Species delimitation analysis indicates cryptic speciation for *Terpios gelatinosus* (Porifera, Demospongiae) from coastal regions of the northeastern Mediterranean Sea

Esra ÖZTÜRK[1], Berivan TEMİZ[2] & Arzu KARAHAN[1,*]

[1]Department of Marine Biology and Fisheries, Institute of Marine Sciences, Middle East Technical University, Erdemli-Mersin, Turkiye

[2]Developmental Biology and Genomics Laboratory, Department of Anatomy, Otago School of Medical Sciences, University of Otago, P.O. Box 56, Dunedin 9054, New Zealand

*Correspondence: arzukarahan@ims.metu.edu.tr, phone no:+905326462547

Abstract

Sponges, comprising over 9000 recorded species, represent one of the most primitive groups of metazoans. Given the importance of species distribution records and the identification of new species in scientific research, these endeavors play a crucial role in enhancing ecological insights, conserving biodiversity, facilitating a better understanding of the relationships between various groups of organisms, advancing our knowledge of evolution, and potentially expanding biomedical implications. In this study, we utilized basic morphological data, mitochondrial Cytochrome Oxidase I (COI) gene analysis, and ITS2 regions to evaluate taxonomy of *Terpios* samples collected from four coastal sites along the northeastern Mediterranean Sea, spanning a distance range of approximately 450 km. Eighty-one COI records and eleven ITS2 records of the order Suberitida were mined from NCBI and species delimitation analysis was performed using both the Automatic Barcode Gap Discovery method and the Poisson Tree Process (PTP), together with 11 samples from the present study. While we noted slight differences in spicule sizes, the general morphologies of all samples from our study were strikingly similar. Within the scope of this research, we report the first-ever presence record of *T. gelatinosus* in the northeastern Mediterranean Sea.

Furthermore, we document evidence pointing to the potential existence of cryptic speciation in the region.

Keywords: Cytochrome Oxidase I, Internal Transcribed Spacer, *Terpios*, morphology, species delimitation, cryptic speciation

1. Introduction

Sponges (Porifera), with 9,590 recorded species, are the most primitive group of metazoans (Müller *et al.*, 2007, World Porifera Database (2023)[1]). They are classified into four classes; the Calcarea, Demospongiae, Hexactinellida, and Homoscleromorpha (van Soest *et al.*, 2012). Demospongiae is the largest class of Porifera, including nearly 90% of all living sponges with ~8,000 species worldwide (Lavrov *et al.*, 2023). They live on different substrates and in diverse habitats, from the tropics to polar seas (Wörheide *et al.*, 2012). High species abundance was reported from the eastern Mediterranean Sea due to varied suitable coastal rocky shores (Gerovasileiou and Voultsiadou-Koukoura, 2012).

Morphology-based taxonomy is used for species classification around three centuries (Dunn, 2003). However, the identification of primitive marine animals based on only classical morphology has been an ongoing problem due to cryptic speciation, convergent evolution, simple and non-uniform body shape (Solé-Cava and Thorpe, 1986; Park *et al.*, 2007; Morrow *et al.*, 2013). One of main morphological character of sponges are spicules, but most of them are quite simple and their size can easily change even under relatively low environmental variation (Solé-Cava and Thorpe, 1986). Thus, molecular markers were suggested to support morphology-based taxonomy (Solé-Cava *et al.*, 1991). DNA barcoding is being used for this purpose for around two decades (Hebert *et al.*, 2003a; Hebert and Gregory, 2005). Almost a million species have been barcoded by now ([2]BOLD, 2023) and this number is increasing daily. Besides, there are certain DNA barcoding initiatives which focus on only certain

marine group like 'Sponge Barcoding Project' (Vargas *et al.*, 2012; Wörheide and Erpenbeck, 2007). Cytochrome Oxidase I gene is the primary gene region used for the barcoding purpose (Hebert *et al.*, 2003b; Karahan *et al.*, 2017), additionally Internal Transcribed Spacer (ITS) region (Wörheide *et al.*, 2004) and many others (van Oppen *et al.*, 2002) are used for different taxa to delimitate the species.

The genus *Terpios*, classified under the Suberitidae family of Demospongiae, is found under diverse temperatures and habitats, from animal shells to the costal and deep waters (van Soest 2002, *et al.*, 2012). *Terpios gelatinosus* (Bowerbank, 1866) displays light brownish yellow or bright brown color. It has been reported that the color alters to deep blue in the presence of a symbiotic alga (Ackers *et al.*, 2007). So far, *T. gelatinosus* records have been given in Italy (Bertolino *et al.*, 2014), Portugal (Monteiro, 2013), Greece (Voultsiadou-Koukoura *et al.*, 2016) and the Mediterranean coast of Israel with single public DNA barcode record in database (Idan *et al.*, 2018). The species was also reported in the eastern Aegean Sea (Topaloğlu and Evcen, 2014).

The primary objective of this study was to delineate the distribution of *Terpios gelatinosus* along the northeastern coasts of Turkiye, spanning a distance of 450 km. This has been achieved through species delimitation analysis, incorporating two molecular markers (COI and ITS2), and also involved an examination of basic morphological characteristics. Additionally, we analyzed database-mined samples to enhance our understanding of the species.

2. Materials and Methods

Eleven samples were collected from rocky coastal areas with a depth of less than 1 meter, between November 2017 and October 2018, along a distance of 450 km of the Turkish Mediterranean coastline, including three sites in Mersin (Mezitli, Kızkalesi, and Tisan) and one site in Antalya (Side, Fig. 1). Two samples were

collected from Kızkalesi, two from Mezitli, three from Side, and four from Tisan. Photographs of all specimens were taken in their natural habitats (under stones), and the colors and general morphologies were recorded. A fragment from each specimen was removed from the surface using a razor blade and transferred to a lysis buffer for DNA extraction (Paz *et al.*, 2003). A fragment from one individual from each site was preserved in formalin (10%) for morphological examination. Basic morphological analysis was performed using stereo and light microscopes (Olympus SZX16-UC30 camera and Olympus CX43-ToupTek camera) following the criteria outlined by Bowerbank (1866). Spicules were prepared for microscopy using the protocol developed by Schwab and Shore (1971).

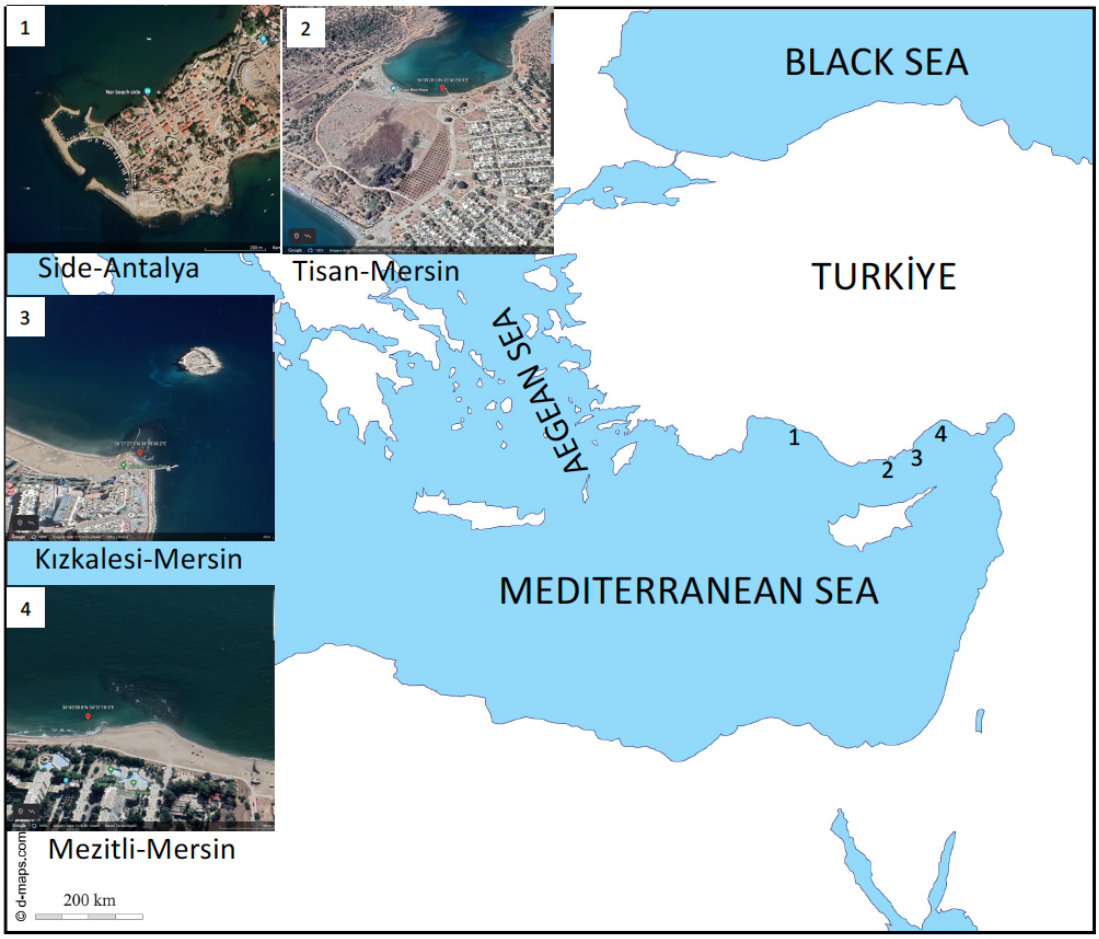

Fig. 1. The sampling sites of the North Eastern Mediterranean coastlines. Distance between the locations; Mezitli-Kızkalesi 72 km; Kızkalesi-Tisan 70 km; Tisan- Side 285 km.

The COI region was amplified using Folmer *et al. (*1994) primer set (HCO2198; 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ and LCO1490; 5′-GGTCAACAAATCATAAAGATATTGG-3′) and the ITS2 region was amplified using White *et al.* (1990), ITS3-ITS4 primer set (5′-GCATCGATGAAGAACGCAGC-3′, 5′-TCCTCCGCTTATTGATATGC-3′). The PCR products were sequenced by Macrogen Inc. (Seoul, South Korea) for both directions.

In total, 81 COI and 11 ITS records of the order Suberitida were retrieved from the NCBI and species delimitation analysis were performed together with 11 samples of the present study. All the sequences were aligned using MAFFT v7 (Katoh *et al*., 2018). The evolutionary distances between the present study and the database-mined samples were computed for both COI and ITS2 markers using the Kimura 2-Parameter and pairwise deletion methods with MEGAX (v. 10.1) software. The correlation between the geographic and genetic distances was tested using the Mantel Test (Mantel, 1967) on the BOLD system by comparing the geographic distance matrix (in kilometers) with the genetic divergence matrix.

2.1. Species delimitation analysis

We conducted species delimitation analyses using two distinct approaches: the Automatic Barcode Gap Discovery method (also known as Assemble Species by Automatic Partitioning - ASAP) developed by Puillandre et al. (2021), which employs sequence similarity clustering, and the Poisson Tree Processes (PTP) method introduced by Zhang et al. (2013), which is based on tree-based coalescent processes. ASAP analyses were carried out using the web-based interface available at https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html. We employed two metric options offered by ASAP for pairwise distance calculations: Jukes-Cantor (JC69) (Jukes and Cantor, 1969) and Kimura 2 parameter (K80) (Kimura, 1980). This approach was

chosen to mitigate any potential bias resulting from the selection of an evolutionary model on the delimitation of Operational Taxonomic Units (OTUs).

PTP analyses (Zhang *et al.*, 2013) were conducted using the Bayesian implementation (bPTP) which is available on the web-based interface (http://species.h-its.org/ptp/). For each alignment, a Bayesian tree was employed as input (Tang, 2014). Bayesian trees were generated using the best-fit substitution models determined by Smart Model Selection (SMS) in PhyML (http://www.atgc-montpellier.fr/sms/execution.php). According to SMS, the optimal model for ITS2 was HKY85+G+I, and for COI, it was GTR+G+I. MrBayes v3.2 was utilized with these models. Two parallel analyses were carried out; 50,000 generations for ITS2 and 1,000,000 generations for COI. Trees were sampled every 100 generations, and the results of the initial 12,500 generations for ITS2 and 250,000 generations for COI were excluded (burn-in fraction of 25%) after confirming the stationarity of the lnL. bPTP analyses employed the following parameter values: 50,000 MCMC generations, thinning every 100 generations, and a burn-in fraction of 0.30 for ITS2, and 1,000,000 MCMC generations, thinning every 100 generations, and a burn-in fraction of 0.30 for COI. The convergence of the MCMC chains was verified by visually inspecting the likelihood plot, as outlined in the PTP manual (https://species.h-its.org/help/), and then the maximum likelihood solutions were recorded

3. Results

3.1. *Morphological records*

Samples exhibited radiant, smooth, jelly-like surfaces (gelatinoid), thin-encrusting textures, and dark blue colors (Fig. 2a, c), which are characteristic features of *Terpios gelatinosus* (Bowerbank, 1866). Tylostyle types of macroscleres were observed in all samples (Fig. 2b, d). The size range varied between approximately 94-330 µm for Kızkalesi, Mezitli, and Side samples (out of a total of 139 counted spicules,

Fig. 2b) and approximately 152-350 µm for the Tisan samples (out of a total of 61 counted spicules, Fig. 2d).

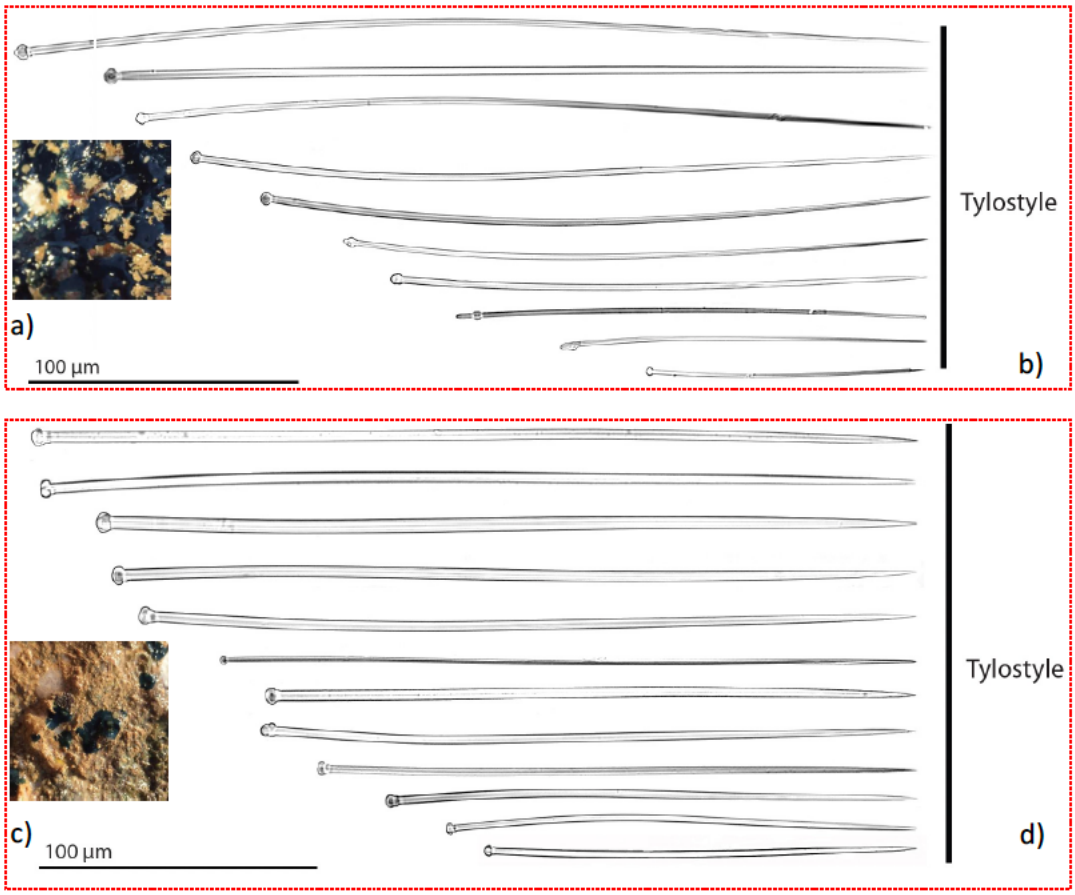

Fig. 2. Basic morphologic records of *Terpios gelatinosus* from a-b) Kızkalesi and c-d) Tisan regions; a-c) General images from field, b-d) Tylostyles under the light microscope (arranged according to sizes).

3.2. ***Data deposition and Blast analysis results***

After alignment and trimming, approximately 570 bp length sequences were used for the analysis. All the sequences were uploaded to the Barcode of Life Data System (BOLD) and NCBI together with sampling areas, coordinates and specimen images information. Two different BOLD BIN IDs were given for the samples; ADY2734 for Tisan and AAK9893 for the rest. The NCBI Accession numbers are:

OQ413306, OQ413307, OQ413308, OQ413309, OQ413310, OQ413311, OQ413312, OQ413313 and OQ413314.

Based on the BLAST results of the COI gene, the Kizkalesi, Mezitli, and Side samples exhibited matches of 99-100% similarity with the *Terpios gelatinosus* species from Israel (KX866738.1, Idan et al., 2018) and *Protosuberites sp.* (AY561979.1, Nichols, 2005) from California, USA. Meanwhile, four Tisan samples showed a coverage of 97.4-97.6% (E-value 0.0) with the same samples. The COI genetic distance between the two congeneric species, *Terpios hoshinota* and *Terpios gelatinosus*, was found to range from 9.2% to 10.9% (Table 1). Unfortunately, due to the absence of an ITS2 reference sequence for *Terpios gelatinosus* in the database, no close matches were obtained in the BLAST analysis (Table 2). The ITS2 distance between the Tisan and Kizkalesi-Mezitli-Side samples was approximately 13%

3.3. Species delimitation for COI

In the species delimitation analyses, the best score was determined based on the ASAP method, where lower scores indicate better partitions (Puillandre et al., 2021). The scores for the Kimura 2 parameter and Jukes-Cantor models were found to be 3.5 and 4, respectively, resulting in the assignment of 23 OTU (Fig. 3). Additionally, genetic distances within the range of 0.005 to 0.05 were considered during the partitioning process.

On the other hand, according to the PTP partition score in total 33 OTUs were assigned (Fig. 4). Notably, the Tisan samples were assigned to different OTUs compared to the Kızkalesi-Mezitli-Side samples in the PTP analysis. In the ASAP analysis, all samples from the present study were grouped within the same OTU, along with 18 database samples representing *Terpios gelatinosus, Protosuberites sp., Protosuberites mereui, Halichondria sp., Pseudosuberites nudus, and Protosuberites denhartogi* (Fig. 3).

It's worth noting that the congeneric species *Terpios hoshinota* was assigned to a different OTU by both ASAP and PTP analyses, with approximately a 10% genetic distance from the samples in the present study (Fig. 3, Table 1). The mean genetic distance among the 29 samples was calculated to be 0.04, with pairwise distances ranging from 0.0 to 0.083.

Table 1. COI Kimura 2 parameter pairwise genetic distances (%) of *Terpios* genus members.

	Kizkalesi- *T. gelatinosus*	Kizkalesi- *T. gelatinosus*	Mezitli- *T. gelatinosus*	Mezitli- *T. gelatinosus*	Side- *T. gelatinosus*	Side- *T. gelatinosus*	Side- *T. gelatinosus*	Tisan- *T. gelatinosus*	Tisan- *T. gelatinosus*	Tisan- *T. gelatinosus*	Tisan- *T. gelatinosus*	KP764915.1-*T. hoshinota*-Indonesia	MN507873.1 *T. hoshinota*-WA	MN507872.1 *Terpios hoshinota*-WA	GBMAA460-14 *T. hoshinota*-China	KX866738.1 *T. gelatinosus-Israel*	AY561979.1 *Protosuberites sp.*
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		0,2	0	0	0	0	0	2,4	2,4	2,4	2,4	9,3	9,3	9,5	9,5	0	0,5
2			0,2	0,2	0,2	0,2	0,2	2,6	2,6	2,6	2,6	9,2	9,2	9,5	9,5	0,02	0,7
3				0	0	0	0	2,4	2,4	2,4	2,4	9,2	9,2	9,5	9,5	0	0,5
4					0	0	0	2,4	2,4	2,4	2,4	9,2	9,2	9,5	9,5	0	0,5
5						0	0	2,4	2,4	2,4	2,4	9,2	9,2	9,5	9,5	0	0,5
6							0	2,4	2,4	2,4	2,4	9,2	9,2	9,5	9,5	0	0,5
7								2,4	2,4	2,4	2,4	9,2	9,2	9,5	9,5	0	0,5
8									0	0	0	10,7	10,7	10,9	10,9	2,4	2,2
9										0	0	10,7	10,7	10,9	10,9	2,4	2,2
10											0	10,7	10,7	10,9	10,9	2,4	2,2
11												10,7	10,7	10,9	10,9	2,4	2,2
12													0,2	0,4	0,4	9,2	9,5
13														0,4	0,4	9,2	9,7
14															0,6	9,5	9,7
15																9,5	9,7
16																	0,5

Table 2. ITS Kimura 2 parameter pairwise genetic distances (%) between the present study samples and *Suberites ficus.*

	AJ627184.1 *Suberites ficus*	Kizkalesi – *T. gelatinosus*	Kizkalesi – *T. gelatinosus*	Mezitli-*T. gelatinosus*	Mezitli -*T. gelatinosus*	Side – *T. gelatinosus*	Side-*T. gelatinosus*	Tisan - *T. gelatinosus*	Tisan - *T. gelatinosus*	Tisan - *T. gelatinosus*	Tisan - *T. gelatinosus*
	1	2	3	4	5	6	7	8	9	10	11
1		17,5	17,5	17,5	17,5	17,5	17,5	21,2	21,2	21,2	21,2
2			0	0	0	0	0	12,8	12,8	12,8	12,8
3				0	0	0	0	12,8	12,8	12,8	12,8
4					0	0	0	12,8	12,8	12,8	12,8
5						0	0	12,8	12,8	12,8	12,8
6							0	12,8	12,8	12,8	12,8
7								12,8	12,8	12,8	12,8
8									0	0	0
9										0	0
10											0

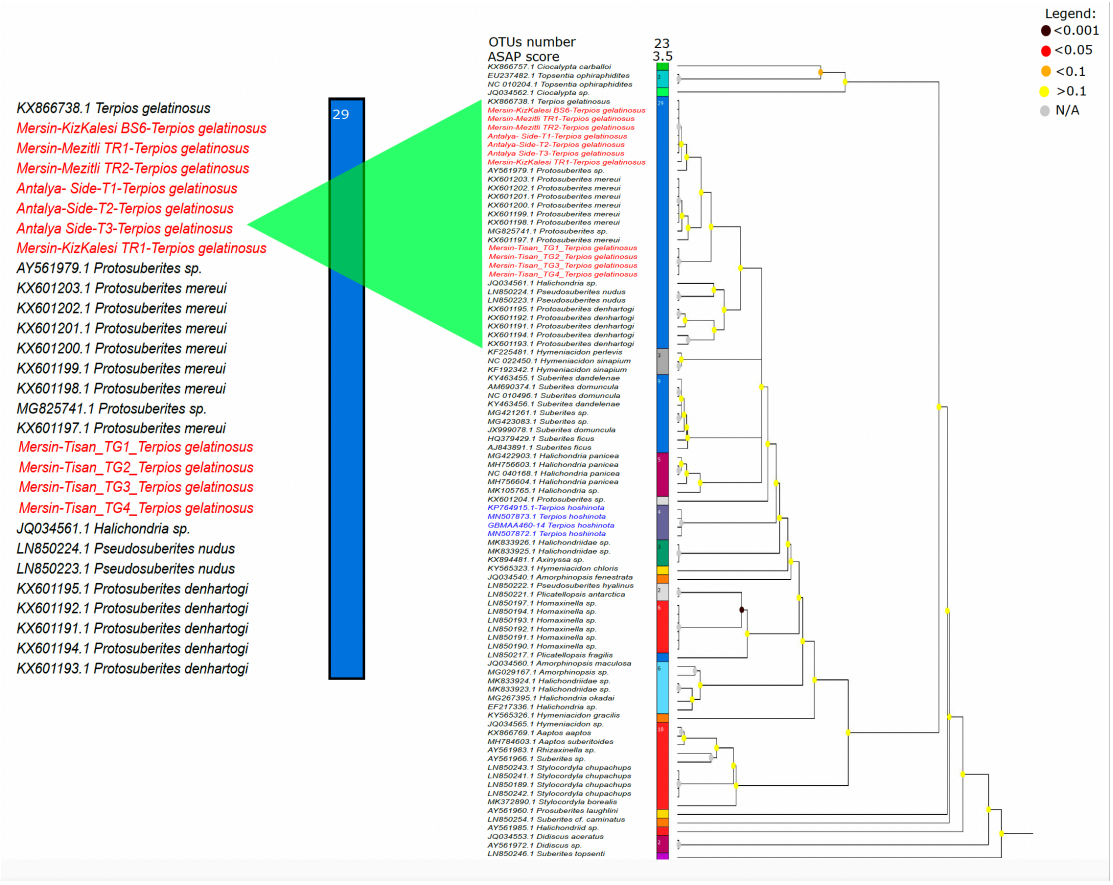

Fig. 3. The figure displays the ASAP scores of Suberitida specimens' COI. Different OTUs are represented by colors on the bar, and the number inside each color bar corresponds to the assigned specimen number for that OTU. The number of subsets, assigned total OTU number, ASAP score (where lower scores indicate better partitions, Puillandre et al., 2021), and the best rank column (1) are included. Samples from the present study are marked in red letters. Legend; Darker colors in the figure indicate lower probabilities, while a grey dot signifies that the probability was not computed. When a probability is very low (dark color), it suggests that the groups within the node likely correspond to different species. The blue letters represent the congeneric species, *Terpios hoshinota*.

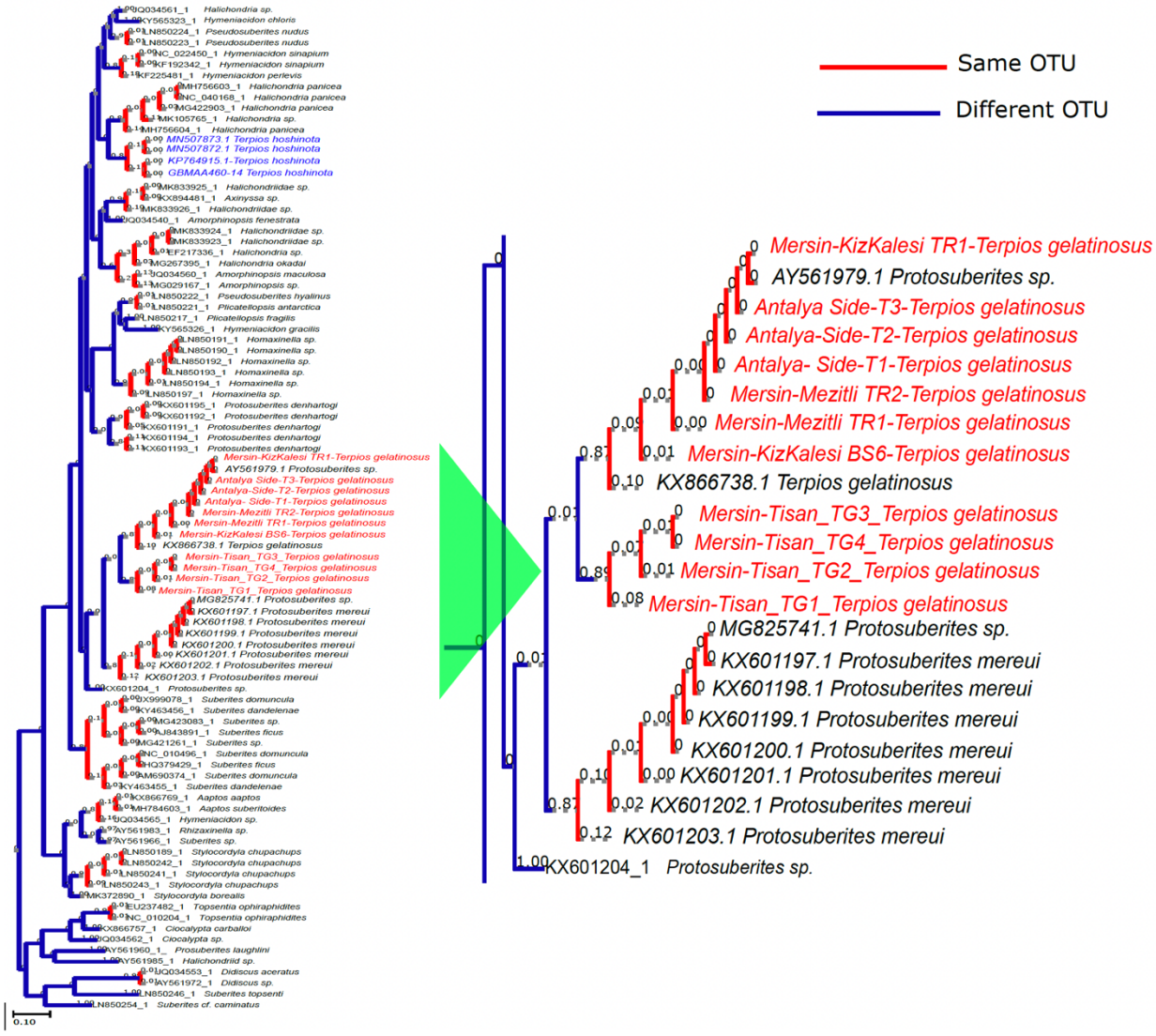

Fig. 4. PTP score of Suberitida specimens' COI; Blue lines represent different OTUs, red lines represent the same OTUs. Red letter represents present study samples. The blue letters represent the congeneric species, *Terpios hoshinota*.

3.4. Mantel Test

According to the Mantel test, no correlation was recorded between the geographic distance and the genetic divergence (Rsq=0.001, P-value =0.37).

3.5. *Species delimitation for ITS2*

The aligned and trimmed sequence length was 240 bp. In the species delimitation analysis, the lowest ASAP score was consistently 1.5 for both the Kimura 2 parameter and Jukes-Cantor models. As a result, eleven OTUs were assigned by both ASAP (Fig. 5a) and PTP (Fig. 5b) analyses. These two analyses revealed that all the Kızkalesi-Mezitli-Side samples were grouped within one OTU, while the Tisan samples formed another distinct OTU. The genetic distance between these two OTUs was approximately 13% (Table 2).

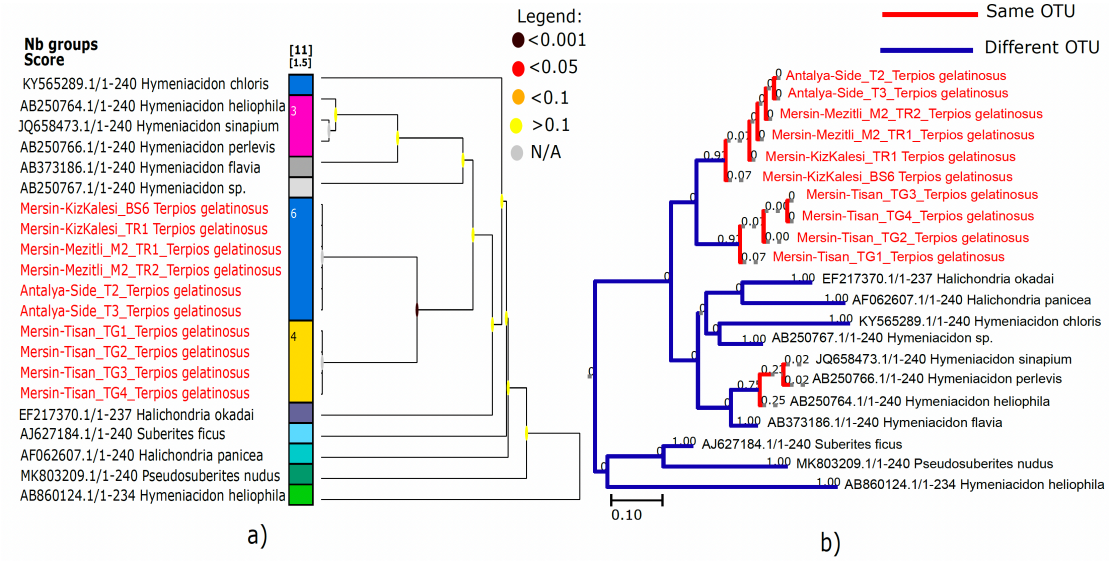

Fig. 5. a) The figure displays the ASAP scores of Suberitida specimens' ITS. Different OTUs are represented by colors on the bar, and the number inside each color bar corresponds to the assigned specimen number for that OTU. The number of subsets, assigned total OTU number, ASAP score (where lower scores indicate better partitions, Puillandre et al., 2021), and the best rank column (1) are included. Samples from the present study are marked in red letters. Legend; Darker colors in the figure indicate lower probabilities, while a grey dot signifies that the probability was not computed. When a probability is very low (dark color), it suggests that the groups within the node likely correspond to different species. **b)** PTP score of Suberitida specimens' ITS;

Blue lines represent different OTUs, red lines represent the same OTUs. Red letter represents present study samples.

3.6. Species assignment

Species assignment was done based on a combination of basic morphological data, BLAST score, ASAP (Assemble Species by Automatic Partitioning), and PTP (Poisson Tree Processes) analyses. Despite minor variations in spicule sizes, both morphological analysis and COI ASAP scores suggested the presence of a single species, identified as *Terpios gelatinosus*, in the area. However, COI's PTP score suggested the presence of two distinct species. Furthermore, ITS results from both ASAP and PTP analyses indicated the potential existence of cryptic speciation in the area. Following a comprehensive morphological examination, it is possible that the Tisan samples may be classified as a different species within the *Terpios* genus.

4. Discussion

Recording species distribution and identifying new species are crucial aspects of scientific research. These endeavors contribute significantly to our understanding of ecology, evolution, inter-organism relationships, biodiversity conservation, and even the potential biomedical applications (Mora *et al*., 2011). In this study, we have successfully unveiled the presence and spatial distribution of the *Terpios gelatinosus* species, along with indications of possible cryptic speciation, in the shallow waters along the northeastern Mediterranean coastlines. Our approach involved the utilization of basic morphological data, a distance matrix incorporating two molecular markers, as well as PTP and ASAP analysis scores for species identification.

In practice, both COI and ITS are frequently used in combination for species delimitation. Each marker offers distinct advantages and disadvantages depending on the research objectives and the organisms under investigation. COI, with its rapid

evolutionary rate, is conserved across a broad spectrum of taxa, making it a valuable tool for identifying species across diverse groups (Hebert *et al*., 2003b). Conversely, ITS is advantageous due to its high variability, enabling the differentiation of closely related species when used alongside other data, such as morphological characteristics or additional genetic markers (de Goeij *et al*., 2013).

While a 2.5% divergence in the mitochondrial DNA COI marker is typically an accepted threshold for most eukaryotes, it is important to note that different taxa, including Porifera, can exhibit varying divergence ranges due to their unique evolutionary histories (Hajibabaei *et al*., 2006; Núñez-Pons *et al*., 2017). As a result, ITS has become a valuable supplementary marker for Porifera identification alongside morphological data (Wörheide *et al*., 2002; Erpenbeck *et al*., 2006, 2007). Furthermore, ITS DNA sequences have been proposed as a potential alternative to the COI barcode region for sponges. This is attributed to their low evolutionary pressure and high variation rates (Wörheide and Erpenbeck, 2007; Song *et al*., 2012). For our species delimitation analysis, we employed both COI and ITS markers in conjunction with basic morphological data. While morphological data and the ASAP score of COI did not suggest discrepancies, the PTP score of COI, as well as both the ASAP and PTP scores of the ITS marker, indicated the potential presence of cryptic speciation in the studied area.

Cryptic speciation, which involves the existence of two or more species that closely resemble each other but are genetically distinct and unable to interbreed, can arise due to various factors such as geographical isolation, habitat differentiation, or ecological adaptation differences (Rützler *et al*., 2009). Identifying and distinguishing cryptic species can be a challenging task due to their similar external appearance. However, genetic techniques are increasingly being employed to uncover hidden biodiversity in sponges and other organisms (Hooper and van Soest, 2002). Despite low genetic divergence cryptic speciation was reported for the *Hemimycale columella* species through the utilization of genetic markers such as COI, 18S, and 28S gene

regions (Uriz *et al*., 2017). On the other hand beside of COI and ITS markers were used to elucidate the morphospecies complex of *Inianthella basta* and identify cryptic speciation in northern Australia (Andreakis *et al*., 2012). In our case we used COI and ITS to reveal a possible cryptic speciation in the Tisan region.

Many cryptic sponge species, which are geographically separated, are believed to have undergone allopatric speciation (Uriz and Turon, 2012). Sponges are sessile organisms with limited dispersal capabilities during their juvenile stage. As a result, gene flow between sponge populations is not solely reliant on larval dispersal but is also influenced by geographical distance (Maldonado, 2006). In our study, we conducted a Mantel test and found no significant correlation between genetic connectivity and geographic distance. Interestingly, both COI's PTP analysis and ITS's ASAP-PTP analyses revealed that the Kizkalesi-Mezitli-Side samples were grouped under a different OTU compared to the Tisan samples, despite the Tisan site being located between the Kizkalesi and Side sites. This unexpected distribution pattern might be explained by marine traffic, which plays a role in the dispersal of this organism. The Kizkalesi-Mezitli-Side sites are situated near heavy shipping routes (Alexopoulos, 2013), while the Tisan site is a relatively protected area, distant from ports. This leads us to consider the possibility of allopatric cryptic speciation in the studied area

In their previous study, Park *et al*. (2007) documented low intraspecific and high interspecific variation in the ITS region. Building upon this knowledge, the high genetic distance observed between the Tisan samples and those from other regions strongly suggests the presence of a potential cryptic species in the area. Given the improved resolution achieved within the *Terpios* genus through the use of the ITS region, it emerges as a promising candidate for a potential barcode site for species within the Suberitidae family.

Terpios fugax Duchassaing and Michelotti, 1864 on the other hand is one of the closest relatives of *T. gelatinosus.* It was recorded in the Atlantic Ocean (de

Laubenfels and Hindle, 1950), Indian Ocean (Pattanayak, 1999), Pacific Ocean (de Laubenfels, 1954) and European seas (Voultsiadou-Koukoura and van Soest, 1993). According to Araya and Rützler (2017), all European records of *T. fugax* are most probably misidentified individuals of *T. gelatinosus*. But we could not compare our samples because there is no public DNA record in any database of the species.

Another significant outcome of the present study is the revelation of the potential polyphyletic origin of the *Terpios* genus. Polyphyly refers to a taxonomic group that lacks monophyly, incorporating members from multiple, distinct ancestral lineages (Sperling *et al*., 2010). In our study, we observed that the two congeneric species, *T. gelatinosus and T. hoshinota*, were found to cluster far apart from each other on separate branches. This pattern is consistent with observations in other sponge species (Borchiellini *et al*., 2001; Hooper and van Soest, 2002; Wörheide and Hooper, 2002; Sperling *et al*., 2010)

In conclusion, this study marks the first reported distribution of *Terpios gelatinosus* in the study regions. Additionally, the results from species delimitation analyses, employing two DNA markers and basic morphological data, strongly suggest the existence of possible cryptic speciation along the Mediterranean coasts of Turkiye. Furthermore, this study raises the intriguing possibility of polyphyly within the *Terpios* genus.

[1]World Porifera Database (2023) [online]. Website https://www.marinespecies.org/porifera (accessed 29 August 2023).

[2]BOLD (2023) [online]. Website https://boldsystems.org/index.php

Acknowledgments

We would like to express our sincere thanks to Erdemli and Manavgat municipalities, as well as to Fatima Nur Oğul and Begüm Ece Tohumcu for their invaluable assistance

during the sampling process.This study was financially supported by the YÖP-701-2018-2666 grant from Middle East Technical University and the IMS-METU DEKOSIM (BAP-08-11-DPT2012K120880) projects

Authors' contributions: A.K. conceived and planned research; E.Ö., B.T., and A.K., carried out the experiments; E.Ö., B.T., and A.K. wrote the manuscript.

Availability of data and material: The datasets generated during and/or analyzed during the current study are available in the BOLD system BOLD (BIN IDs: ADY2734 and AAK9893) and NCBI (for COI: OQ413306, OQ413307, OQ413308, OQ413309, OQ413310, OQ413311, OQ413312, OQ413313 and OQ413314, for ITS OQ507665, OQ507666, OQ507667, OQ507668, OQ507669, OQ507670, OQ507671, OQ507672, OQ507673, OQ507674, OQ507675, ITS: OQ507665, OQ507666, OQ507667, OQ507668, OQ507669, OQ507670, OQ507671, OQ507672, OQ 507673, OQ507674, OQ507675)

Conflicts of interest: The authors declare that they have no conflict of interest.

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

References

Ackers RG, Moss D, Picton, BE, Stone SMK, Morrow CC. Sponges of the British Isles (“Sponge V”). A colour guide and working document 1992 EDITION, reset with modifications. Marine Conservation Society, Belfast, UK, 2007.

Alexopoulos A. Particularly Sea-Sensitive Areas and Marine Protection Zones. A Controversial Issue that needs Interpretation. Spoudai J. Econ. Bus. 63 (3-4): 51-59, 2013.

Andreakis N, Luter HM, Webster NS. Cryptic speciation and phylogeographic relationships in the elephant ear sponge *Ianthella basta* (Porifera, Ianthellidae) from northern Australia. Zool. J. Linn. Soc. 166 (2): 225-235, 2012.

Araya JF, Rützler K. First record of *Terpios fugax* Duchassaing and Michelotti, 1864 (Demospongiae: Suberitidae) in the Eastern Pacific Ocean. N. Z. J. Zool. 44: 86-90. 2017.

Bertolino M, Calcinai B, Cattaneo-Vietti R, Cerrano C, Lafratta A, Pansini M *et al.* Stability of the sponge assemblage of Mediterranean coralligenous concretions along a millennial time span. Mar. Ecol. 35: 149-158, 2014.

Borchiellini C, Peixinho S, Boury-Esnault N, Vacelet J, Le Parco Y. Phylogenetic relationships of tetractinellid sponges (Porifera: Demospongiae: Astrophorida) inferred from mitochondrial DNA sequences. Mol. Phylogenet. Evol. 20 (1): 41-50, 2001.

Bowerbank JS. A Monograph of the British Spongiadae. Vol. II. Ray Society, London, UK, 1866.

de Goeij JM, Vermeij MJA, Osinga R, Middelburg JJ. Spatial patterns in sponge biodiversity in a Caribbean coral reef. PloS one. 8 (3): e57620, 2013.

de Laubenfels MW, Hindle DE. The Porifera of the Bermuda Archipelago. Proc. Zool. Soc. Lond. 27: 1-154, 1950.

de Laubenfels MW. The sponges of the west-central Pacific (No. 7). Oregon State College Publishers, Corvallis, US, 1954.

Dunn CP. Keeping taxonomy based in morphology. Trends Ecol. Evol. 6 (18): 270-271, 2003.

Erpenbeck D, Hooper JNA, Wörheide G. CO1 phylogenies in diploblasts and the "Barcoding of Life" - Are we sequencing a suboptimal partition? Mol. Ecol. Notes. 6: 550-553, 2006.

Erpenbeck D, Voigt O, Adamski M, Adamska, Hooper JNA, Wörheide G *et al.* Mitochondrial diversity of early-branching metazoa is revealed by the complete mt genome of a haplosclerid demosponge. Mol. Biol. and Evol. 24: 19-22, 2007.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek RC. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 3: 294-299, 1994.

Gerovasileiou V, Voultsiadou-Koukoura E. Marine Caves of the Mediterranean Sea: A sponge biodiversity reservoir within a biodiversity hotspot. PLoS One. 7: e39873, 2012.

Hebert PDN, Cywinska A, Ball SL, de Waard JR. Biological identifications through DNA barcodes.
Proc. R. Soc. B: Biol. Sci. 270: 313-321, 2003a.

Hebert PDN, Ratnasingham S, de Waard JR. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. R. Soc. B: Biol. Sci. 270: 96-99, 2003b.

Hebert PDN, Gregory TR. The promise of DNA barcoding for taxonomy. Syst. Biol. 54: 852-859, 2005.

Hajibabaei M, Singer GAC, Hickey DA, Heneghan L. DNA barcoding: How it complements taxonomy, molecular phylogenetics and genetics. Trends Ecol. Evol. 21 (8): 354-359, 2006.

Hooper JNA, van Soest RWM. Systema Porifera: a guide to the classification of sponges. Kluwer Academic/Plenum Publishers, New York, USA, 2002.

Idan T, Shefer S, Feldstein T, Yahel R, Huchon Di, Ilan M. Shedding light on an East-Mediterranean mesophotic sponge ground community and the regional sponge fauna. Mediterr. Mar. Sci. 19: 84-106, 2018.

Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN (ed). Mammalian Protein Metabolism, Vol. III., Academic Press, New York-London, pp 22-126, 1969.

Katoh K, Rozewicki J, Yamada KD. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief. Bioinformatics, 20: 1160-1166, 2018.

Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111-120, 1980.

Karahan A, Douek J, Paz G, Stern, N, Kideys AE, Shaish L *et al.* Employing DNA barcoding as taxonomy and conservation tools for fish species censuses at the southeastern Mediterranean, a hot-spot area for biological invasion. J. Nat. Conserv. 36: 1-9, 2017.

Lavrov DV, Diaz C, Maldonado M, Morrow C, Perez T, Pomponi SA *et al.* Phylomitogenomics bolsters the high-level classification of Demospongiae (phylum Porifera). bioRxiv 2023-06, 2023

Maldonado M. The ecology of the sponge larva. Can. J. Zool. 84: 175-194, 2006.

Mantel N. The detection of disease clustering and a generalized regression approach. Cancer Res. 27 (2): 209-220, 1967.

Monteiro P, Bentes L, Oliveira F, Rangel MO, Afonso CML, Rodrigues J *et al.* An overview of the submerged sea caves of Sagres (South of Portugal-Algarve). MeshAtlantic Technical Report No 2. Universidade do Algarve-CCMAR. 2013.

Mora C, Tittensor DP, Adl S, Simpson AGB, Worm B. How many species are there on Earth and in the ocean? PLoS Biol. 9 (8): e1001127, 2011.

Morrow CC, Redmond NE, Picton BE, Thacker RW, Collins AG *et al.* Molecular phylogenies support homoplasy of multiple morphological characters used in the taxonomy of heteroscleromorpha (Porifera: Demospongiae). Integr. Comp. Biol. 53: 428-446, 2013.

Müller WE, Wang X, Zeng L, Schröder HC. Phylogenetic position of sponges in early metazoan evolution and bionic applications of siliceous sponge spicules. Chinese Sci. Bull. 52 (22): 3029-3040, 2007.

Nichols SA. An evaluation of support for order-level monophyly and interrelationships within the class Demospongiae using partial data from the large subunit rDNA and cytochrome oxidase subunit I. Mol. Phylogenet Evol. 34: 81-96, 2005.

Núñez-Pons L, Calcinai B, Gates RD. Who's there? - First morphological and DNA barcoding catalogue of the shallow Hawai'ian sponge fauna. PLoS One, 12: e0189357, 2017.

Pattanayak JG. Annotated checklist of marine sponges of the Indian region. Mem. Queensl. Mus. 44: 439-456, 1999.

Park MH, Sim CJ, Baek J, Min GS. Identification of genes suitable for DNA barcoding of morphologically indistinguishable Korean Halichondriidae sponges. Mol. Cells. 23 (2): 220-227, 2007.

Paz G, Douek J, Mo C, Goren M, Rinkevich B. Genetic structure of *Botryllus schlosseri* (Tunicata) populations from the Mediterranean coast of Israel. Mar. Ecol. Prog. Ser. 250: 153-162, 2003.

Puillandre N, Brouillet S, Achaz G. ASAP: assemble species by automatic partitioning. Mol. Ecol. Resour. 21 (2): 609-620, 2021.

Rützler K, Piantoni C, Pérès JM. Cryptic species diversity in marine sponges: Challenges and opportunities. Invertebr. Biol. 128 (3): 167-182, 2009.

Schwab DW, Shore RE. Fine structure and composition of a siliceous sponge spicule. Biol. Bull. 140 (1): 125-136, 1971.

Solé-Cava AM, Thorpe JP. Genetic differentiation between morphotypes of the marine sponge *Suberites ficus* (Demospongiae: Hadromerida). Mar. Biol. 93: 247-253, 1986.

Solé-Cava AM, Thorpe JP, Manconi R. A New Mediterranean Species of *Axinella* Detected by Biochemical Genetic Methods. In: Reitner J, Keupp H (eds). Fossil and Recent Sponges, Springer, Berlin, Heidelberg, Germany, pp 313-321, 1991.

Song J, Shi L, Li D, Sun Y, Niu Y, Luo H *et al.* Extensive Pyrosequencing Reveals Frequent Intra-Genomic Variations of Internal Transcribed Spacer Regions of Nuclear Ribosomal DNA. PLoS One. 7: e43971, 2012.

Sperling EA, Petersen JM, González A, Collins AG, Pisani D. Polyphyly and rapid diversification in the marine sponge suborder Haplosclerina. Mol Phylogenet Evol. 56 (2): 655-661, 2010.

Tang CQ, Humphreys AM, Fontaneto D, Barraclough TG. Effects of phylogenetic reconstruction method on the robustness of species delimitation using single-locus data. Methods Ecol. Evol. 5: 1086-1094, 2014.

Topaloğlu B, Evcen A. Updated checklist of sponges (Porifera) along the coasts of Turkiye. Turk. J. Zool.38: 665-676, 2014.

Uriz MJ, Turon X. Sponge ecology in the molecular era. Adv. Mar. Biol. 61: 345-410, 2012.

Uriz MJ, Garate L, Agell G. Molecular phylogenies confirm the presence of two cryptic *Hemimycale* species in the Mediterranean and reveal the polyphyly of the genera *Crella* and *Hemimycale* (Demospongiae: Poecilosclerida). PeerJ. 5: e2958, 2017.

van Oppen MJH, Wörheide G, Takabayashi M. Nuclear markers in evolutionary and population genetic studies of Scleractinian corals and sponges. In: Proceedings 9th International Coral Reef Symposium 2000; Bali. ICRS. pp 131-138, 2002.

van Soest RWM. Family Suberitidae Schmidt, 1870. In: Hooper JNA, van Soest RWM, Willenz P (eds). Systema Porifera, Springer, Boston, US, pp 227-244, 2002.

van Soest RW, Boury-Esnault N, Vacelet J, Dohrmann M, Erpenbeck D *et al.* Global diversity of sponges (Porifera). PLoS one. 7 (4): e35105, 2012.

Vargas S, Schuster A, Sacher K, Büttner G, Schätzle S, Läuchli B *et al.* Barcoding sponges: an overview based on comprehensive sampling. PloS One. 7 (7): e39345, 2012.

Voultsiadou-Koukoura E, van Soest RWM. Suberitidae (Demospongiae, Hadromerida) from the North Aegean Sea. Beaufortia. 43: 176-186, 1993.

Voultsiadou-Koukoura E, Gerovasileiou V, Bailly N. Porifera of Greece: An updated checklist. Biodivers. Data J. 4: e7984, 2016.

White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds). PCR Protocols: a guide to methods and applications, Academic press, London, United Kingdom, pp 315-322, 1990.

Wörheide G, Hooper JNA. A molecular framework for the phylogeny of Sponges (Porifera). Mar. Biol. 140 (1): 185-198, 2002.

Wörheide G, Degnan BM, Hooper JNA, Reitner J. Phylogeography and taxonomy of the Indo-Pacific reef cave dwelling coralline demosponge *Astrosclera 'willeyana'*: new data from nuclear internal transcribed spacer sequences. In: Proceedings 9th International Coral Reef Symposium 2000, Bali, ICRS, pp 339-346, 2002.

Wörheide G, Nichols SA, Goldberg J. Intragenomic variation of the rDNA internal transcribed spacers in sponges (Phylum Porifera): Implications for phylogenetic studies. Mol. Phylogenet. Evol. 33: 816-830, 2004.

Wörheide G, Erpenbeck D. DNA taxonomy of sponges - Progress and perspectives. J. Mar. Biol. Assoc. U.K. 87: 1629-1633, 2007.

Wörheide G, Dohrmann M, Erpenbeck D, Larroux C, Maldonado M, Voigt O *et al.* Deep Phylogeny and Evolution of Sponges (Phylum Porifera). In: Becerro MA, Uriz MJ, Maldonado M, Turon X (eds), Advances in Marine Biology, vol. 61. Academic Press, Amsterdam, The Netherlands, pp 1-78, 2012.

Zhang J, Kapli P, Pavlidis P, Stamatakis A. A general species delimitation method with applications to phylogenetic placements. Bioinform. 29: 2869-2876, 2013.