı yapıdaki siyah yüzeyler denenmelidir.

Materyal dokusu

Doygun ışık altında özellikle mat yüzeyli küçük boyutlu Gastropoda numunelerinin fotoğraflarının çekiminde aydınlatma kabininin kullanılması diğer numunelere göre daha başarılı sonuçlar vermiştir. Şekil 10'da yer alan (MOLDRY000563) *Galeodea echinophora* (Linnaeus, 1758) bu durum için iyi bir örnektir. Boyutunun küçük oluşu, alan derinliği içine sığan yüksekliği nedeniyle numuneye net bir görsel elde edilmesine olanak tanımıştır. Netliğin tüm numune üzerinde neredeyse eşit sağlanması görselde dokunun belirginliğini arttırmış ve fiziki nesneyle yüksek düzeyde örtüşmesini sağlamıştır. Ayrıca görsel içinde yer alan ölçek doğruluğu da elde edilmiştir. Bu numune koyu bir arka planda daha belirgin hale gelecektir.

Eklembacaklıların Crustacea alt şubesi üyesi (CRSDRY000068) *Astacus leptodactylus* Eschscholtz,

Şekil 10. MOLDRY000563 *Galeodea echinophora* (Linnaeus, 1758); Olympus E300 body, Zuiko 50 mm 1:2, objektif, diyafram f/14, pozlama süresi 1/30 sn, odak uzunluğu 39

1823'da vücut yapısı olarak Şekil 10'daki *Galeodea echinophora* gibi alan derinliği içinde kalabilecek yüksekliğe sahip olan bir numune olarak bu çalışmada seçilmiştir. Şekil 11'de numunenin sağ lateralinden yaklaşık 45º ye yakın bir açıyla çekilen görselde sağ ve sol kelipedleri arasında 7 cm'lik bir mesafe bulunmaktadır. Numunenin zeminde geniş yer kaplaması ve 45º açıyla pozlama yapılmasına rağmen netlik başarısı yüksektir. Ancak 45º açıyla yapılan çekimde sağ keliped soldakinden kalın ve büyük gözükmüştür. Belirli açılardan numuneye yaklaşmak ve pozlama yerine olabildiğinde dorsalden pozlamak bu tür optik bozulmaların önüne geçecektir. Ölçek odak noktasına yakın yerleştirilmiş ve veri etiketi ile numunenin görseldeki yerleşimi belirlenen standarda uygun olmuştur.

Tartışma ve Sonuç

Ülkemizde az sayıda olan biyoçeşitlilik müzeleri, sahip oldukları koleksiyonlarla evrenimizin en önemli zenginliği olan yaşama ait taksonlara ev sahipliği yapmaktadırlar. Biyoçeşitliliğe ait numunelerin ülkemizde kayıtlarının oluşturulması, farklı koleksiyonlardakilerin birleştirilmesi ve açık erişime sunulması Anadolu'nun ve gezegenimizin yaşam zenginliğinin yorumlanmasında büyük önem taşımaktadır. Çağımızın teknolojisi olan dijitalleşme bu kapsamda kullandığımız önemli bir araçtır. Bu konudaki ilk büyük zorluk, numunelerin dijital görsellerinin elde edilmesi için yüksek maliyetli teknolojiler, cihazlar ve teknik donanımlara duyulan ihtiyaçtır.

Çalışmada DSLR fotoğraf makinesi olarak 2004 yılı üretimi olan 8 MP Olympus E300 kullanılmıştır. Dijital görselin fiziki numuneler ile birebir olmasını sağlamak için fotoğraf makinesiyle birlikte uygun ışıklandırmanın da bulunması gerekmektir. Hauser *vd.* (2005) ile Arino & Galicia (2005)'in çalışmalarında yer alan ekonomik uygulanabilen ve verimli sonuçlar veren tasarımlar iyi çözümler olarak karşımıza çıkmaktadır. Numunelerin özelliklerine bağlı olarak her tasarım ya da çözümün kullanılması mümkün olmayabilir. Burada bizlerin de önerdiği gibi çalışmaya uygun özellikte materyaller için uygun çözümlerle başlamak iyi bir seçenek olacaktır.

ESOGÜ Zooloji Müzesinde başlangıç olarak sergilenen taksonların dijital fotoğraflarının çekilmesi planlanmıştır. Bunun için bu çalışmada biyoçeşitlilik müzelerinde fotoğraf çekiminde kullanılabilecek ekonomik ve uygulanabilir bir çözüm önerisi olarak ışıklandırma kabini tasarlanmıştır (Şek. 5). Tam spektrumlu led aydınlatma panel kullanılamaması, beyaz denge ayarında renk sıcaklığı değerinin yüksek oluşu, her numunenin fotoğraflanmasında

Şekil 11. CRSDRY000068 *Astacus leptodactylus* Eschscholtz, 1823; Olympus E300 body, Zuiko 50 mm 1:2 objektif, diyafram f/16, pozlama süresi 1/25 sn, odak uzunluğu 33 mm, ölçek 50 mm.

arzu edilen renksel detayların fotoğrafta aslına sadık kalınmasında zorluklar oluşturmuştur. Ancak yüzeyleri mat ve zemin üzerindeki yüksekliği ile birlikte optik düzeneğin alan derinliği içinde kalan Gastropoda ve Crustacea üyelerine ait numunelerin dijital görsellerinin alınmasında uygun sonuçlar elde edilmiştir. Aydınlatma kabini fotoğraf çekimi için geliştirilebilir bir platform niteliğindedir.

Bu çalışmada müze numuneleriyle ilgili kayıtlı verileri filtrelerken, numunelerin sıralanmalarını ve sınıflandırılmalarını kolaylaştıracak harf ve sayılarla kodlanmış kimlik numarası oluşturulması için bir öneri yer almaktadır. Numunelere verilen kayıt numaralarının üretiminde uygulanan altı harfli kodun ilk üç harfi veri tabanında örneklerin filum ya da sınıf düzeyinde ayrımını kolaylaştırırken diğer üç harfi ise materyalin durumunu, yani kuru (DRY), sıvı (LIC), iskelet (SKL) ya da tahnit (TAX) olduğunu belirtir. Biyoçeşitlilik müzelerinde filum, sınıf bilgisi ve materyalin durumu önemli ayrım ve müze içinde yerleştirilmelerinde önemli rol oynarlar. Burada önerilen etiket üzerine de yerleştirilen envanter kodlama tasarım fiziki örneklerin analiz ve yerleşimini de kolaylaştıracaktır. Elde edilen dijital görsellerin adlandırılmasında Hauser *vd.* (2005)'in tasarımı dikkate değerdir. Dijital veriden oluşan görselin dosya adı bir anlamda fiziki örneklerdeki etiketlerin görevini üstlenmektedir. Biz bu çalışmada Hauser *vd.* (2005)'den farklı olarak dosya adının içinde dijital görseli elde eden, yani fotoğrafı çeken kişinin ad soyadının da tıpkı fiziki materyalde olduğu gibi yer alması gerektiğine inanıyoruz. Buna göre oluşturulan dosya adı düzeninde fotoğrafı çeken kişinin adının ilk harfi ve soyadı yer almaktadır. Numune kodunun dosya adının başlangıcında yer alması, dijital dosya görüntülemede ve listelemede müze envanter kodunun kullanılabilmesini sağlamak içindir. Veri kayıtları içinde dosya adından yararlanarak dosya tarama işlemleri için kolaylık sağlayacak farklı düzenlemeler de uygulanabilir.

İklim değişikliğinin yaşandığı bu yüz yılda gezegenimizin sahip olduğu biyoçeşitlilik ne yazık ki büyük bir tehdit altındadır. Var olan çeşitliliğin kayıtlarının alınması ve müzelerde tutulan geçmiş yıllara ait numunelere ait bilgilerin açık erişime sunulması için yapılacak her türlü çalışma gelecek için önem taşımaktadır. Ülkemizin biyolojik çeşitliliğinin envanteri çıkarılmalı, koleksiyonlardaki fiziki numuneler bir an önce güvenli ortamlarda korumaya alınmalı ve dijitalleştirilmelidirler. Bu amaca ulaşmak için hem fiziki hem de dijital numunelerin standart yöntemlerle kaydedilmesine ihtiyaç vardır.

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RESEARCH ARTICLE

Antimicrobial Potential and Molecular Characterisation of Endophytic Fungi Isolated from *Conyza bonariensis* from Tanzania

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Abstract

Objective: The harnessing of medicinal plants for the treatment of infectious diseases has an environmental concern, and therefore environmentally friendly alternative sources like harnessing the potential of endophytes are of paramount importance. The present study aimed to evaluate chemical composition and antimicrobial activity of crude extracts of endophytic fungi isolated from *C. bonariensis* (L.) in Tanzania.

Materials and Methods: Initially, endophytic fungi were isolated from *C. bonariensis* (L.) and characterized, followed by mass cultivation and the harvesting of crude extracts. Then crude extracts were tested against selected microorganisms: Two Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis;* Two Gram-negative bacteria, *Escherichia coli* and *Salmonella typhi*, and a yeast *Candida albicans*. Gas Chromatography-Mass Spectrometry analyzed the chemical composition of crude extracts.

Results: Five endophytic fungi (*Talaromyces* sp., *Penicillium daleae*, *Neurospora crassa, Talaromyces radicus* 1 and *Talaromyces radicus* 2) were isolated. Furthermore, crude extract of *N. crassa* had the highest antimicrobial activity with the range of MIC found to be 1.5 µg/mL > MIC > 0.78 µg/mL for all tested microorganisms except for *S. aureus* where the range of MIC was 40 µg/mL>MIC>20 µg/mL. On the other hand, the crude extract of *Talaromyces radicus* 1 had the lowest MIC range (100 mg/mL>MIC>50 mg/mL). The GC-MS results demonstrated variation in chemical composition with proved various biological properties.

Conclusions: Ethyl acetate crude extracts of *N. crassa* demonstrated the highest antimicrobial activity, therefore warranting further exploration of the endophyte and their crude extracts for various medicinal and industrial applications. Further studies on the characterization of pure compounds, which may be responsible for the antimicrobial activity that was observed, are urgently needed.

Keywords: Endophytic fungi, Internal transcribed spacer, Minimum inhibitory concentration, Tanzania, Gas chromatography mass spectrometry, *Conyza bonariensis*

Introduction

Human beings have been interacting with their natural environment as a source of food, medicine, fertilizers, fragrances, and clothing (Fatemeh, 2018; Okot *et al*., 2020). Worldwide, medicinal plants continue to have the potential in producing secondary metabolites (Kianfé *et al*., 2020). Medicinal plants are plants that possess therapeutic or exert a beneficial pharmacological effect on the human or animal body (Kilonzo & Munisi, 2021). Indigenous people have been using the traditional medicinal plant for the treatment of various diseases. For example, medicinal plants *Achillea millefolium, Ipomoeae pandurata*, and *Hieracium pilosella* have been used as an antioxidant, antifungal and antimicrobial, respectively (Candan *et al*., 2003; Frey & Meyers, 2010; Abdollahi *et al*., 2012). However, the direct harnessing of medicinal plants for the treatment of infectious diseases has an environmental concern, and therefore an environmentally friendly alternative source like harnessing the potential of endophytes is of paramount importance (Ibrahim *et al*., 2021).

Endophytic microorganisms may be fungi or bacteria that live inside the tissue of plants for the whole or part of

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their life cycle and have no negative effects on their host plants (Manyahi *et al*., 2014; Wu *et al*., 2020). Endophytes have been reported to produce more or less secondary metabolites as their host medicinal plants (Ancheeva *et al*., 2020; Ibrahim *et al*., 2021; Mwanga *et al*., 2019). Endophytic fungi crude extracts have been demonstrated to contain bioactive secondary metabolites such as alkaloids, phenols, flavonoids, tannins, terpenoids, and saponins, which have antimicrobial, antioxidant, anti-inflammatory, and anti-diabetic activity (Ancheeva *et al*., 2020; Patil *et al*., 2016). For example, a chemical analysis of a crude extract of *Nigrospora* sp. from *Moringa oleifera* demonstrated to contains griseofulvin with strong antifungal activities while dechlorogriseofulvin and mellein showed weak antifungal activity (Zhao *et al*., 2012). Similarly, crude extracts of *Nigrospora* sp., *Aspergillus fumigates Fusarium oxysporum* from *Leucas martinicensis, Sceletium tortuosum* and *Moringa oleifera*, were demonstrated to contain bioactive secondary metabolites with biological activities as their host plants (Manganyi *et al*., 2019).

In Tanzania, various communities including the Safwa people of the Mbeya region have been using *Conyza bonariensis* leaves and other plants for the treatment of fungal, bacterial, and inflammatory diseases (Araujo *et al*., 2013; Espinoza *et al*., 2020; Ghwanga & Chacha 2019). In Tanzania, antimicrobial activity of *C. bonariensis* has been tested experimentally, and the results were promising (Ghwanga & Chacha 2019). Although endophytic fungi isolated from *C. bonariensis* had been reported in some other parts of the world (Alsini *et al.,* 2022), such information remained to be evaluated in Tanzania taken into consideration that biological activity of crude extract of endophytic fungi may be influenced by geographical conditions like temperature, humidity and soil properties of a particular location (Langenfeld, 2013; Fang *et al*., 2019) where the host plant is found. Since the antimicrobial potential of the crude extract obtained from endophytic fungi isolated from *C. bonariensis* had not been assessed in Tanzaia, this study was conducted to investigate both the chemical composition and antimicrobial effects of these crude extracts derived from *C. bonariensis* endophytic fungi in Tanzani.

Materials and Methods

Sample collection

Fresh and healthy leaves of C*onyza bonariensis* were collected from the Mbeya region located between latitude 08°54′00″S and longitude 33°27′00″E by purposive random sampling for isolation of endophytic fungi. The voucher specimen sample was taken to the University of Dar Es Salaam at the Botany Department for identification and was provided with voucher specimen number FMM4139.

Test organisms

Five test microorganisms were involved in the present study as stipulated in Table 1. Four test microorganisms were bacteria and one was fungus. The test microorganisms were obtained from the microbial strains Library of the Department of Molecular Biology and Biotechnology (MBB), University of Dar es Salaam. The test microorganisms were sub-cultured and reconstituted as previously described (Mpinda *et al*., 2018).

Isolation of endophytic fungi from C. bonariensis

Dust and debris were removed from the leaves by washing with tap water and left to dry. The leaves were decontamination with 70% ethanol for 2 minutes, then dipped in 0.4% sodium hypochlorite (NaOCl) for 1 minute, followed by dipping in 70% ethanol for two minutes, and finally rinsed twice with sterile distilled water to remove ethanol leftover. Surface-sterilized leaves were placed on sterile aluminum foil and allowed to dry in a biosafety cabinet (Nxumalo *et al*., 2020). Leaves were then cut into small pieces of about 1 cm^2 by using a sterile surgical blade, and then placed in potato dextrose agar (PDA; containing potato 200 g/L), dextrose (20 g/L), and agar (15 g/L, pH 6.0) in petri dishes containing chloramphenicol (0.25 mg/mL) to prevent the growth of bacteria. Also, to test for the efficiency of surface sterilization, water from the last washing step was inoculated on PDA media with the same chemical composition. The inoculated petri-dished were incubated for 3-5 days at 30°C. After obtaining pure isolates, the colony diameter of each fungal endophyte was measured every day for five days. The colony diameter

Table 1. List of selected test microorganism used for screening of antimicrobial activity in the study

Serial number	Microorganism name	Culture number	Gram stain
1	*Staphylococcus aureus*	ATCC29213	Gram-negative
2	*Escherichia coli*	ATCC8736	Gram-negative
3	*Salmonella typhi*	ATCC 6539	Gram-positive
4	*Bacillus subtilis*	ATCC6051	Gram-positive
5	*Candida albicans*	DSM1665	-

information was used to establish the growth rate of each fungal endophyte. The fungal growth rate was calculated by dividing the growth difference between day 5 and day 4 to the past (day 4) growth as stipulated in the formula below:

$$\text{Fungal growth rate} = \frac{\text{Present growth (mm)} - \text{Past growth (mm)}}{\text{Past growth (mm)}}$$

Morphological and molecular characterization of endophytic fungi

Morphological characterization was performed by considering a variety of parameters such as the color of the colony (front and back view of the plate on PDA medium), texture, colony topology and spore formation of pure isolates (Paul *et al*., 2012).

Molecular characterization was performed by assessing genetic variation of the internal transcribed spacer (ITS) region of genomic DNA. Initially, genomic DNA was extracted by using a CTAB-based protocol as previously described by Umesha *et al*. (2016). Then, polymerase chain reaction (PCR) was done by using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC -3') primers as previously described by Mpenda & Mkangara (2022). The amplicons were then sent to Inqaba Biotech Company in South Africa for Sanger sequencing by using the same primers (ITS1 and ITS4).

Endophytic fungi mass cultivation and crude extracts harvesting

Endophytic fungi were cultivated in a 5000 mL conical flask containing 2000 mL of sterile malt extract broth (MEB) as previously described (Jaronski & Mascarin, 2016; Mwanga *et al*., 2019). Briefly, pure endophytic fungi mycelia were cut from PDA media and placed in a conical flask containing MEB broth and were left stationary at 30˚C for four weeks. After growth, fungal mycelia were filtered by using Whatman No. 1 filter paper with a pore size of 11 µm. The filtrate was mixed with an equal volume of ethyl acetate and shaken for 10 minutes to allow the absorption of secondary metabolites (Ancheeva *et al*., 2020). The top ethyl acetate solvent layer was collected by using a separating funnel and then was concentrated by using a rotary evaporator (Model No RE-501) at 40˚C at a pressure of 100 mbar. The crude extract was obtained by allowing evaporation of the remaining solvent from concentrate in a dark ventilated room. The crude extracts were preserved at 4˚C for further analysis (Santos *et al*., 2015).

Screening for antimicrobial activity

The test microorganisms described in Table 1 were grown on nutrient and potato dextrose agar for bacteria and fungus, respectively. The nutrient agar inoculated with bacteria was incubated at 37˚C for 24 hours; whereas, the PDA medium inoculated with fungus was incubated at 30˚C for 48 hours. Bacterial and fungal colonies were re-suspended in physiological saline solution, which was then compared with 0.5 McFarland standards (prepared by adding 0.05 mL of 1.175% $BaCl_2$ to 9.95 mL of 1% H_2SO_4).

Endophytic fungi crude extracts were screened by a disc diffusion method as previously described by Khaki *et al*. (2014). Briefly, a paper disc (5-mm-diameter) was soaked with 20 µL of 100 mg/mL for each crude extract. To ensure proper soaking before transferring to petri dishes, discs were kept in a refrigerator at 4°C for 30 minutes. Pre-soaked discs with a crude extract were placed on Petri dishes that had previously inoculated with test microorganism suspension using sterile cotton swabs. The incubation conditions were the same as discussed above. Also, chloramphenicol (0.25 mg/mL) and fluconazole (0.25 mg/mL) were employed as positive controls for bacteria and fungi, respectively and 10% dimethyl sulfoxide (DMSO) was utilized as a negative control. The experiment was performed in duplicate, and zones of growth inhibition were measured in mm after 24 hours of incubation for bacteria, and after 48 hours of incubation hours for fungi (Selvi, 2014).

Minimum inhibitory concentrations

Minimum inhibition concentration (MIC) was established for test organisms that were inhibited by endophytic fungi crude extracts by the disc diffusion method, as previously reported by Khaki *et al*. (2014). The ability of endophytic fungi crude extracts to suppress the growth of test organisms was evaluated for each of the two-fold serial dilution concentrations. Inoculum for the assays was prepared in 0.5 McFarland standards (approximately 1.5×10^8 CFU/mL). Minimum inhibition concentration in mg/mL was reported as a range between the last concentration that showed inhibition and the immediate subsequent concentration to that did not show inhibition.

Chemical analysis of endophytic fungi crude extract

Chemical analysis was performed by Gas Chromatography-Mass Spectrometry recorded in GCMS-2010 Shimadzu instrument operating in Electron Ionization (EI) mode (MS) at 70 ev, and Flame Ionization Detector (FID) for GC. A Restek-5MS column (30 m × 0.25 mm × 0.25 µm) was used. The oven temperature program was 90˚C to 280˚C

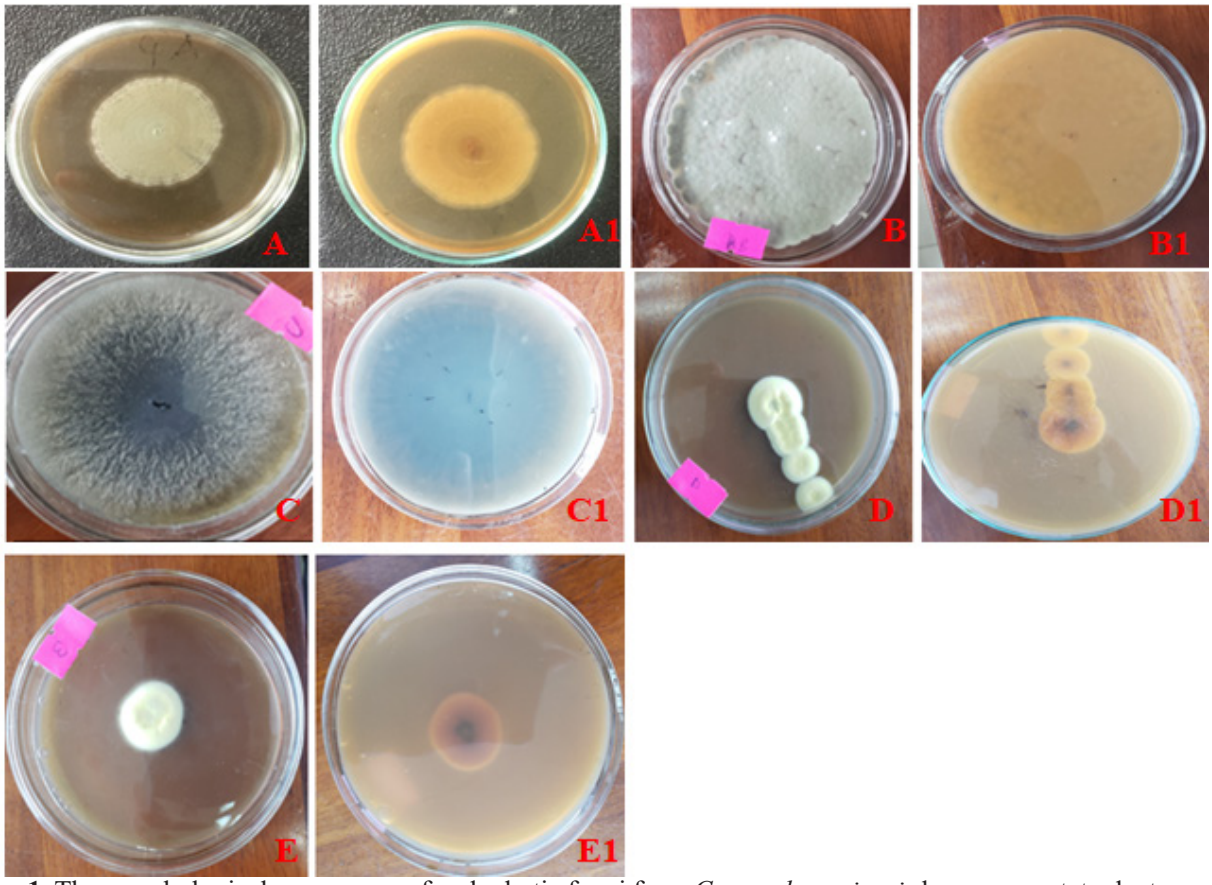

Figure 1. The morphological appearance of endophytic fungi from *Conyza bonariensis* leaves on potato dextrose agar: A and A1 is front and back view of *Talaromyces* sp. (ON394877) colony; B and B1 is front and back view of *Penicillium daleae* (ON394878) colony; C and C1 is the front and back view of *Neurospor acrassa* (ON394879) colony; D and D1 is the front and back view of *Talaromyces radicus* (ON394880) colony of isolate D; E and E1 is the front and back view of *Talaromyces radicus* (ON394881) colony of isolate E.

and held at 90˚C for two minutes. The temperature was increased to 28˚C for 10 minutes (hold time) at the rate of 15˚C per minute. The injection temperature was 250˚C with split injection mode. The flow rate of carrier gas helium was 1.21 mL min^{-1}. The ion source temperature and interface temperature in MS was 230˚C and 300˚C, respectively. The identification of chemicals in the extracts was done by scan method, which involves the use of Mass Spectral Library & Search Software (NIST 11). Quantification of chemicals in the extracts was done using the Peak Integration method (area normalization) whereby ion allowance was 20%, target ion and other five quantitation ions were used on quantitative analysis. 10 µL of the sample was dissolved in dichloromethane to make 1 mL and injected in GC (Da Silva *et al*., 2021), The results were reported as percentage compositions derived from the peak area of all scanned compounds in the extracts (Da Silva *et al*., 2021; Espinoza *et al*., 2020).

Data analysis

Descriptive and inferential statistics were performed using R software (Hackenberger, 2020). For descriptive statistics, bar graphs were used to summarize the statistics; for inferential statistics, two-way analysis of variance (ANOVA) was employed to tell whether there was a difference between the means of the zone of inhibition index of test organisms. Following ANOVA, we used Tukey HSD to perform a pairwise comparison, with a significant difference set at ($p<0.05$) (Gelfond *et al*., 2018; Leppink *et al*., 2017).

Raw DNA sequences were obtained electronically and by using Geneious (BIOMATTERS& geneious) bioinformatics software was use to generate consensus sequences. Similarity search was done using Basic Local Alignment Search Tool (BLAST) on National Center for Biotechnology Information (NCBI). Closely related sequences were retrieved from GenBank of NCBI, and multiple sequence alignment was performed by using MUSCLE algorithm on Molecular Evolution Genetic

Table 2. The morphological feature and growth rate of endophytic fungi isolated from *C. bonariensis* leaves. Number one to five in the figure represent days of endophytic fungi growth diameter (mm). The numbers in the growth rate column that bear different superscript letters (a, b, c) are significantly different ($p<0.05$).

Isolate	Texture	Front view	Back view	Spore	1	2	3	4	5	Growth rate (%)
Talaromyces sp.	Powder	White green	Yellow	Yes	1	3	6	12	15	25^{a}
Penicillin daleae	Powder	Greenish white	Yellow	No	1	2	4	5	6	20^{b}
Neurospora crassa	Cottony	Pale dark blue	Black	No	10	15	35	45	60	33^{c}
Talaromyces radicus	Velvety	Mint green	Pale yellow	Yes	1	2	4	5	6	20^{b}
Talaromyces radicus	Velvety	Mint green	Pale yellow	No	1	5	7	11	13	18^{b}

Table 3. Antimicrobial activities of endophytic fungi crude extracts from *Conyza bonariensis* leaves represented in activity indices and standard error of mean. Along the rows, the activity index values that bear different superscript letters are significantly different ($p<0.05$); whereas, across the column, the activity index values that bearing different superscript numbers are significantly different ($p<0.05$) The concentrations were 0.25 mg/mL and 100 mg/mL for positive control and crude extracts, respectively.

Isolate ID	Crude extract/ positive controls	Accession numbers	*E. coli*	*C. albicans*	*S. aureus*	*S. typhi*	*B. substilis*
			Mean values of activity indexes ± Standard error of mean				
-	Chloramphenicol / Fluconazole	-	$1.00\pm0.0^{a,1}$	$1.00\pm0.0^{a,1}$	$1.00\pm0.0^{a,1}$	$1.00\pm0.0^{a,1}$	$1.00\pm0.0^{a,1}$
A	*Talaromyces* sp.	ON394877	$0.47\pm0.0^{a,2}$	$0.46\pm0.0^{a,2}$	$0.46\pm0.0^{a,2}$	$0.47\pm0.0^{a,2}$	$0.4\pm0.0^{a,2}$
B	*Penicillin daleae*	ON394878	$0.55\pm0.0^{a,2}$	-	-	-	$0.4\pm0.0^{a,2}$
C	*Neurospora crassa*	ON394879	$0.76\pm0.0^{a,1}$	$1.19\pm0.0^{b,1}$	$0.76\pm0.1^{a,1}$	$1.28\pm0.0^{b,1}$	$0.98\pm0.0^{a,1}$
D	*Talaromyces radicus*	ON394880	$0.34\pm0.1^{a,2}$	-	$0.35\pm0.0^{a,2}$	$0.35\pm0.0^{a,2}$	$0.3\pm0.01^{a,2}$
E	*Talaromyces radicus*	ON394881	$0.48\pm0.1^{a,2}$	$0.48\pm0.1^{a,2}$	$0.63\pm0.0^{b,1}$	$0.54\pm0.0^{b,1}$	$0.52\pm0.0^{b,2}$

Analysis (MEGA X) software. Then, using aligned sequences, a UPGMA phylogenetic tree was constructed using MEGAX to depict genetic relatedness between isolated found in the present study and the previous one retrieved from GenBank of NCBI.

Results

Morphology features and growth rate of endophytic fungi

Five endophytic fungi were isolated and purified, and there was high morphological variation between isolates from *C. bonariensis* in the present study. The morphological features and growth rate of isolates are presented in Figure 1 and Table 2.

Identification of endophytic fungi

Isolates found in the present study were identified by a molecular-based approach in addition to morphological features. Following the submission of DNA sequence in GenBank of NCBI, the accession numbers of isolates were obtained and are presented in Table 3. Furthermore, the evolutionary relationship between isolates of the present study and previously reported isolates is portrayed in Figure 2.

Antimicrobial activity of endophytic fungi crude extracts

The antimicrobial activity of endophytic fungi crude extract from *Conyza bonariensis* leaves was investigated using the disc diffusion method. Figure 3 below displays zones of inhibition against tested microorganisms for some of the crude extracts (crude extracts of isolate A and C) evaluated in the present study.

Crude extract of endophytic fungi from *C. bonariensis* had antimicrobial activity against selected tested microorganisms (Fig. 3). In Table 4, the results clearly show that the antimicrobial activity of crude extract of endophytic fungi in the present study has significant antimicrobial activity. Specifically, *N. crassa* crude extract had the best antimicrobial activity as compared to other crude extracts (Table 3). Furthermore, the antimicrobial activity of crude extract of *N. crassa* had higher antimicrobial activity against some of the test microorganisms as compared to the positive control (chloramphenicol and fluconazole, 0.25 mg/mL) based on the activity indexes information (Table 2).

The minimum inhibition concentration

The MIC values for each of the test organisms as portrayed in Table 4. In summary, endophyte C crude extract had highest MIC range of 1.5 µg/mL>MIC>0.78 µg/mL in all (*B. subtilis, E. coli, S. typhi, C. albicans*) tested microorganisms with the exception of *S. aureus* (Table 4).

Chemical composition of endophytic fungi crude extracts

Chemical profiles of crude extracts of endophytic fungi isolated from *C. bonariensis* were established by

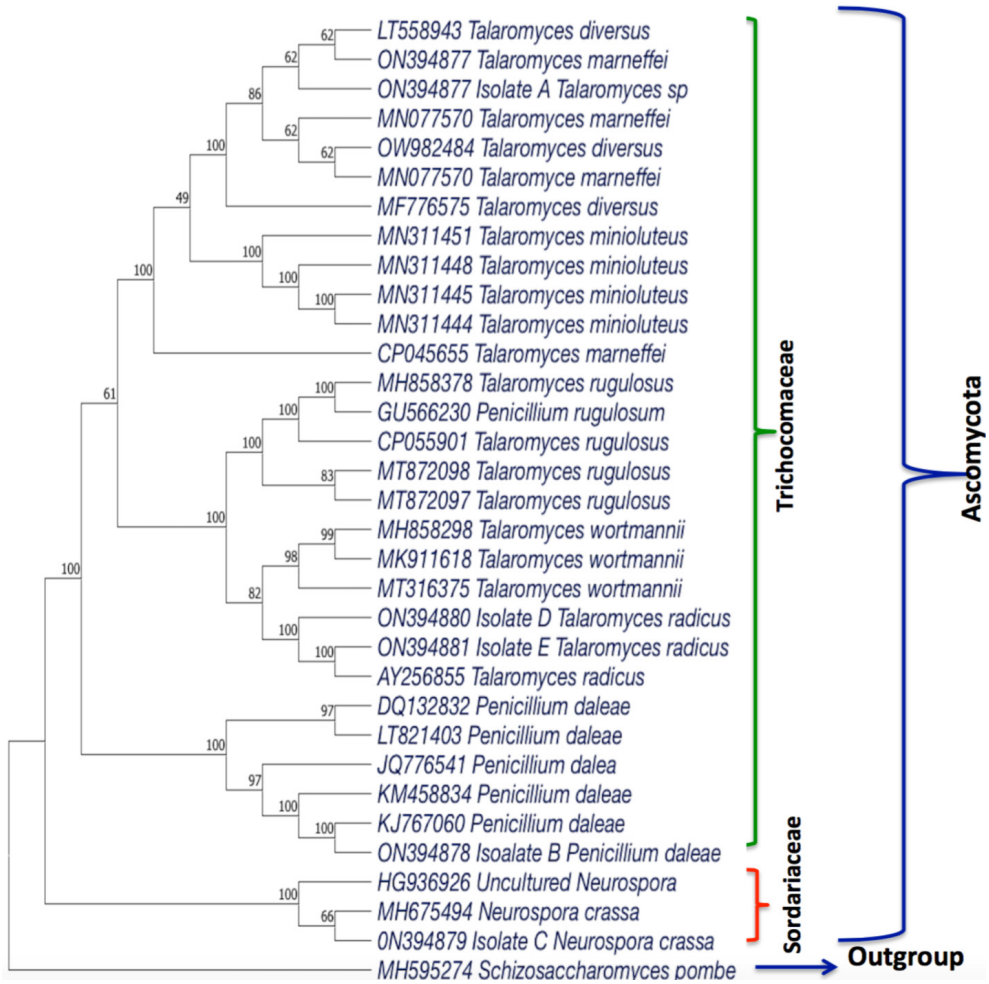

Figure 2. A UPGMA phylogenetic tree depicting the relationship of *Talaromyces* sp. (ON394877), *Talaromyces radicus* (ON394880), *Talaromyces radicus* (ON394881), *Penicillium daleae* (ON394878) and *N. crassa* (ON394878) with other related fungi obtained from GenBank. The *Schizosaccharomyces pombe (*MH595274.1) was used as an out-group. Numbers depicted on the branches represent values based on 1000 replications of Felsenstein's Bootstrap method.

performing chemical analysis using GC-MS. Table 5-9 display the percentage composition based on peak area of various compounds that were detected in the study. Also, biochemical information outlined in Table 5-9 is complimented by information found in the corresponding chromatograms (Fig. 4-8).

Chemical profiles of crude extracts were highly variable among endophytic fungi crude extracts (Fig. 4-8). Specifically, high (49%) composition of long-chain alkenes was observed in ethyl acetate crude extract of *Talaromyces* sp. (Table 5, Fig. 4). On the other hand, the high composition of fatty alcohols and alkenes was observed in crude extract of *Penicillium daleae* (Table 6, Fig. 5). In crude extract of *Penicillium daleae*, the 11-octadecenoicacid, methyl ester had a higher percentage composition (20.52%), and cis-10-nonadecenoic acid methyl ester had the lowest composition (3.61%) as shown in Table 6. Likewise, long-chain fatty alcohols and alkenes were predominated compounds in ethyl acetate crude extract of *N. crassa* (Table 7, Fig. 6). The percentage (24%) compositions of n-nonadecanol-1 was the highest; whereas, 11-octadecenoic methyl ester (3.8%) composition was the lowest (Table 7). However, some compounds had no match with the reference ion shown in Table 7 indicating the presence of novel compounds in crude extract of *N. crassa*.

Although the two extracts of *Talaromyces radicus* (*Talaromyces radicus* 1 and *Talaromyces radicus* 2*)* had antioxidant No. 33 compound (Table 8-9), but the chemical composition profiles of the two extracts were not similar (Fig. 7-8). The antioxidant No. 33 percentage composition was higher (45%) in *T. radicus* 2 as compared to percentage composition (33%) of *T. radicus* 1. For *T. radicus* 1 crude extracts, pentadecanoic acid was the second in composition proportion (33%); whereas, for *T. radicus* 2, Acetamide, 2,2-dichloro-N-[2-hydroxy-1-

Table 4. *Conyza bonariensis* endophytic fungi crude extracts minimum inhibitory concentrations (mg/mL) for all except C (µg/mL) observed in the present study (A: *Talaromyces* sp. (ON394877); B: *Penicillium daleae* (ON394878); C: *N. crassa* (ON394878); D: *T. radicus* (ON394880); E: *T. radicus* (ON39881)).

Crude extract	Test organism				
	S. aureus **(ATCC29213)**	***B. subtilis*** **(ATCC6051)**	***E. coli*** **(ATCC8736)**	***S. typhi*** **(ATCC 6539)**	***C. albicans*** **(DSM 1665)**
A	25>MIC>12.5	25>MIC>12.5	25>MIC>12.5	25>MIC>12.5	25>MIC>12.5
B	-	25>MIC>12.5	25>MIC>12.5	-	-
C	4010^{-3}>MIC>2010^{-3}	$15x10^{-4}$>MIC>7.810^{-4}	$15x10^{-4}$>MIC>7.810^{-4}	$15x10^{-4}$>MIC>7.810^{-4}	$15x10^{-4}$>MIC>7.810^{-4}
D	100>MIC>50	100>MIC>50	100>MIC>50	100>MIC>50	-
E	50>MIC>25	25>MIC>12.5	50>MIC>25	25>MIC>12.5	25>MIC>12.5

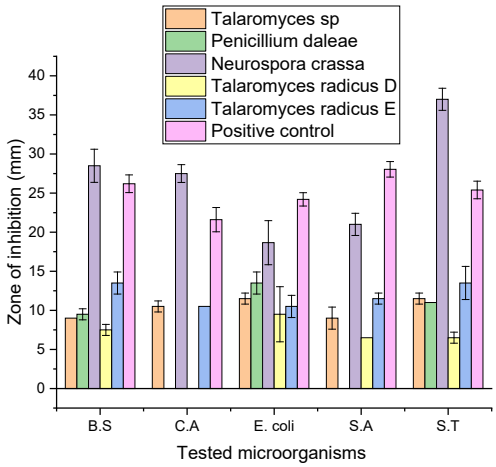

Figure 3. A bar graph portraying inhibition zones (mm) of endophytic fungi ethyl acetate crude extracts in the present study; error bars represent standard deviation; Positive controls were 0.25 mg/mL of chloramphenicol and fluconazole for bacteria and fungi, respectively**.**

(hydroxymethyl)- 2-(4-nitrophenyl)ethyl] was second in composition proportion (33%).

Discussion

Medicinal plants have been reported as the major source of secondary metabolites (Abdollahi *et al*., 2012; Amoateng *et al*., 2018; Frey & Meyers, 2010). Studies have been performed to identify novel compounds with biological potentials such as antimicrobial, antioxidant and anti-inflammatory (Kianfé *et al*., 2020). However, the direct harnessing of medicinal plants for the treatment of microbial infections has environmental implications. Therefore, environmentally friendly sources of secondary metabolites from endophytic fungi are highly desirable. In

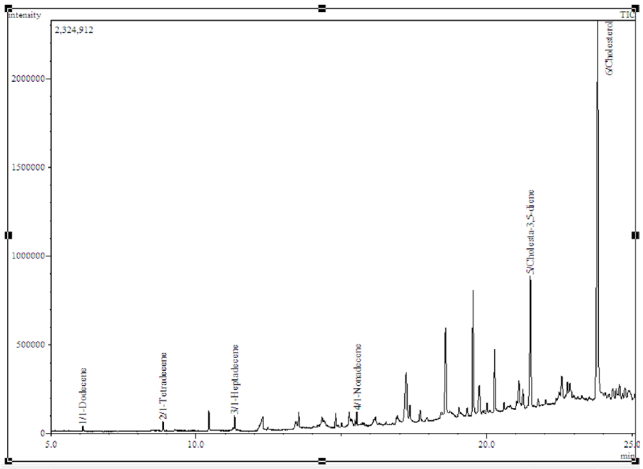

Figure 4. Chromatogram of isolate A, *Talaromyces* sp. (ON394877), ethyl acetate crude extract from *Conyza bonariensis* leaf.

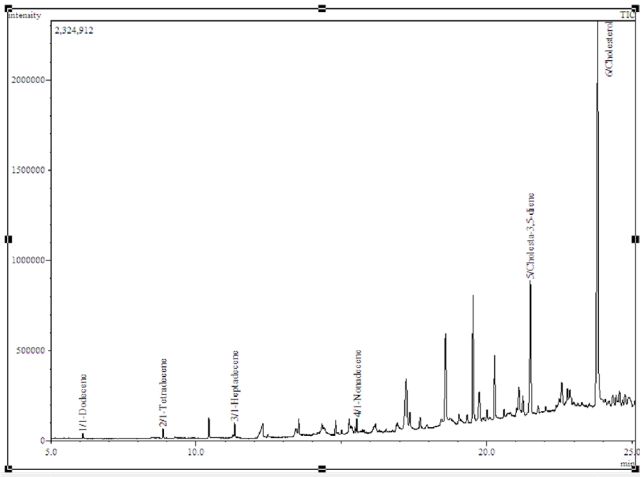

Figure 5. Chromatogram of crude extracts from isolate B, *Penicillin daleae* (ON394878), from *Conyza bonariensis* leaf

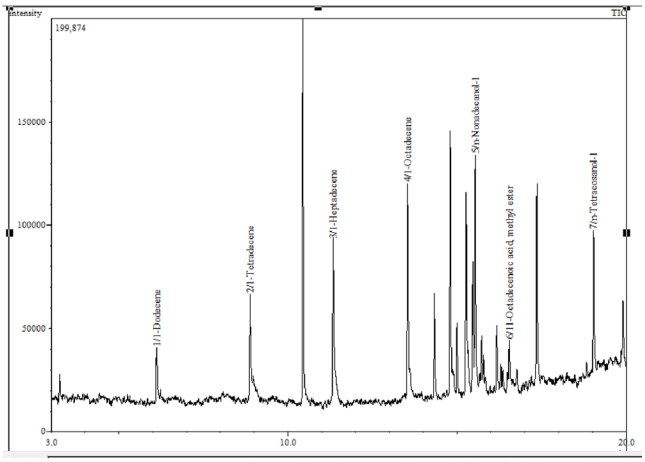

Figure 6. Chromatogram of crude extracts from isolate C, *Neurospora crassa* (ON394879) from *Conyza bonariensis* leaf.

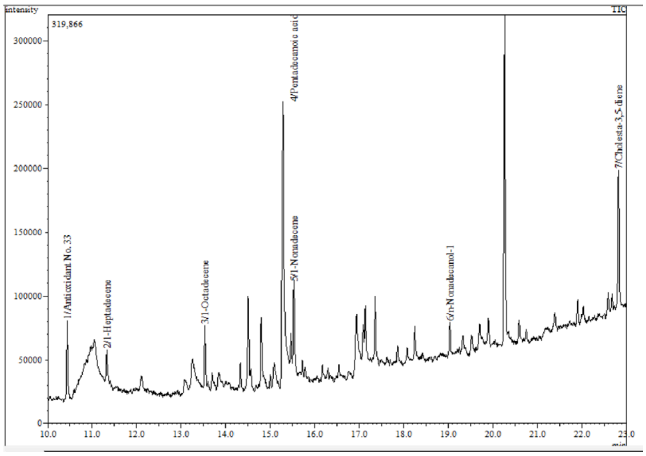

Figure 7. Chromatogram of ethyl acetate crude extracts of isolate D, *Talaromyces radicus* 1 (ON394880) from *Conyza bonariensis* leaf.

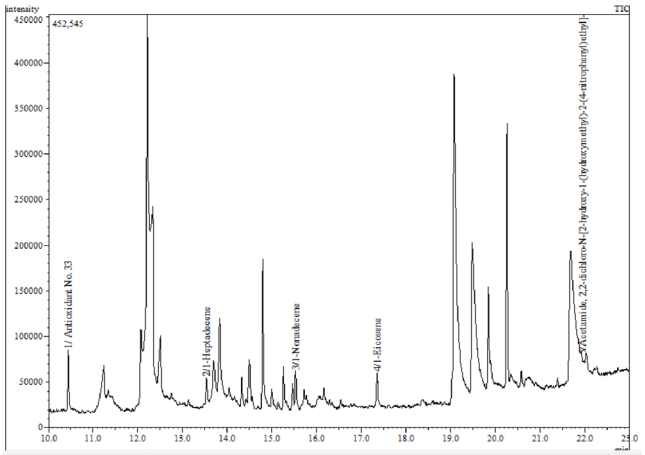

Figure 8. Chromatogram of crude extracts of isolate E, *Talaromyces radicus* (ON39488) from *Conyza bonariensis* leaf.

the present study, it was hypothesized that crude extract of endophytic fungi from *Conyza bonariensis* crude may have secondary metabolites with antimicrobial activity because the Safwa tribe of the Mbeya region of Tanzania has long traditionally used *C. bonariensis* leaves for treatment of microbial infections. Also, the same plant, experimentally, has been demonstrated to have antimicrobial activity (Araujo *et al*., 2013; Ghwanga & Chacha, 2019; Girma & Jiru, 2021). Indeed, results from the present study have supported this hypothesis because crude extracts of endophytic fungi from *C. bonariensis* had antimicrobial activity even higher compared to the antimicrobial activity of positive controls (Table 3, Fig. 3).

In this study, five endophytic fungi *(Talaromyces* sp., *Penicillum dalea*, *Neurospora crassa, Talaromyces radicus,* and *Talaromyces radicus*) were isolated. Based on the genetic relationship as depicted in Figure 2, all five isolates belong to the phylum Ascomycota. Furthermore, except for one isolate (*N. crassa*), which belongs to the family Sordariaceae, all other four isolates (*Talaromyces* sp., *Penicillum dalea*, *Talaromyces radicus* 1 and *Talaromyces radicus* 2*)* are members of the family Trichocomaceae (Fig. 2). Interestingly, all isolates are a member of the division Pezizomycotina of the Phylum Ascomycota (Amiri & Tibuhwa, 2020). Also, two isolates (D and E) were of the same species *(Talaromyces radicus),* which suggests that the two isolates may be of different strains, and because in the present study genotyping involved only the ITS region for identification of isolates, it is challenging to use the same information to decipher the two isolates to strain level. Furthermore, endophytic fungi isolated in the present study belong to the division Pezizomycotina not surprising because it is well known that most of the endophytes belong to the division Pezizomycotina (Amiri & Tibuhwa, 2020). In addition, the number of endophytes isolated from the present study is relatively low as compared to other studies (Mwanga *et al*., 2019; Yoon *et al*., 2018). This difference may be caused by the nature of the experiment. In the study, only *C. bonariensis* leaves were involved in the isolation of endophytes, and may be *C. bonariensis* leaves have a relatively lower richness of endophytic fungi.

In the present study antimicrobial activity of crude extracts of endophytic fungi was highly variable (Fig. 3). The variability in antimicrobial activity may be explained by the nature of endophytes isolated in the present study. The information generated in the present study indicates that endophytes were able to generate a variety of secondary metabolites with different levels of antimicrobial activity. Interestingly, taken as an example, the two species

Table 5. Chemical composition of isolate A, *Talaromyces* sp. (ON394877), crude extract from *Conyza bonariensis* leaves (R. time: Retention time, P. area: Peak area, P. height: Peak height).

Name of compounds	(% Composition P. area)	R. time	Chemical formula	Target peak	P. area	P. height	Activity
1-Dodecene	2.9206	6.107	$C_{12}H_{24}$	55.05	6723	3749	Antibacterial (Al-Abd *et al*., 2015; Khan *et al*., 2021; Xiong *et al*., 2013)
1-Tetradecene	4.90402	8.869	$C_{14}H_{28}$	55.1	11371	6295	Antimicrobial and antioxidant (Gautam *et al*., 2018)
1-Heptadecene	6.74488	11.329	$C_{17}H_{34}$	57.1	16273	8658	Antifungal (Mou *et al*., 2013)
1-Nonadecene	6.22916	15.533	$C_{19}H_{38}$	97.2	15001	7996	Anti-tuberculosis and antifungal (El-fayoumy *et al*., 2021)
Cholesta-3,5-diene	30.15565	21.505	$C_{27}H_{44}$	368.5	103942	38709	Wound healing (Bae *et al*., 2018)
Cholesterol	49.04568	23.813	$C_{27}H_{46}O$	386.5	210838	62957	Anticancer, antimicrobial, anti-psychotic, antioxidant (Albadawi *et al*., 2017)

Table 6. Chemical composition of crude extracts from isolate B, *Penicillin daleae* (ON394878), from *Conyza bonariensis* (R. time: Retention time, P. area: Peak area, P. height: Peak height).

Name of compounds	(% composition P. area)	R. time	Chemical formula	Target peak	P. area	P. height	Activity
1-Dodecene	3.61388	6.115	$C_{12}H_{24}$	55.1	9982	4083	Antibacterial (Al-Abd *et al*., 2015; Khan *et al*., 2021; Xiong *et al.,* 2013)
1-Tetradecene	6.5188	8.874	$C_{14}H_{28}$	55.1	16716	7365	Antimicrobial and antioxidant (Gautam *et al*., 2018)
1-Heptadecene	10.65312	11.332	$C_{17}H_{34}$	57.1	24598	12036	Antifungal
1-Octadecene	17.14182	13.535	$C_{18}H_{36}$	57.1	37753	19367	Antibacterial, antioxidant anticancer (Tonisi *et al*., 2020)
9-Hexadecenoic acid, methyl ester, (Z)-	5.62041	14.682	$C_{17}H_{32}O$	55.1	11971	6350	Antioxidant (Rahman *et al*., 2014)
11-Hexadecenoic acid, methyl ester	4.14494	14.729	$C_{17}H_{32}O_2$	55.1	8759	4683	Antioxidant, antimicrobial and ant-inflammatory (Yang *et al*., 1999)
n-Nonadecanol-1	19.11383	15.533	$C_{19}H_{40}O$	57.1	39401	21595	Antimicrobial and cytotoxicity (Begum *et al*., 2016)
11-Octadecenoic acid, methyl ester	20.52469	16.593	$C_{19}H_{36}O_2$	55.1	43122	23189	Antioxidant and antimicrobial (Rahman *et al*., 2014)
cis-10-Nonadecenoic acid, methyl ester	3.61211	17.551	$C_{20}H_{38}O$	55.1	7862	4081	Antibacterial and antioxidant (Gıdık, 2021)
n-Tetracosanol-1	9.05639	19.038	$C_{24}H_{50}O$	97.15	21446	10232	Antioxidant (Begum *et al*., 2016)

of *T. radicus*, which are said to might be different at the strain level, had different levels of antimicrobial activity, whereas *T. radicus* of isolate E had higher antimicrobial activity as compared to *T. radicus* isolate D (Table 3). A similar observation has been reported in other studies (Amiri & Tibuhwa, 2020; El-Said *et al*., 2016; Mwanga *et al*., 2019). The variation in antimicrobial activity may be explained by the difference in the chemical composition of secondary metabolites found in crude extract from particular endophyte. The GC-MS results from this study clearly show the difference in chemical composition and abundance of crude extracts (Table 5-9).

Table 7. Chemical composition of ethyl acetate crude extracts of isolate C, *Neurospora crassa* (ON394879), from *Conyza bonariensis* (R. time: Retention time, P. area: Peak area, P. height: Peak height).

Name of compounds	(% Composition P. area)	R. time	Chemical formula	Target peak	P. area	P. height	Activity
1-Dodecene	7.22334	6.12	$C_{12}H_{24}$	55.1	9695	3481	Antibacterial (Al-Abd *et al*. 2015; Khan *et al*., 2021; Xiong *et al*., 2013)
1-Tetradecene	11.83416	8.879	$C_{14}H_{28}$	55.1	15471	5703	Antimicrobial and antioxidant (Gautam *et al*., 2018)
1-Heptadecene	16.76869	11.338	$C_{17}H_{34}$	57.1	22560	8081	Antifungal (Mou *et al*., 2013)
1-Octadecene	22.28217	13.542	$C_{18}H_{36}$	57.1	25050	10738	Antibacterial, antioxidant anticancer (Tonisi *et al*., 2020)
9-Hexadecenoic acid, methyl ester, (Z)-	Ratio of reference ion does not match.	0	$C_{17}H_{32}O$	55.1	0	0	Antioxidant (Rahman *et al*., 2014)
11-Hexadecenoic acid, methyl ester	Ratio of reference ion does not match.	0	$C_{17}H_{32}O_2$	55.1	0	0	Antioxidant and antimicrobial (Rahman *et al.,* 2014)
n-Nonadecanol-1	24.00863	15.537	$C_{19}H_{40}O$	57.1	24599	11570	Antimicrobial and cytotoxicity (Begum *et al*., 2016)
11-Octadecenoic acid, methyl ester	3.84097	16.547	$C_{19}H_{36}O_2$	55.1	4433	1851	Antioxidant and antimicrobial (Mazumder *et al*., 2020)
cis-10-Nonadecenoic acid, methyl ester	Ratio of reference ion does not match.	0	$C_{20}H_{38}O_2$	55.1	0	0	Antibacterial and antioxidant (Gıdık, 2021)
n-Tetracosanol-1	14.04204	19.039	$C_{24}H_{50}O$	97.15	13554	6767	Antioxidant (Begum *et al*., 2016)

Table 8. Chemical composition of ethyl acetate crude extracts of isolate D, *Talaromyces radicus* (ON394880), from *Conyza bonariensis* (R. time: Retention time, P. area: Peak area, P. height: Peak height).

Name of compounds	(% composition P. area)	R. time	Chemical formula	Target peak	P. area	P. height	Activity
Antioxidant No. 33	32.64824	10.437	$C_{14}H_{22}O$	191.15	41231	22239	Antioxidant (Kahkönen & Heinonen, 2003)
1-Heptadecene	3.66428	11.33	$C_{17}H_{34}$	57.1	4922	2496	Antifungal (Mou *et al*., 2013)
1-Octadecene	6.872	13.533	$C_{18}H_{36}$	57.1	8420	4681	Antibacterial, antioxidant, anticancer (Tonisi *et al*., 2020)
Pentadecanoic acid	33.26189	15.289	$C_{15}H_{30}O_2$	73.05	69544	22657	Antibacterial and antifungal (Yoon *et al*., 2018)
1-Nonadecene	10.35424	15.532	$C_{19}H_{38}$	57.1	15721	7053	antimicrobial properties (El-fayoumy *et al*., 2021)
n-Nonadecanol-1	4.49814	19.039	$C_{19}H_{40}O$	57.1	6455	3064	Antimicrobial and cytotoxicity (Begum *et al*., 2016)
Cholesta-3,5-diene	8.70121	22.825	$C_{27}H_{44}$	368.35	11788	5927	Antimicrobial (Al-Hassan *et al*., 2020)

In the present study, *N. crassa* ethyl acetate crude extract shows the highest antimicrobial activities compared to other ethyl acetate crude extracts of endophytic fungi (*Talaromyces* sp., *Talaromyces radicus* 1, *Talaromyces radicus* 2, and *Penicillum dalea*) as shown in Figure 3 and Table 3. Furthermore, the MIC of *N. crassa* is highest compared to the positive control against several test microorganisms as shown in Figure 3. The differences in MIC among endophytic fungi crude extracts may be due to differences in chemical compositions (Santos *et al*., 2015; Selvi, 2014). The antimicrobial performance of crude extracts of *N. crassa* reported in the present study was even higher compared to the positive control (20 µL of 0.25 mg/mL of chloramphenicol). Furthermore, crude extracts of *N. crassa* had a higher performance as compared to previous studies (Ashok *et al*., 2015; Duhan *et al*., 2020). The variation in antimicrobial activity performance between studies exhibited by *N. crassa* has a multifaceted explanation. The genetic variability of *N. crass* in different studies is one of the possible explanations. Also, differences in tested organisms

Table 9. Chemical composition of ethyl acetate crude extracts of isolate E, *Talaromyces radicus* (ON39488), from *Conyza bonariensis* (R. time: Retention time, P. area: Peak area, P. height: Peak height).

Name of compounds	(% composition P. area)	R. time	Chemical formula	Target peak	P. area	P. height	Activity
Antioxidant No. 33	45.33531	10.442	$C_{14}H_{22}O$	191.15	48469	23850	Antioxidant (Kahkönen & Heinonen, 2003)
1-Heptadecene	6.52372	13.537	$C_{17}H_{34}$	57.1	8633	3432	Antifungal (Mou *et al.*, 2013)
1-Nonadecene	7.84862	15.534	$C_{19}H_{38}$	97.1	9012	4129	Antimicrobial (El-fayoumy *et al*., 2021)
1-Eicosene	6.94571	17.358	$C_{20}H_{40}$	57.1	7580	3654	Antioxidant and antimicrobial (Hsouna *et al*., 2011)
Acetamide, 2,2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]-	33.34664	21.693	$C_{11}H_{12}Cl_2N_2O_5$	153.05	107771	17543	Antimicrobial (Abu-Khadra *et al*., 2016)

and extracting solvents used in studies are other possible explanations for the observation. Furthermore, a growth difference in growth condition may be another possible reason for difference in antimicrobial activity between the present study and previous reports.

The results from this study have shown the potential use of endophytic fungi from *C. bonariensis* as an alternative source of bioactive secondary compounds with antimicrobial activity. Ethyl acetate crude extracts of *N. crassa* demonstrated the highest antimicrobial activity, therefore warranting further exploration of the endophyte and their crude extracts for various medicinal and industrial applications. Specifically, studies on the characterization of pure compound(s) that may be responsible for antimicrobial activity are urgently needed. This may result in the discovery of novel drugs.

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RESEARCH ARTICLE

ISSR-Based Population Genetic Structure of Some Turkish Honeybee (*Apis mellifera* L., 1758) Populations

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Abstract

Objective: *Apis mellifera*, in the order Hymenoptera, are social insects, also known as honey bees. Türkiye has many different honey bee ecotypes and subspecies. Examination of genetic diversity and evolutionary relationships of colonies in nine different locations in Türkiye was carried out using ISSR primers.

Materials and Methods: *Apis mellifera* samples were collected from Çorum, Elazığ, Eskişehir, Kütahya, İzmir, Manisa, Antalya, Samsun, and Muğla. The Lifton method was used for DNA extraction from 180 honey bees. DNA samples were amplified using six ISSR primers.

Results: From the amplification of 180 individuals with 6 ISSR primers, 283 polymorphic loci were identified. Genetic diversity data (*Na, Ne, h, I*) were obtained for nine populations from 283 loci. In all data on the calculated genetic diversity, the lowest values were found in the Samsun population, and the highest values were found in the Manisa population. The gene flow level calculated from the genetic differentiation value was found to be 0.22.

Conclusion: Genetic diversity has a role in the adaptation of species to changing environmental conditions and it is one of the raw materials of evolution. Herein, we preferred ISSR markers to identify the genetic structure of honey bees. The genetic diversity of honey bees has been found to be lower compared to previous studies. This variability may be a result of the ecological, climatic conditions, and biogeographic differences of Anatolia. The studies to be carried out with more examples from more locations related to honey bees in Türkiye will contribute to the clear identification of the genetic structure of this organism. Additionally, using other marker systems will help to clarify the status of populations in Türkiye.

Keywords: *Apis mellifera*, Genetic diversity, ISSR, Türkiye

Introduction

Honey bees, *Apis mellifera* Linnaeus, 1758 are social insects belonging to Hymenoptera and live as colonies. Hymenoptera includes more than 19,000 identified bee species (Cane, 2008). *Apis* are named true honey bees and they have very important economic and ecological roles (Kilani, 1999). Bees pollinate plant species and produce honey, bee gum, pollen, and royal jelly. Early studies including morphometric and molecular characterization of *Apis mellifera* L. showed that they are divided into five evolutionary strain groups.

The groups belonging to 33 subspecies are Group A (central and southern Africa), Group C (eastern Europe and the northern Mediterranean), Group M (northern Africa, northern and western Europe), Group O (the Middle East and the eastern Mediterranean) and Y (east Africa) (Ruttner, 1988; Franck *et al.,* 2000; Ilyasov *et al.*, 2020). Ruttner (1988) has determined different honey bee ecotypes and subspecies from Türkiye have a wide range of habitats

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and climates. Almost all the Türkiye honey bees, except for the northeast and southeast, are *A. mellifera anatolica* Maa. (Anatolian), and the others are *A. mellifera caucasica* Gorbachev (Caucasian) and *A. mellifera meda* Skorikov (Iranian), respectively.

Amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), and simple sequence repeats (SSR) are PCR-based markers that are very suitable for determining phylogenetic relationships and genetic diversity of plants, animals, and particularly insects. ISSR markers have been essentially built up to distinguish plant genetic diversity but confirmed to be highly beneficial for population studies of other organisms, especially insects (Paplauskienė *et al.,* 2006; Shouhani *et al.,* 2014; Rahimi *et al.,* 2016). ISSR is a PCR-based dominant marker and it is used to investigate population structure analysis, phylogenetic analysis, identification of species, genetic mapping, and genetic variation of closely related species (Zietkiewicz *et al.,* 1994). This technique has a great advantage in that it does not need preliminary DNA sequence knowledge for primer design, and it gives enough variable and sensible data, so researchers can spend a short time and an acceptable amount of money (Sheppard & Smith, 2000). For these reasons, the ISSR marker system might be reasonable and beneficial for the determination of target genomic positions (Radjabi *et al.,* 2012). Some studies have been done to determine the population genetic structure of honey bees using ISSR markers (Ceksteryte *et al.,* 2012; Shouhani *et al.,* 2014; Rahimi *et al.,* 2016; Ahmad, 2018).

The present study primarily aimed to characterize the phylogenetic relationships and polymorphism of *A. mellifera* populations from nine different regions of Türkiye using ISSR markers, and for data analysis, POPGEN version 1.32 (Yeh *et al.,* 1999) and GenAlEx version 6.5 (Peakall & Smous, 2012) computer programs were used.

Material and Methods

***Honey bee samples*:** 180 worker honey bees were collected from different regions (20 individuals from each of the Çorum, Elazığ, Eskişehir, Kütahya, İzmir, Manisa, Antalya, Samsun, Muğla populations) of Türkiye in nine *A. mellifera* colonies from June to August 2021.

***DNA extraction*:** Total DNA was obtained from honey bee samples collected from 9 different locations by the "Lifton DNA extraction" method (Bender *et al*., 1983).

***PCR amplification*:** PCR was performed in a total volume of 25 µl. The reaction content was established with 12.3 µl dH2O, 10.5 µl of PCR Master Mix (Thermo Scientific™ Catalog number: K0171), 1 µl ISSR primer (base sequences are given in Table 1), 0.2 µl Tween®-20 and 1 µl DNA. A reaction of 35 cycles was prepared. Each cycle was optimized with denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and elongation at 68°C for 3 minutes. In addition, pre-denaturation at 95°C for 3 minutes and final elongation at 72°C were added to the reaction cycles. The amplified products were run at a 70 V constant current for three hours on a 1% agarose gel containing ethidium bromide prepared with 1X TBE buffer. PCR products were electrophoresed on a 1% agarose gel in 1X TBE buffer and stained with ethidium bromide.

***Statistical analysis*:** Thermo Scientific™ GeneRuler 1 kb DNA Ladder was used as a reference to interpret the gel image obtained as a result of the electrophoresis of PCR products. The band sizes of each individual were

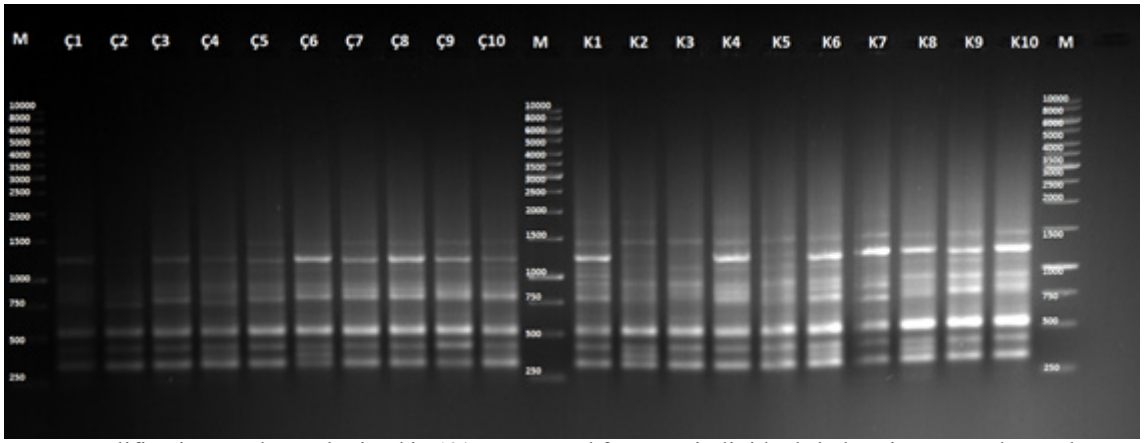

Figure 1. PCR amplification products obtained in 1% agarose gel from ten individuals belonging to two honey bee populations with CCA$(TG)_7$T primer [Ç1-10, 'Çorum 1-10'; K1-10, 'Kütahya 1-10'; M, 1kb ladder (GeneRulerTM)].

scored according to the band sizes created by the Thermo Scientific™ GeneRuler 1 kb DNA Ladder. By examining the corresponding band lengths on the DNA ladder, the presence of a band was scored with 1, and absence with 0 on Microsoft Excel (Fig. 1). Bands that did not show a clear image were not included in the scoring. The generated Microsoft Excel data were analyzed with PopGene ver. 1.32 software. As a result of the analysis, genetic diversity parameters, genetic differentiation values, and gene flow level values were obtained. In addition, the phylogenetic tree structure between populations was revealed with the UPGMA dendrogram by using the genetic distance value between the populations. Finally, the generated scoring table was analyzed with GenAlEx 6.503 software, and the main sources of genetic variation were revealed by molecular variance analysis.

Results

Molecular finger printings of 180 worker honey bees collected from different regions of Türkiye were carried out using six ISSR primers. All primers used produced polymorphic bands in DNA amplification. The number of total bands was 283 and their fragment size ranged from 150 to 10000 bp. Using six ISSR primers, 283 polymorphic loci were identified. The average number of polymorphic loci detected per primer used is approximately 47. All 283 loci identified with the six primers used were polymorphic, and no monomorphic locus was detected (Table 1).

All primers used produced the observed number of bands in populations between 107 (Manisa) and 65 (Samsun). On the other hand, polymorphic locus percentages were between 30.04% (Manisa) and 8.48%

Table 1. Information on primer sequences provided by Baysal *et al*. (2011) and information on band profiles created by these primers.

Primer Numbers	ISSR Primer Sequence (5'_3')	Number of total bands	Percent of polymorphic bands	Range of fragment size (bp)
1	$(ACC)_6CC$	53	100	300-8100
2	$CCA(TG)_7T$	67	100	400-10000
3	$CCA(TGA)_5TG$	29	100	400-2900
4	$GCA(AC)_7$	44	100	400-8000
5	$GGG(AC)_7$	52	100	300-6500
6	$(GA)_6GG$	38	100	150-4000
	Total	283	-	-
	Mean	47.16	100	325-6583

Table 2. Band profiles and percent polymorphism rates of *A. mellifera* populations.

Population	Sample Size	Number of bands	Number of polymorphic bands	Number of private bands	% Polymorphism
Corum	20	81	58	4	20.49
Elazığ	20	104	70	12	24.73
Eskişehir	20	100	70	12	24.73
Kütahya	20	85	55	11	19.43
İzmir	20	102	76	16	26.86
Manisa	20	107	85	23	30.04
Antalya	20	83	62	1	21.91
Samsun	20	65	24	0	8.48
Muğla	20	71	39	5	13.78
Mean	20	88.66	59.88	9.33	21.16

Table 3. Genetic diversity values of *A. mellifera* populations. *Na*: the mean number of observed alleles; *Ne*: the mean number of effective alleles; *He*: Nei's gene diversity; *I*: Shannon's information index.

Population	*Na*	*Ne*	*He*	*I*
Çorum	1.20±0.40	1.12±0.28	0.06±0.15	0.10±0.22
Elazığ	1.24±0.43	1.13±0.29	0.08±0.16	0.12±0.23
Eskisehir	1.24±0.43	1.13±0.29	0.08±0.16	0.12±0.23
Kütahya	1.19±0.39	1.10±0.26	0.06±0.14	0.09±0.21
İzmir	1.26±0.44	1.15±0.29	0.08±0.16	0.13±0.23
Manisa	1.30±0.45	1.17±0.31	0.10±0.17	0.15±0.25
Antalya	1.21±0.41	1.12±0.26	0.07±0.15	0.11±0.22
Samsun	1.08±0.27	1.04±0.16	0.02±0.09	0.03±0.14
Muğla	1.13±0.34	1.09±0.25	0.05±0.14	0.08±0.20
Mean	1.20±0.39	1.11±0.26	0.06±0.14	0.10±0.21

(Samsun). The number of polymorphic and specific bands in the populations ranged from 24 to 85 and 0 to 23, respectively (Table 2).

The average number of effective alleles (*Ne*) was found to be 1.12, 1.13, 1.13, 1.10, 1.15, 1.17, 1.12, 1.04, 1.09 in Çorum, Elazığ, Eskişehir, Kütahya, İzmir, Manisa, Antalya, Samsun, and Muğla, respectively. The average number of observed alleles (*Na*) were found to be 1.20, 1.24, 1.24, 1.19, 1.26, 1.30, 1.21, 1.08, 1.13 in Çorum, Elazığ, Eskişehir, Kütahya, İzmir, Manisa, Antalya, Samsun, and Muğla, respectively. Nei's gene diversity (*He*) values ranged between 0.10 (Manisa) and 0.02 (Samsun). Shannon's information index (*I*) values ranged from 0.15 (Manisa) to 0.03 (Samsun). Generally, the highest values of genetic diversity were found in the Manisa population, and the lowest values in the Samsun population (Table 3).

Total genetic variation (H_T) was 0.22 ± 0.02 based on the POPGENE ver. 1.32 analysis in all populations studied. The total genetic variation originated from approximately 32% within the populations (H_S: 0.07 ± 0.003) and approximately 68% among populations (D_{ST}: 0.15). Tests of homogeneity among the studied nine populations were made using analysis of molecular variance (AMOVA). The results are summarized in Figure 2. Similar to the POPGENE ver. 1.32 analysis, in the AMOVA analysis, a major part of the total genetic variation is due to variation among populations, while a minor part is due to variation within populations.

The genetic differentiation value (G_{ST}) was 0.68 and using the GST value, the gene flow level (N_M) was calculated as 0.22. Genetic distance values (D_N) ranged from 0.0337 to 0.3252 among population pairs. Independent with geographic distances, the minimum distance was detected between Antalya and Samsun, and the maximum distance was detected between Elazıg and Samsun populations (Table 4).

The UPGMA dendrogram was created using Nei's (1987) genetic distance values (D_N). According to the dendrogram given in Figure 3, the tree structure is divided into three main branches. Similar to the UPGMA dendrogram in the baseline coordinate analysis (PCoA), all studied populations were clearly separated, and the relationship between the populations was shown once again using PCoA (Fig. 4).

Table 4. Estimates of Nei's (1987) genetic distance (*DN*) coefficients between the *A. mellifera* populations.

Population	Çorum	Elazığ	Eskişehir	Kütahya	İzmir	Manisa	Antalya	Samsun	Muğla
Çorum	*****	0.7965	0.8049	0.9019	0.8062	0.7998	0.8228	0.7872	0.7864
Elazığ	0.2275	*****	0.8785	0.7953	0.8933	0.8589	0.7617	0.7224	0.7439
Eskişehir	0.2170	0.1295	*****	0.7959	0.8708	0.8624	0.7818	0.7446	0.7754
Kütahya	0.1033	0.2291	0.2283	*****	0.8008	0.7913	0.7988	0.7654	0.7871
İzmir	0.2154	0.1129	0.1383	0.2222	*****	0.8770	0.7640	0.7242	0.7458
Manisa	0.2235	0.1521	0.1480	0.2341	0.1312	*****	0.7722	0.7365	0.7582
Antalya	0.1951	0.2722	0.2462	0.2247	0.2691	0.2585	*****	0.9669	0.9299
Samsun	0.2392	0.3252	0.2949	0.2673	0.3227	0.3059	0.0337	*****	0.8908
Muğla	0.2403	0.2959	0.2544	0.2395	0.2933	0.2768	0.0726	0.1156	*****

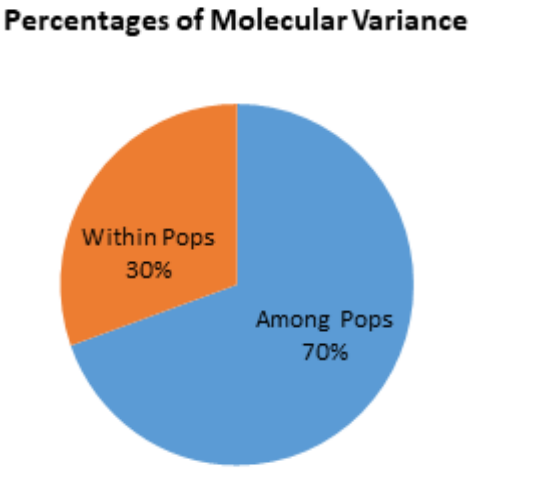

Figure 2. AMOVA results of all studied *A. mellifera* populations.

Discussion

The honey bee (*Apis mellifera L.*) is a globally important species of certain economic and ecological significance. Data we obtained as a result of the study indicated that the ISSR method is an appropriate technique for the determination of genetic polymorphism in *A. mellifera* populations. ISSR bands are highly repeatable in comparison with other dominant marker systems.

The aim of this study was the molecular characterization and determination of the genetic polymorphism of nine different *A. mellifera* populations using six ISSR primers. ISSR markers have been chosen in this study, because of giving a high level of polymorphic bands. When we compare data in our investigation with data in other honey bee studies in which the polymorphism levels were

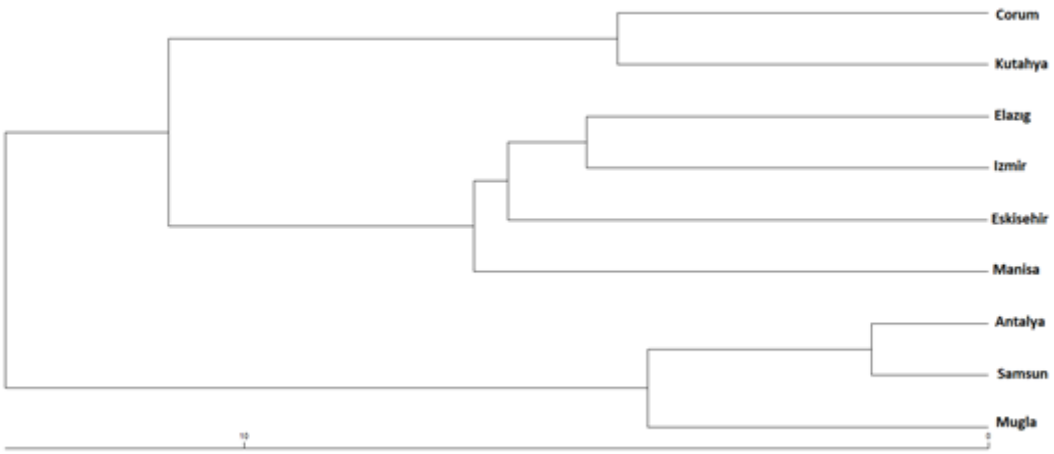

Figure 3. UPGMA dendrogram of *A. mellifera* populations revealed by Nei's (1987) genetic distance values (DN).

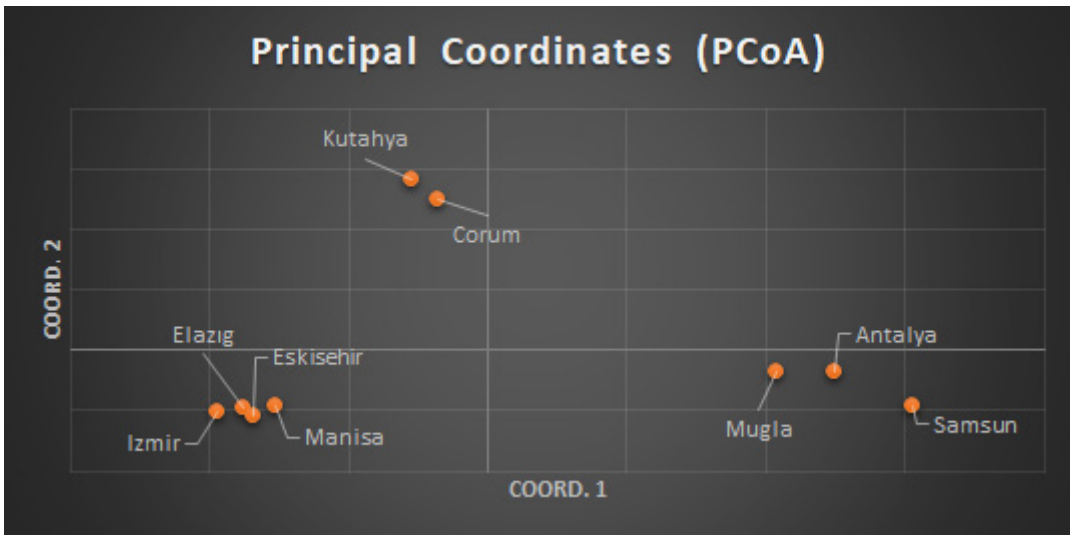

Figure 4. PCoA graph of *A. mellifera* populations

detected using ISSR markers (Al-Otaibi, 2008; Ceksteryte *et al.,* 2012; Karakaş, 2013; Shouhani *et al.,* 2014; Rahimi *et al.,* 2016; Ahmad, 2018), the polymorphism levels were found 81.6 %, 38.27 %, 51.33 %, 53.66 %, 61.56 %, and 55.52 %, respectively. Our polymorphism level (21.16%) was found to be less than in other studies.

We found 283 reproducible bands, ranging from 150 to 10000 bp. Al-Otaibi (2008), Rahimi *et al*., (2016), and Ahmad (2018), reported different results. Rahimi *et al*. (2016) and Ahmad (2018) determined the genetic relationship of different honey bee populations using ten ISSR primers. Rahimi *et al*. (2016) obtained 40 polymorphic bands ranging from 150 bp to 1500 bp while Ahmad (2018) obtained 50 polymorphic bands ranging from 100 bp to 850 bp. On the other hand, Dušinsk *et al*. (2006) determined polymorphism of some blackflies species (*Simulium* sp.) having many taxonomic problems using five ISSR primers and they obtained 55 polymorphic bands ranging from 230 bp to 1400 bp. The same differences were revealed in the results of other studies using ISSR primers for the determination of genetic polymorphism (Hundsdoerfer *et al.,* 2005; Al-Otaibi, 2008; Shouhani *et al.,* 2014).

Expected heterozygosity (*He*) and Shannon's information index (*I*) values, it was seen that Manisa population values were greater than the other populations. Moreover, the percentage of polymorphic loci and private bands have the highest in the Manisa population according to other populations. In addition to these results, the average number of observed alleles (1.30) and the average number of effective alleles (1.17) values were found high in the Manisa population. The average values of genetic diversity parameters were 1.20 for *Na*, 1.11 for *Ne*, 0.06 for *He,* and

0.10 for *I* and these results were consistent with the previous studies. These values were found in 1.38, 1.22, 0.13, and 0.19 (Ceksteryte *et al.,* 2012), 1,51, 1.29, 0.17 and 0.25 (Karakaş, 2013), respectively. These results show that the genetic diversity of the honey bees studied is relatively less. In all studied populations, H_T, H_S, $G_{ST,}$ and N_M values were found as 0.22, 0.07, 0.68, and 0.22, respectively. These results are relatively lower than Ceksteryte *et al*., (2012) (0.22, 0.42, 0.42, and 0.68 respectively) and Karakaş (2013) (0.29, 0.17, 0.39, and 0.76 respectively).

G_{ST} reveals genetic differentiation and takes a value between 0 and 1. A G_{ST} value greater than 0.25 indicates high differentiation between populations. The genetic differentiation value determined in nine honey bee populations screened with six ISSR primers was 0.68. The gene flow level (N_M) calculated based on the genetic differentiation value was found to be 0.22. According to Wright (1951), a gene flow level higher than 0.5 is considered a limit to prevent genetic drift of populations. If the Nm value is above 1, it is accepted that it prevents the local differentiation of populations (Slatkin, 1987). The calculated level of genetic differentiation and gene flow indicates high inbreeding in the studied populations. This indicates that existing populations are at risk of genetic drift.

In the studies conducted by Ceksteryte *et al*. (2012) and Karakaş (2013), the G_{ST} value is above the critical value and the genetic differentiation level is high. However, contrary to the work we have done in both studies, the gene flow level (N_M) is high. Therefore, the probability of genetic drift in the populations used in this study is higher than in the other two studies.

According to the data obtained as a result of POPGENE ver. 1.32 software and AMOVA analysis, it was determined that the main contribution to genetic variation was caused by population genetic variation. In parallel with low gene flow and high genetic differentiation values among populations, this situation may cause irreversible significant gene losses in the gene pool in case of colony losses.

According to the percentages of molecular variance obtained from the AMOVA analysis, it was determined that the main contribution to genetic variation originates among populations (%70). These results are relatively compatible with Karakaş (2013) (54.4%) using the ISSR markers. Kükrer (2013) and Ahmad (2018) reported that, unlike this study, the main contribution to total genetic variation originates from within the studied populations.

PCoA analyses confirmed the grouping. Our principal component analysis (PCoA) results in our study were compatible with the results of Kükrer (2013), Karakaş (2013), and Tunca & Kence (2011). The UPGMA dendrogram was created using Nei's (1987) genetic distance values (DN). According to the dendrogram given in Figure 3, the tree structure is divided into three main branches. The first branch consists of Çorum and Kütahya populations, the second branch consists of Elazığ, İzmir, Eskişehir, and Manisa populations, and the third branch structure consists of Antalya, Samsun, and Muğla populations. This is an unexpected result because groups formed a tree structure independent of their geographical distance. This situation can be explained by the transportation of honey bee colonies living in a certain region to different cities and the creation of new bee colonies there. Also, Ruttner (1988), almost all of the honey bees located outside the northeastern and southeastern regions in Türkiye "*A. mellifera anatolica* Maa. (Anatolian)" was stated. Since there is no population in the northeast and southeast regions among the studied populations, it is thought that a branching structure independent of the geographical location may have occurred.

Conclusion

Knowledge of the genetic diversity of the populations is very important. Genetic diversity has a role in the adaptation of species to changing environmental conditions, and it is one of the raw materials of evolution. Herein, we preferred ISSR markers to identify the genetic structure of honey bees. The genetic diversity of honey bees has been found to be lower compared to previous studies. This variability may be a result of the ecological, climatic conditions, and biogeographic differences of Anatolia. The low gene flow identified and the high level of genetic differentiation between populations indicate inbreeding. This combined with low genetic diversity makes it likely that conditions such as disease that may occur will not be enough to save the colony and result in the loss of an entire colony. In this case, it will result in the disappearance of important gene resources. For this reason, it is essential to develop existing protection programs and implement additional protection programs. The studies to be carried out with more examples from more locations related to honey bees in Türkiye will contribute to the clear identification of the genetic structure of this organism. Additionally, using other marker systems will help to clarify the status of populations in Türkiye.

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RESEARCH ARTICLE

The Length-Weight Relationship and Condition Factor of the Red Cornetfish, *Fistularia petimba* Lacepède, 1803 in the Southeastern Mediterranean Coast of Türkiye (İskenderun Bay)

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Abstract

Objective: In fisheries biology, length-weight relationship data are commonly used and helpful for determining the weight of an individual fish of known total length or total weight from length-frequency distribution. The present investigation aims to estimate the length-weight relationships and Fulton's condition factors from the southeastern Mediterranean coast of Türkiye.

Materials and Methods: A total of 368 red cornetfish, *Fistularia petimba* Lacepède, 1803 (203 females and 165 males) specimens were collected from the İskenderun Bay (southeastern Mediterranean, Türkiye) within the fishing periods of April 2019 and March 2020, caught by commercial bottom trawler at 28-42 m depths. Fish samples were measured in total length (TL) to the nearest 0.1 cm, and the total weight was scaled to the nearest 0.01 g.

Results: The total length ranged from 21.50-56.60 cm, and the weight ranged from 8.10-94.04 g for Fistularia petimba. The length-weight relationship was found as the $W = 0.00033 \times L^{3.126}$ ($r^2 = 0.963$) for both sexes. The value of *b* is slightly different than 3 and showed positive allometric growth. The condition factor was found to be 0.539 for females, 0.526 for males, and 0.520 for both sexes.

Conclusion: This study is the first investigation of the length-weight relationship and condition factor for *Fistularia petimba* from İskenderun Bay. The present study provides the basic fisheries parameters for this species in the Mediterranean Sea, which will be useful for fisheries management and fish population dynamics.

Keywords: Cornetfish, Fistulariidae, Regression parameters, Condition, Mediterranean Sea

Introduction

The red cornetfish, *Fistularia petimba* Lacepède, 1803, belongs to the Fistulariidae family, represented by a single genus (Froese & Pauly, 2023). The genus *Fistularia* Linnaeus, 1758 contains *Fistularia commersonii* Rüppell, 1838 and *F. petimba*, distributed in the Mediterranean Sea (Golani, 2000; Stern *et al.,* 2017). *Fistularia petimba* differs from its congeneric species *F. commersonii* by a row of bony plates along its posterior lateral line ossifications and the reddish or orange colour (Froese & Pauly, 2023). Stern *et al.* (2017) and Karan *et al.* (2019) confirmed that *F. petimba* is a genetically distinct species in the Mediterranean Sea.

Fistularia petimba is distributed in the western and eastern Atlantic, including the Mediterranean (Cardenas *et al.,* 1997; Froese & Pauly, 2023) and the Indo-Pacific Ocean (Fricke, 1999). *Fistularia petimba* is a reef species that inhabits coastal areas over soft bottoms at depths ranging from 10 to 200 m (May & Maxell, 1996). It feeds mainly on fish, cephalopods, shrimp, stomapods, other crustaceans, and small fishes (Druzhinin, 1977).

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Fistularia petimba was first reported in Spain (western Mediterranean) in 1996 (Cardenas *et al.,* 1997). Then, this species was identified in the Azores and Galician waters in the Eastern Atlantic Ocean (Azevedo *et al.,* 2004; Bañón & Sande, 2008). Later, this species was also recorded in the waters of Israel in 2017 (Stern *et al.,* 2017), Türkiye in 2018 and 2019 (Ünlüoğlu *et al.,* 2018; Çiftçi *et al.,* 2019), Cyprus (Michailidis & Manitaras, 2019), Syria (Hussein *et al.,* 2019), Egypt in 2019 (Ragheb, 2022), Lebanon and Greece in 2021 (Kondylatos & Nikolidakis, 2021; Sakr & Bariche, 2021). In addition, recent records are reported from Gökova Bay and Güllük Bay for the Muğla coast from northward along the shores of the eastern Mediterranean in the northern Aegean Sea coast of Türkiye by Cerim *et al.* (2021). Lately, this species has been confirmed from Bandırma Bay and distributed in the Marmara Sea, Türkiye (Uyan & Turan, 2021).

In fisheries biology, length-weight relationship data are reasonably helpful for determining the weight of an individual fish of known total length or total weight from length-frequency distribution (Froese, 1998; Koutrakis & Tsikliras, 2003). These data are used initially to obtain information on the condition of fish and to determine whether somatic growth is isometric or allometric (Le Cren, 1951; Ricker, 1975). Besides, the length-weight relationship is the primary value used for evaluating the status of fish stocks. It provides information for fish growth and health, and the length-weight relationship can be helpful in local and regional morphological and life-historical comparisons between different populations and habitats (Erguden *et al.,* 2011; Erguden & Turan, 2017; Erguden *et al.,* 2018).

Fistularia petimba penetrated the eastern part of the Mediterranean from the Red Sea via the Suez Canal (Stern *et al.,* 2017), and several were reported in the Mediterranean Sea six years ago (CIESM, 2022). *Fistularia petimba* is known to have high reproductive success. This species is a potential hazard to the ecological balance as well as economically affecting commercial fishing in the Mediterranean Sea (Çiftçi *et al.,* 2019).

To date, scarce data reports are available on populations of *F. petimba* in the Mediterranean. Only a few data were reported on the morphometric and meristic characteristics in the Egyptian Mediterranean waters (Ragheb, 2022) and a preliminary biological assessment of *F. petimba* from Cyprus (Papageorgiou *et al.*, 2023). In the present study, we first reported the length-weight parameters of *F. petimba* from İskenderun Bay. To the best knowledge, this study presented the first reference on length-weight relationships and condition factors for this species from İskenderun Bay (southeastern Mediterranean, Türkiye).

Materials and Methods

The red cornetfish of *F. petimba* were collected from İskenderun Bay using commercial trawl fisheries at 28-42 m depths within the fishing periods of April 2019 and March 2020 (Fig. 1). The collected fish specimens were transported with ice in a cooler box to the laboratory. Fish samples were measured in total length (TL) to the nearest 0.1 cm, and total weighed (W) was scaled to the nearest 0.01 g. Sexes were identified by macroscopic and microscopic examination of the gonads. The total length and total weight were measured for males and females separately. According to Fritzsche (1990) and Stern *et al*. (2017), all specimens were identified.

The length-weight relationship was calculated by applying an exponential regression equation $W=aL^b$ where; W is the weight (g), L is the total length (cm), and a and b are regression constants. Then, the parameters were estimated by linear regression on the transformed equation: $log\ (W) = log\ (a) + b \times log\ (L)$ (Ricker, 1975). The parameters a and b were calculated using least-squares regression as the coefficient of determination (r^2).

The sex ratio was calculated with $\chi 2$ test performed to check for any significant difference. The student's t-test was used to test for the difference of b values from 3, representing isometric growth (Pauly, 1993). Analysis of

Figure 1. Study area (İskenderun Bay, Türkiye)

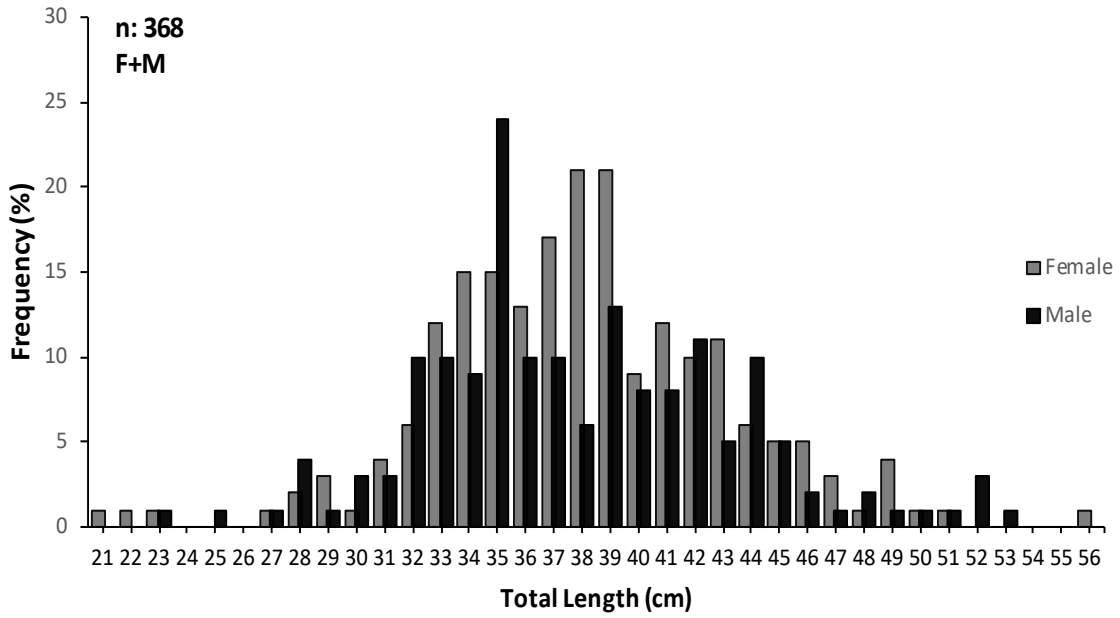

Figure 2. Total length (cm) frequency of males and females specimens of *Fistularia petimba* from İskenderun Bay

variance (ANOVA) was used to test the difference of the *b* values of the length-weight relationship between sexes (Zar, 1999) and statistically analysed using Microsoft Excel 2020 and SPSS Version 26.0 package programs.

Fulton's condition factor (K) was calculated for each sex to assess the condition of specimens and an overall measurement of the fish according to the formula; $K = (W/L^3) \times 100$, where L is length (cm) and W is the weight (g) (Sparre & Venema, 1992).

Results

In the present study, 203 females (55.2%) and 165 males (44.8%) *F. petimba* specimens were collected and investigated during the study period from İskenderun Bay. The sex ratio for female and male samples (M:F) was 1.00:1.23. The male-to-female ratio was not statistically significant at 0.05 ($p>0.05$).

The total length values of females and males ranged from 21.50-56.60 cm and 23.70-53.70 cm, respectively, and also the total weight values of females and males ranged from 8.10-94.04 g and 8.94-86.31 g, respectively (Table 1). There was no significant difference between sexes in overall total length and total weight (t-test, $p>0.05$).

The average total length of the calculated both sexes was found to be 38.31 cm ± 5.319, and the average total weight was 32.15 g ± 14.406. The dominant length class of collected *F. petimba* specimens ranged from 35.00 cm to 35.50 cm for all samples males dominated the 34.00-35.00 cm class, whereas females dominated 38.00-39.50 cm (Fig. 2). The descriptive statistics and estimated parameters of the length-weight relationship values are given in Table 1.

The length-weight relationship parameters were separately evaluated for *F. petimba* population of female, male, and both sexes specimens. The sample size (n), the level of equation parameters, the coefficient of determination (r^2), and the 95% confidence limits and growth type are given in Table 2. The linear regression equations for the length-weight parameters of *F. petimba* were highly correlated ($r^2 > 0.95$).

All values of *b* ranged from 3.106 for females, 3.138 for males, and 3.126 for both sexes for *F. petimba* (Table 2). The value of *b* is slightly different than 3.0, indicating that the species demonstrate positive allometric growth. The slopes (*b* values) of the total length-weight regressions were significantly different between sexes (*t*-test, $p<0.05$).

Table 1. Mean and standard deviation (SD), maximum, minimum for total length (TL) and total weight (TW) parameters of each sex of *Fistularia petimba* (İskenderun Bay, Türkiye)

Sex	n	TL (min-max)	Mean TL ± SD	TW (min-max)	Mean TW ± SD
Female	203	21.50-56.60	38.52±5.151	8.10-94.04	32.74±14.219
Male	165	23.70-53.70	38.28±5.532	8.94-86.31	31.42±14.643
Both Sexes	368	21.50-56.60	38.31±5.319	8.10-94.04	32.15±14.406

Table 2. Length-weight relationships of *Fistularia petimba* from İskenderun Bay, Türkiye (SE: Standard Error, A: Allometric Growth)

n	Sex	*a*	*b*	r^2	SE of *b*	95% CI of *b*	t-test	p	Growth Type
203	Females	0.00036	3.106	0.964	0.042	3.022-3.189	7.604	<0.05	A (+)
165	Males	0.00033	3.138	0.965	0.047	3.045-3.230	7.716	<0.05	A (+)
368	Both Sexes	0.00032	3.126	0.963	0.032	3.073-3.199	10.825	<0.05	A (+)

The length-weight relationships were calculated for females, males, and both sexes and observed as W=0.00036 × $L^{3.106}$ (r^2 = 0.964), W=0.00032 × $L^{3.138}$ (r^2 = 0.965) and W= 0.00033 × $L^{3.126}$ (r^2 = 0.963) respectively (Fig. 3). An ANOVA test calculated a significant statistical difference in length-weight relationship parameters between females and males (p<0.001). The correlation coefficient (r^2) for all relationships is above 0.95, indicating a strong degree of correlation between the variables in all groups.

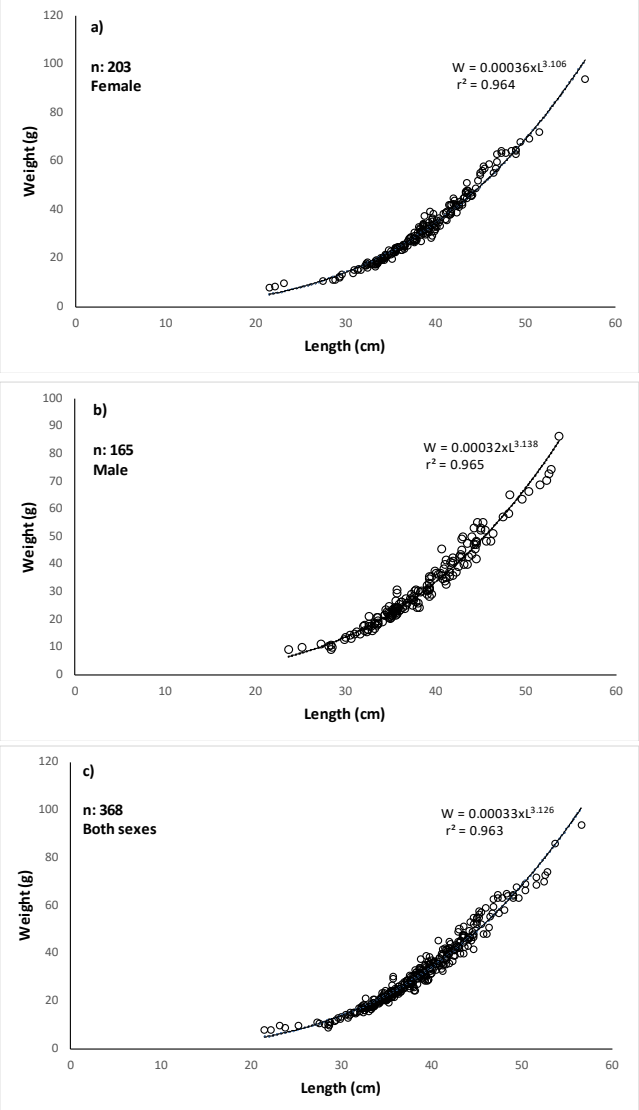

Figure 3. Length-weight relationship of female (a), male (b) and both sexes (c) for *Fistularia petimba* from the İskenderun Bay (south-eastern Mediterranean, Türkiye)

Fulton's condition factor of the present species from the southeastern Mediterranean coast (İskenderun Bay, Turkey) was analysed using the total weight. The condition factor was 0.539±0.057 for females, 0.526±0.047 for males, and 0.520±0.049 for both sexes. Therefore, condition factor values do not show significant variations (p>0.001) for female and male individuals of *F. petimba*.

Discussion

A total of 368 samples of *F. petimba* were collected from İskenderun Bay, southeastern Mediterranean, Türkiye. The population of *F. petimba* comprised 55.2% of female specimens and 44.8% of male specimens. The total length of the examined for both sexes was measured as 27.5-56.6 cm (38.311±5.319) and total weight 8.10-94.04 g (32.747±14.219) (Table 1). Female specimens were found to grow slightly larger than male specimens. Fritzsche (1976) indicated that this species usually has 100 cm and smaller samples, with the mean total length of the samples being 65.9 cm. Durizhinin (1977) reported total lengths of 128.5 cm and weights of 1450 g, with females larger than males for *F. petimba*. Cárdenas *et al.* (1997) similarly reported a large red specimen of 124.0 cm from the Western Mediterranean off Spain in 1996. In our study, the size ranges of the samples consisted of smaller specimens. Thus, maximum length and weight values were not found in the samples collected from İskenderun Bay. So far, the maximum length reported for *F. petimba* is 200 cm (Sanchez, 1991) and the weight is 4.7 kg (Bykov, 1983).

Previously, in Mediterranean studies, Ünlüoğlu *et al.* (2017) reported a total length ranged 35.1-47.4 cm for *F. petimba* from the eastern Mediterranean Sea, Türkiye. Cerim *et al.* (2021) stated that the total length ranged 33.7-50.4 cm for *F. petimba* collected from the South Eastern Aegean Sea (Türkiye). Papageorgiou *et al.* (2023) reported a total length ranged 38.9-43.7 cm for *F. petimba* from the Levantine Sea, Cyprus. In addition, Michailidis & Manitaras (2019) stated a total length of 60.2 cm for *F. petimba* from the Mediterranean waters of Cyprus. On the other hand, Hussein *et al.* (2019) declared that a large specimen of 64.0 cm of total length without filament for *F. petimba was* caught from the Mediterranean coast

Table 3. Previously available length-weight data of *Fistularia petimba* from different regions in the Mediterranean Sea

References	Number of samples	Date	Location/Country	Depth (m)	Fishing Gear	Total Length (cm)	Weight (g)
Cardenas *et al.* (1997)	1	June 1996	Cadiz, Western Mediterranean, Spain	50	Gill net	124.0	950.0
Stern *et al.* (2017)	1	December 2016	Ashdod, Eastern Mediterranean, Israel	80	Trawl net	29.5	65.0
Ünlüoğlu *et al.* (2017)	2	October 2016	Antalya Bay, Mediterranean Sea, Türkiye	30-43	Trawl net	35.1-39.8	-
	2	November 2016	İskenderun Bay, Mediterranean Sea, Türkiye	35-38	Trawl net	43.5-47.4	-
Çiftçi *et al.* (2019)	2	May 2018	Mersin Bay, N.E. Mediterranean Sea, Türkiye	150	Trawl net	46.5-51.3	-
Hussein *et al.* (2019)	1	July 2019	Lattakia, Mediterranean Sea, Syria	45	Gill net	64.2	54.0
Michailidis & Manitaras (2019)	1	September 2019	Gialia, Mediterranean Sea, Cyprus	55	Gill net	60.2	67.0
Cerim *et al.* (2021)	4	October 2019	Gökova Bay, Aegean Sea, Türkiye	15-20	Trawl net	33.7-36.9	-
	1	November, 2019	Güllük Bay, Aegean Sea, Türkiye	65	Trawl net	50.4	-
Sakr & Bariche (2021)	1	November 2017	Tripoli, Eastern Mediterranean, Lebanon	-	Gill net	49.5	-
Kondylatos & Nikolidakis (2021)	1	June 2021	Marathokampos Bay, Aegean Sea, Greece	20	Trammel net	34.2	17.3
Turan & Uyan (2021)	1	October 2021	Bandırma Bay, Marmara Sea, Türkiye	32	Trammel net	34.6	-
Ragheb (2022)	1	March 2019	West of Alexandria, Egypt	40-60	Trawl net	47.3	51.0
Papageorgiou *et al.* (2023)	76	June 2020, March 2021, August 2021 and September 2021	Levantine Sea, Cyprus	-	Trawl net	38.9-43.7	28.1-43.6
This study	368	April 2019 and March 2020	İskenderun Bay, SE. Mediterranean Sea,Türkiye	28-42	Trawl net	21.5-56.6	8.1-94.0

(Lattakia, Syria). The detailed previous Mediterranean studies of length-weight data for *F. petimba* are given in Table 3.

The growth coefficient values *b* ranged from 3.106 (females) to 3.138 (males), and the types of growth are positive allometric. Similarly, the previous studies of positive allometric growth (*b*=3.512) of *F. petimba* have been reported in the Levantine Sea (Cyprus) by Papageorgiou *et al.* (2023). In other studies, *b* values for both sexes of *F. petimba* were estimated as *b*= 3.158 in New Caledonia (Letourneur *et al.*, 1998) and as *b*=3.432 in the southeastern inner continental shelf region (Brazil) (Dias *et al.*, 2014). These previous results are in accordance with the studies performed in İskenderun Bay. Some differences in the *b* exponents could be attributed to the different areas and habitats, environmental conditions, the stomach contents, and stage of gonads as well as to the differences in season, diet, and maturity (Erguden *et al.,* 2011; Erguden *et al.,* 2015; Erguden *et al.,* 2017). In this study, the difference in the exponents may have occurred due to the different sampling regions, seasonal changes, age, maturity, and sex.

The values of *b* equal to 3 indicate that the fish grows isometrically; values different from 3 indicate allometric growth. In the length-weight relationships, the *b* value in the length-weight relationship showed that positive allometry (+) was obtained from the İskenderun Bay populations. According to Froese (2006), an over-proportional increase in length relative to growth in weight is expressed in a value of $b<2.5$, or a value of $b>3.5$ indicates an over-proportional rise in weight relative to growth in length.

The condition factor (*K*) of *F. petimba* from İskenderun Bay (southeastern Mediterranean, Türkiye) was analysed using the body weight. The condition factor value is very important to evaluate the fish health, and the ideal estimated is equal to or close to one. Besides, the condition factors differ from one species to another. In the present study, the condition factor values were estimated at a minimum as 0.520 and a maximum as 0.540 for *F. petimba*. This is because the condition of the fish may differ due to habitat area, diet composition, competition, prey, and top predators (Czudaj *et al.,* 2022; Ragheb, 2022).

The present study is the first reference on length-weight relationships and condition factors according to sex for *F. petimba* inhabiting İskenderun Bay. Determining the population parameters of this species, which will contribute to near-future studies, is very important in terms of the ecological balance in the Mediterranean ecosystem of non-native species entering the Mediterranean waters. In conclusion, this study provides basic information on the length-weight relationships *F. petimba* that would be useful for fishery scientists and managers in the Mediterranean Sea.

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Language

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