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Seven ascidian (Tunicata) species from the North Eastern Mediterranean

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Abstract

Members of the tunicates, a subphylum of marine filter-feeder chordates, inhabit all marine and oceanic habitats from the subtidal to the abyssal. Considered the closest relatives to the vertebrates, the tunicates are widely used as model organisms for evo-devo, allorecognition, senescence, and whole-body regeneration studies. However, species boundaries are poorly understood due to the high morphological and genetic plasticity that characterizes many tunicate taxa. Here, we present findings on seven tunicate species (*Botrylloides israeliense, Botrylloides* sp.*, Botryllus humilis*, *Botryllus schlosseri, Symplegma brakenhielmi, Polyclinum constellatum* and *Didemnum perlucidum*) sampled from six Turkish sites at the North Eastern Mediterranean Sea and employed the mitochondrial barcoding marker (COI) for evaluating the relationships among geographically restricted and widely spread ascidian species. Species delimitation was conducted using sequences generated in the current study in addition to sequences obtained from GenBank. General morphological features and colors of colonies were recorded at sampling sites. Then, all Styelidae colonies were attached and cultured on slides in an aquaculture room, enabling the study of other features, such as zooid distributions and sizes, oral tentacle numbers, and life cycles, using stereo and light microscopes. The spicules of formalin-fixed *Didemnum perlucidum* samples were examined under a light microscope. Then, scientific names were assigned to all species based on the results of the species delimitation and on comparisons of the obtained COI sequences with GenBank sequences. A putative new *Botrylloides* species (*Botrylloides* sp.) from the Antalya region was revealed, with a 99% match with the COI gene of another specimen from Saudi Arabia; further waiting for detailed traditional taxonomy.

Keywords: Botryllid ascidians; *Didemnum*; *Polyclinum; Symplegma*; COI; species delimitation.

Introduction

The ascidians (Phylum: Chordata, Subphylum; Tunicata) are a class of marine filter feeder organisms, with ca. 3000 described species that inhabit all marine and oceanic habitats from the subtidal to the abyssal zone (Shenkar *et al.,* 2023). As the closest relatives of the vertebrates, tunicates are widely used as model organisms for evo-devo research analyses (Ferrier, 2011), for elucidating the evolution of immunity (Mueller & Rinkevich, 2020; Ballarin *et al.,* 2021), senescence and aging processes (Ben-Hamo *et al.,* 2018, 2023), whole-body regeneration phenomena (Rosner *et al.,* 2019), stem cell biology (Rinkevich *et al.,* 2022) and more. However, the inadequate comprehension of species boundaries in many clades can be attributed to their rapid evolution rate and high genome plasticity (Berná & Alvarez-Valin, 2014; Holland, 2016) and the existence of similar morphological characteristics across different taxa (Saito & Okuyama, 2003), unveiling many instances of cryptic diversity (Reem *et al.,* 2017a, 2022; Viard *et al*., 2019). Furthermore, the traditional classification of ascidians heavily relies on skilled taxonomists (Rubinstein *et al.,* 2013). This field is diminishing in its availability, on top of challenges associated with distinguishing taxonomic traits among closely related species (Rocha *et al*., 2012).

Nowadays, alongside the traditional taxonomy, researchers utilize a diverse range of molecular tools to unravel the intricacies of biodiversity (Brunetti *et al.,* 2017; Viard *et al.,* 2019; Reem *et al.,* 2017a, b, 2022) and tackle emerging challenges in species delineation (Nydam *et al.,* 2021; Temiz *et al.,* 2023). One commonly used marker for this purpose is the cytochrome oxidase subunit 1 (COI) gene (Hebert *et al.,* 2003). In this study, we introduce preliminary findings on the delineation of species for seven ascidian species found along the Turkish

Mediterranean coast, to our knowledge, presenting this information for the first time.

While the distribution and characteristics of *Botryllus schlosseri* (Pallas, 1766) clades are well-known (Reem *et al.,* 2022), there are limited data available from the Turkish coastline, with the exception of a presence record from the Black Sea coast of Türkiye (Kayış, 2011). Moreover, with the exception of *Botrylloides niger* (Temiz *et al.,* 2023), to our knowledge, no other records of botryllid ascidian have been reported from the Mediterranean coast of Türkiye. Although the occurrence of *Symplegma brakenhielmi* (Michaelsen, 1904) on the Mediterranean coast of Türkiye has been reported (Çınar *et al.*, 2006), no genetic data about its taxonomy has been provided thus far. Additionally, despite their extensive distribution and dense population, there are no records of *Didemnum perlucidum* Monniot F., 1983 in this region. Furthermore, the presence of *Polyclinum constellatum* Savigny, 1816 has only been documented on the Aegean coast of Türkiye, indicating its existence in the area (Aydın-Önen, 2018). The lack of comprehensive studies in this region raises the question of how many ascidian species inhabit the Mediterranean coast of Türkiye. Given the close proximity of the northeastern Mediterranean to the Suez Canal, documenting biodiversity becomes increasingly crucial from both a biodiversity and conservation perspectives (López-Legentil *et al.,* 2015; Zenetos *et al.,* 2017; Galil *et al.,* 2018).

In this initial study, our objective was to provide records of seven ascidian species (*B. schlosseri*, *B. israeliense* Brunetti 2009*, Botrylloides* sp.*., B. humilis* Monniot C., 1988 *S. brakenhielmi, P. constellatum* and *D. perlucidum*) found along the coastal shelves of the Mediterranean coast of Türkiye. To accomplish this, we utilized the COI gene and employed species delimitation to establish the boundaries of these species. Furthermore, we cultivated certain Styelidae specimens in an aquaculture room to document specific biological characteristics.

Materials and Methods

Sampling and general morphological examinations

Specimens were collected from the shallow waters (<1 m). Sample collection was conducted at six sites along the Mediterranean coastline of Türkiye (Fig. 1, Table S1) using a single-edged razor blade. All ascidian samples found under each stone, including various species and different color morphs of the same species, were collected. The specimens were labeled, and their color morphs were recorded. Then, an overhead picture was taken, and two fragments were obtained from each colony. One fragment was placed in a 1.5 mL tube containing 70% (v/v) ethanol for DNA extraction, while the other fragment was placed in a 4% formaldehyde solution for morphological examination and stored at room temperature until further use. The field-fixed samples were not relaxed during fixation. Additionally, fragments of *Botryllus, Botrylloides,* and *Symplegma* colonies were placed on a microscope

Fig. 1: Map and Google Earth captures of the sampling sites. Red triangles point to the center of collection sites. Credits: d-maps.com.

slide and secured with a sewing thread. These colonies were cultured in the IMS-METU aquaculture room under constant temperature (26-28°C) and salinity (38-40 ppt) conditions (Karahan *et al.,* 2022). Cultured colonies were subjected to a one-hour menthol crystal relaxing protocol (Williams &Van Syoc, 2007) for examination, except for those colonies that were being monitored for their life cycle or blastogenesis. Detailed information regarding the sampling sites, dates, coordinates and salinity information are found in Table S1. A total of 25 samples were included in the present study (Table S2).

For the examination of zooid distribution, colonial structure, oral tentacle numbers, and blastogenesis of the Styelidae family members, Stereo microscopes (Olympus SZX16 with UC30 camera) and light microscopes (Olympus CX43 with ToupTek camera) were employed. To identify Didemnid spicules, a fragment of an individual was treated by immersing it in ethanol and then burned until it was completely black. The burned piece was gently dissolved in 5% bleach. After a 15-minute incubation period, the spicules were rinsed with tap water three times and stored in ethanol at room temperature for subsequent microscopic analysis. Pictures of the spicules were captured using a light microscope at magnifications of 10X20 and 10X100.

DNA extraction, Polymerase Chain Reactions (PCR), and data analysis

DNA extraction from colony fragments was carried out using a modified phenol-chloroform protocol (Karahan *et al.,* 2022) and quantified using a Nanodrop spectrophotometer and, if needed, diluted to a concentration of 20 ng/µl. PCR amplification was conducted in a total volume of 50 µL ready-to-use PCR Master Mix (Thermo Scientific) with 0.5 µM forward and reverse primers and approximately 10-20 ng/µl of DNA. The primers used were based on those described by Reem *et al.* (2017a) for the mitochondrial cytochrome oxidase subunit I (COI) gene. The forward primer sequence used was F2: 'AMWAATCATAAAGATATTRGWAC' and the reverse primer sequence was R2: 'AARAARGAMGTRTTRAAATTHCGATC.' The PCR products were purified and sequenced in both forward and reverse directions by Macrogen Inc. (Seoul, South Korea). Detailed data regarding the DNA of the vouchered specimens (stored in the IMS-METU genetic laboratory) were uploaded to the Barcode of Life Data System (BOLD, http://www.boldsystems.org; Table S2).

Bioinformatic Analyses

A total of 51 Styelidae family members (15 from the present study and 36 from the database), 47 Polyclinidae family members (8 from the present study and 39 from the database), and 57 Didemnidae family members (2 from the present study and 55 from the database) were included in the analyses. A total of 130 NCBI ascidian sequences were mined in March 2023. Initially, the samples from the current study were compared to identify the closest matches. The selection process involved the application of specific criteria: preference was given to sequences with a voucher record, approved by a taxonomist, and a minimum length of 500 bp. Furthermore, to increase the efficacy of species delimitation, species belonging to the same family as the samples in the present study were included in the analysis. The IDs of the sequences from the database are found in Figures 2-4.

The sequences were aligned using MAFFT version 7 (Katoh *et al*., 2018) and subsequently trimmed using Jalview version 2.11.1.7 (Waterhouse *et al*., 2009). After the alignment and trimming process, the final sequence lengths were 506 bp for Styelidae, 552 bp for Polyclinidae, and 512 bp for the Didemnidae family members, respectively. The Blast analysis was performed utilizing the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) engine. The best model for the MrBayes (Ronquist *et al.,* 2012) was selected using PhyML-SMS v3 software (SMS: Smart Model Selection in PhyML; Lefort *et al.,* 2017). MrBayes was run under the GTR + I + Γ model (a General Time Reversible model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites) for approximately 2.000.000 combined states (two independent runs) for each tunicate family, resulting in a high effective sample size value (ESS= over 1000 for each, >100). A minimum of 7500 trees were sampled for each family after discharging a burn-in fraction of 25% and confirming LnL stationarity. As convergence diagnostic, an average standard deviation of split frequencies below 0.01 (<0.007) and PSRFs (Potential Scale Reduction Factors) close to 1.0 (Ronquist *et al.,* 2012) were verified. The final trees were visualized using FigTree v.1.4.4 (Rambaut, 2018, http://tree.bio.ed.ac.uk/software/figtree). MEGA11 (Tamura *et al.,* 2021) was used to calculate the Kimura 2-P distance with 1000 bootstrap replications and Gamma-Distributed rates (Kimura, 1980).

Species delimitation was conducted using two methods: the Automatic Barcode Gap Discovery method (Also known as ASAP, assemble species by automatic partitioning, Puillandre *et al*., 2021) and the Poisson Tree Processes (PTP, Zhang *et al*., 2013). ASAP is a sequence similarity clustering method, while PTP is a tree-based coalescence method. The hypothetical species identified by these methods were assigned as the Operational Taxonomic Unit (OTU).

The ASAP analysis was performed using the web-based interface available at https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html (accessed in March 2023). For pairwise distance calculations, Jukes-Cantor (JC69, 1969) metric options provided by ASAP were used.

The PTP analysis was conducted using the Bayesian implementation, available on the web-based interface at http://species.h-its.org/ptp/ (accessed in March 2023). The trees constructed using MrBayes were used in the analyses. Default parameter values were utilized for the analyses.

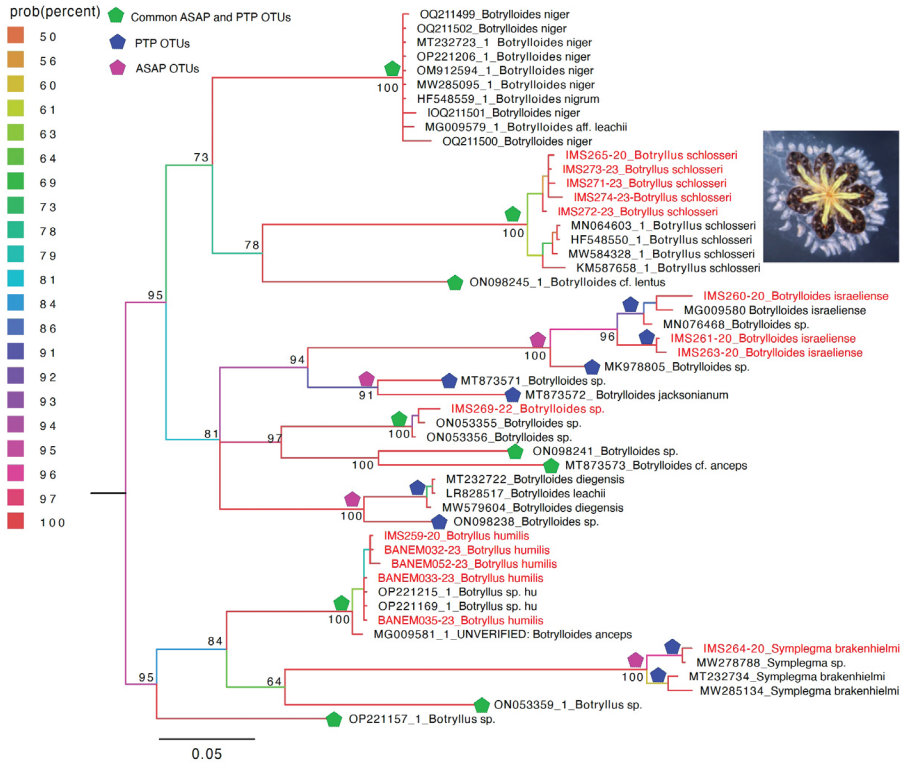

Fig. 2: Bayesian majority rule consensus tree, reconstructed from the 506 bp long COI sequence alignment of Styelidae specimens. The distance scale is given under the tree; branch colors represent bootstrap probability, the database samples' NCBI IDs are provided before the species name. Green pentagons show common ASAP and PTPs OTUs, pink pentagons are for the ASAP OTUs and the blue pentagons depict the PTPs OTUs. The red colored letters indicate present study samples. Node bootstrap values are given on the left side of each node. The image of *Botryllus schlosseri* (sample L6) colony is inserted right side of the species OTU.

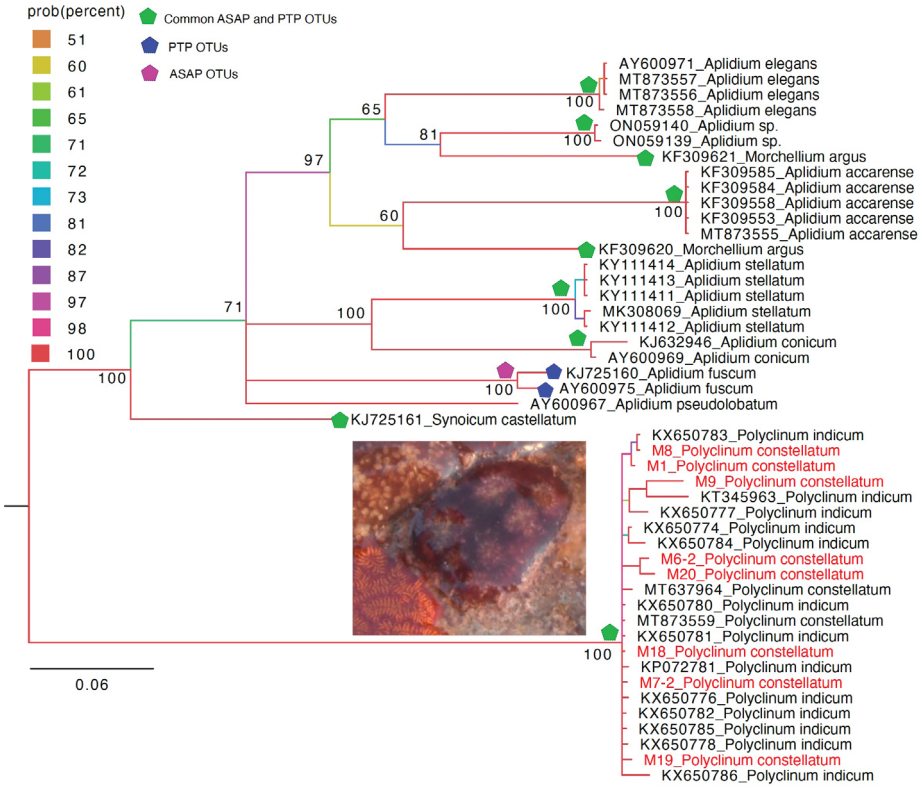

Fig. 3: Bayesian majority rule consensus tree, reconstructed from the 552 bp long COI sequence alignment of *Polyclinum* specimens. The distance scale is given under the tree; branch colors represent bootstrap probability, the database samples' NCBI IDs are provided before the species name. Green pentagons show common ASAP and PTPs OTUs, pink pentagons are for the ASAP OTUs and the blue pentagons depict the PTPs OTUs. The red colored letters indicate present study samples. Node bootstrap values are given on the left side of each node. The image of *Polyclinum constellatum* (sample M20) colony is inserted right side of the species OTU.

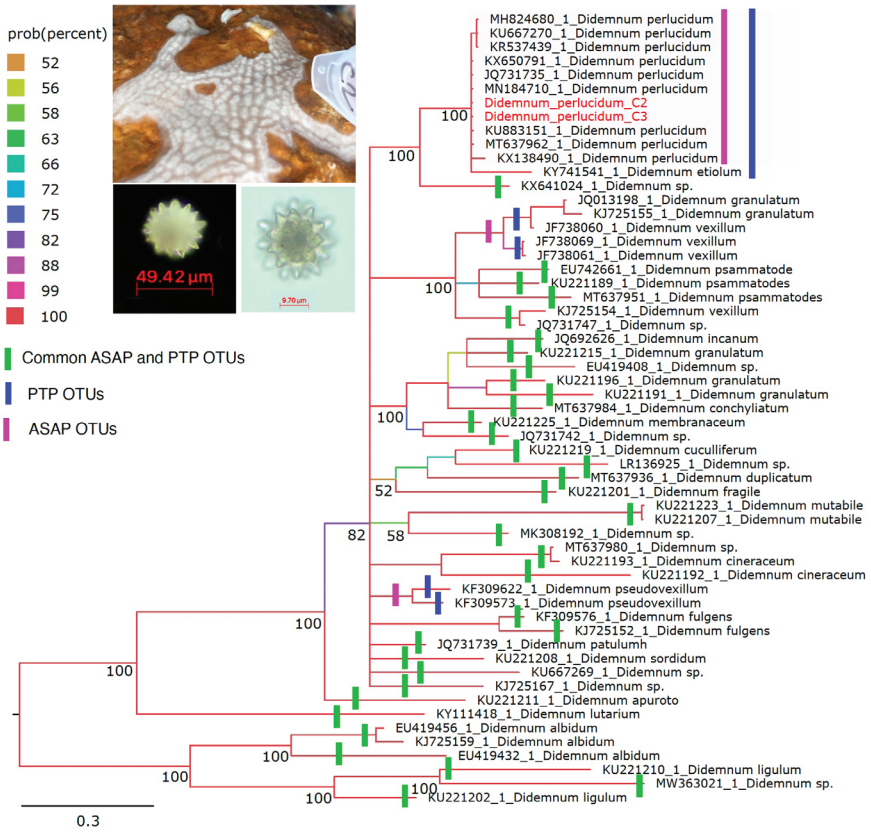

Fig. 4: Bayesian majority rule consensus tree, reconstructed from the 515 bp long COI sequence alignment of Didemnidae specimens. The distance scale is given under the tree; branch colors represent bootstrap probability, and the database samples' NCBI IDs are provided before the species name. Green bars show common ASAP and PTPs OTUs, pink bars are for the ASAP OTUs and the blue bars depict the PTPs OTUs. The red colored letters indicate present study samples. Node bootstrap values are given on the left side of each node. The image of *Didemnum perlucidum* (sample C2) colony is inserted right side of the species OTU. Top image; general view in the colony in field, below image; spicules with different magnifications (10x20 and 10x100).

Results

In this study, 25 ascidian sequences from Türkiye, and 130 sequences obtained from NCBI, were used in the analysis. The sequences obtained from the present study exhibited a length greater than 500 bp (~ 600 bp) and can be accessed using BOLD IDs (Table S2). Blast results of the present study samples are provided in Table S3, and genetic distances between samples assigned to the same OTU are provided in Tables S4-10. Additionally, for samples identified at the genus level, the genetic distances to the closest assigned samples were also included. The Bayesian trees showing ASAP and PTP scores are presented in Figures 2-4, the ASAP scores in Figures S1-3, and PTP scores in Figures S4-6. The logarithm likelihood (LnL) values of the Bayesian analyses are displayed in Figure S7.

Botryllus schlosseri. Five colonies of *B. schlosseri* were collected from the Hatay-Konacık region under two rocks (Table S1, S2). These colonies displayed four distinct haplotypes; L12, L13, L14 and L19 (Fig. 2, Table S2). Haplotype L12, characterized by a brown color with yellow stripes, was cultured in the IMS-METU aquaculture room, where its subclones successfully survived under *in situ* conditions for approximately three years. Over time, we observed an increase in pigmentation, and the blastogenic cycles of the colonies became irregular. ASAP and PTP scores revealed that all haplotypes from the present study clustered together with specimens from Clade A collected from the English Channel (MN064603.1), South Taranto-Italy (HF548550.1, Griggio *et al*., 2014), Israel (KM587658.1, Reem *et al*., 2017b), and from South Korea (MW584328.1, Lee & Shin 2021) (Fig. 2). Our samples exhibited a similarity of over 98% with the NCBI *B. schlosseri* samples (Table S3). The maximum genetic distance recorded among all the samples was 2% (Table S4).

Botrylloides israeliense. A total of 37 colony fragments of *B. israeliense* were collected exclusively from Mersin sites, specifically Mezitli, Tisan and Kızkalesi, with no records from other regions (Table S1, Table S2). For species delimitation, we used three sequences; two belonged to a common haplotype (from Kızkalesi samples, Fig. 5a-c) and one was a single unique haplotype (from a Tisan sample, Fig. 5d). These colonies displayed a range of colors, from creamy to black, with distinct radial color patterns observed (Table S2). The zooids, with an average length of approximately 2.3 mm, were arranged in a 'leachii type' system (Fig. 5b). Within the colony, they were oriented vertically, while the ampullae were positioned horizontally (Fig. 5b). The oral siphons were fringed by eight tentacles, with two and three size orders observed (LSLSLSLS-LSMSLSMS) (Fig. 5c).

Based on the ASAP score, all the samples from the present study, a sample from the Mediterranean coast of

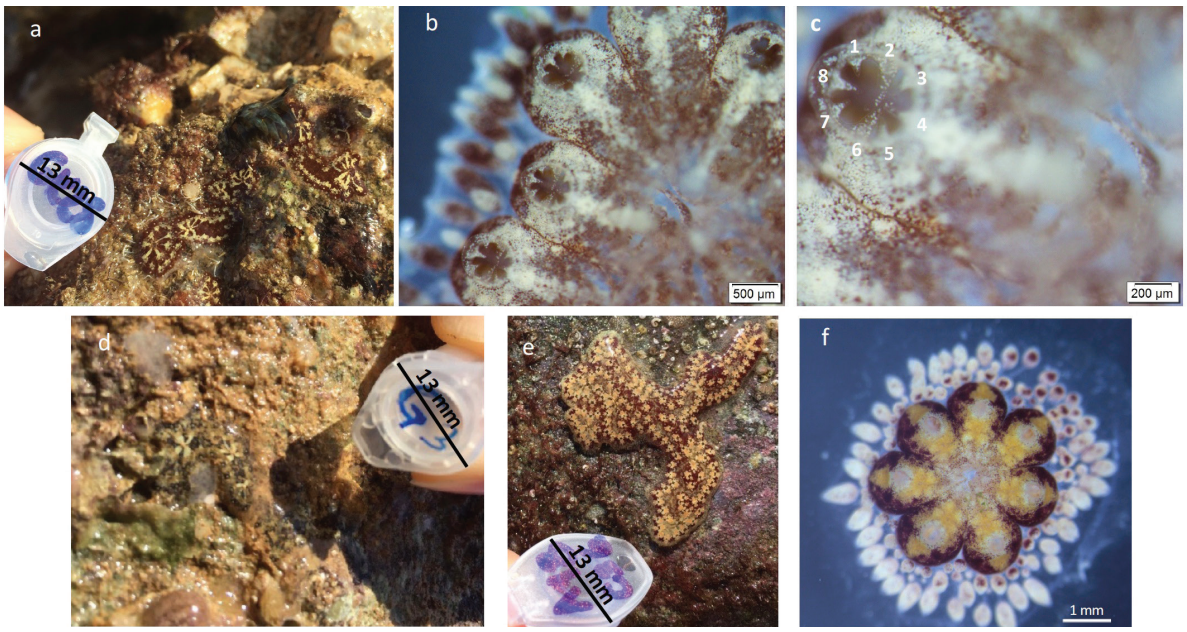

Fig. 5: a-c) *B. israeliense* images (sample GK28), a colony growing under a stone in the field, b) Closer zooids images of colonies growing on slides in the aquaculture room. c) Image of oral siphon, numbers depict oral siphons tentacles. L; long, S: short, M: middle (order from the number 2 to the clockwise direction; LSMSLSMS). d) *B. israeliense* colonies (sample G3) growing under a stone in Tisan site. e-f) *Botrylloides* sp. images (sample C64); e) the colony image under a stone in the field, f) in the aquaculture room on slide.

Israel (MG009580, Reem *et al.,* 2017a), and two *Botrylloides* sp. samples, one from Italy (Miseno Lagoon, Naples, MN076468, Viard *et al.,* 2019) and the other from the English Channel (MK978805, Viard *et al.,* 2019), were categorized within the same OTU (Fig. 2, Fig. S1). However, according to the PTP result, while the Tisan sample from the present study was assigned to the same OTU as the Israeli and Italy samples, the two Kızkalesi samples were assigned to a different OTU, and the English Channel sample (MK978805) was assigned to another OTU (Fig. 2, Fig. S4).

Using the NCBI BLAST analysis, it was observed that the Tisan sample displayed a similarity above 97% with *B. israeliense* (MG009580, Reem *et al.,* 2017a) and *Botrylloides* sp. (MN076468, Viard *et al.,* 2019), while the coverage decreased to around 95% for the Kızkalesi samples (Table S3). The genetic distance between the Tisan and Kızkalesi samples was measured at 5%, while the distances between the Tisan, Israeli, and Italy samples were 3%, and between the Kızkalesi, Israeli, and Italy samples were 4%. The highest genetic distance was recorded between the Tisan sample and the English Channel sample (MK978805), with a value of 7% (Table S5).

***Botrylloides* sp.** From the Antalya-Kemer region, a single colony (IMS269-22) of *Botrylloides* sp. was collected. This particular colony is characterized by its brownish color with a yellow strip around the oral siphon. It exhibits a 'leachii type' system arrangement (Fig. 5e, Table S1, S2). A colony fragment was cultured for a duration of one month, leading to the growth of zooids with a length of approximately 1.7 mm (Fig. 5f). The blastogenic cycles of the colony lasted for five days under 26-28°C water temperature and 38-40 ppt salinity (Fig. S8). Both the ASAP and PTP analyses assigned the colony from the present study to the same OTU as two samples from Yanbu, Saudi Arabia (*Botrylloides* sp.; ON053355-ON053356) (Fig 2, Fig. S1, S4). Based on the NCBI Blast result, this colony exhibited a coverage of 98.81% with the other samples (Table S3). The closest OTU was determined to have a genetic distance of 17% (MT873573, *Botrylloides cf. anceps*, Table S6).

Botryllus humilis. A total of 27 colony fragments of *B. humilis* were collected from the Hatay (Konacık), Mersin (Mezitli), and Antalya (Alanya) regions (Fig. 1, Table S1, S2). In larger colonies, the zooids exhibited a ladder-like systems, while in smaller colonies, they formed oval systems (Fig. 6, Fig. S9). The zooids had a relaxed length ranging from 1.5 to 2 mm (Fig. 6b, c, d-Fig. S10. The color of the colonies varied from very light to dark (Fig. 6, Fig. S9). The pigmentation pattern and color around the oral siphon also varied depending on the color morphs of the colonies. Specifically, a particular colony named AC1, collected from the Mersin-Mezitli region in October 2021, displayed a dark purple color with a light purple edge in its natural habitat (Fig. 6a). The color gradually faded in the aquaculture room (Fig. 6b, c), eventually turning brownish-orange over time (Fig. 6d). Initially, a purple pigment spot was observed below the oral siphon, which later transformed into a light brownish. Additionally, numerous pigment spots were recorded around the oral siphon (Fig. 6d, captured in April 2023). An image of the abdomen is provided in Figure 6e, showing a cylindrical stomach. The oral siphons in AC1 zooids were fringed by twelve oral tentacles, which were arranged in three size orders (LSMSMSMSLSMS), along with some very small tentacles in between (Fig. 6f).

Under laboratory conditions, it was observed that sexual reproduction occurred at a salinity of 38-40 ppt and 26-28°C seawater temperatures. Video and figures of colony L6 (BANEM035-23-BOLD: AEE3749), includ-

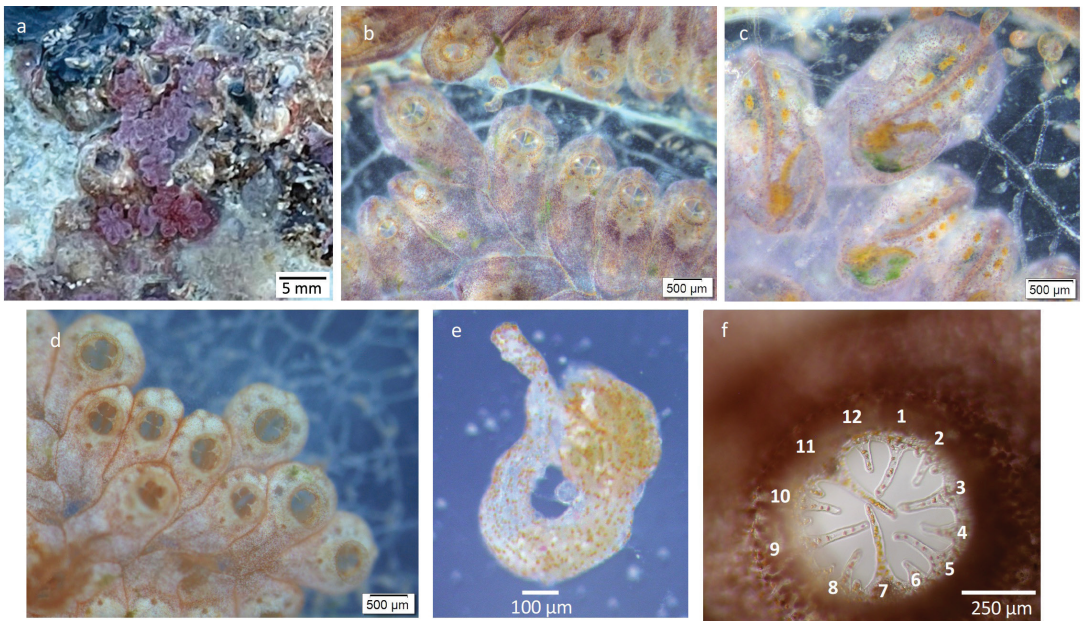

Fig. 6: *Botryllus humilis* (sample AC1): a) a colony growing under a stone in the field, b-c) images of colony cultivated in the aquaculture room on a slide, d) a year-old colony in the aquaculture room, e) an isolated stomach and intestine, and f) an oral siphon with numbers depicting the oral siphon tentacles. L; long, S: short, M: middle, order from the number 11 to the clockwise direction; LSMSMSMSLSMS.

ing ovaries and testes, are presented in Figure S11a-b and Suppl. Movie-1. These recordings were taken after 15 months of cultivation in the aquaculture room. Many ovaries were observed on zooids and primary buds (Sup. Movie-1). A testis was visualized at the left ovary site of the zooid, overlapping the anterior part (Fig. S11a). The testis exhibited six follicles, with two regressed follicles and one larger than the others (Fig. S11b). While most of the offspring were observed on the slides, unfortunately, most died at the oozooid stage. However, a portion of the progeny managed to survive and lived for 2-3 years under the IMS-METU aquaculture conditions. Regarding aged colonies (over 2,5-3 years old), some exhibited enlarged and condensed ampullas, along with formless and small zooids and irregular blastogenic cycles (Fig. S12). However, no sign of morphological hibernation, as defined by Hyams *et al.* (2017, 2022), was observed, even when the colonies were subjected to low temperature of 16°C (38-40 ppt).

All 27 samples of *B. humilis* examined in this study shared a single haplotype, and five samples were utilized for species delimitation. Both ASAP and PTP scores indicated that all samples clustered together within a single OTU along with three database samples: two *B. humilis* samples (OP221215, OP221169 '*Botrylus* sp. *hu*', Palomino-Alvarez *et al.,* 2022) from Mexico (Hunucma) and one *Botrylloides anceps* sample (MG009581, Reem *et al.,* 2017a) from the Mediterranean coast of Israel (Fig. 2, Fig. S1, S4). The NCBI Blast analysis revealed that all samples from the present study exhibited a coverage above 99% with the *B. humilis* samples, which were referred to as *Botryllus* sp. *hu* in the NBCI record (Table S3, Palomino-Alvarez *et al.,* 2022). The genetic distance between the Mexico samples and the present study samples was less than 1%, and a 1% distance was observed between the samples of the present study and the Israeli *B. anceps* sample (Fig. 2, Table S7).

***Symplegma brakenhielmi* (Ascidiacea; Stolidobranchia; Styelidae; Symplegma).** A single red colony was collected from the Mersin-Mezitli region (Fig. 1, Fig. 7a, Table S1, S2). A ramet of this genotype was cultured in the aquaculture room (Fig. 7b-d), showing a transparent tunic with dispersed zooids not arranged within systems. The lengths of adult zooids ranged from 1 to 3 mm. Both pallial and vascular budding were observed, with the latter being more prominent.

Species delimitation of the specimen from the present study was conducted in conjunction with three database samples: a *Symplegma* sp. (MW278788) from Hawaii (Oahu), a *S. brakenhielmi* (MT232734, Nydam *et al.,* 2021) from Panama (Bocas del Toro), and another *S. brakenhielmi* from Florida (MW285134). Based on the ASAP scores, all samples clustered together within the same OTU (Fig. 2, Fig. S1). However, PTP analysis resulted in two OTUs: the present study sample and MW278788 clustered together (100 identities), while the other two database samples formed a separate OTU with ca. 4% genetic distance (Fig. 2, Fig. S4, Table S8). According to NCBI BLAST results, the sample from the present study exhibited 97.22% coverage with the *S. brakenhielmi* (MT232734) sample in the database. Furthermore, the coverage increased to 99.60% when compared to the *Symplegma* sp. sample (MW278788, Table S3).

Polyclinum constellatum (Ascidiacea: Aplousobranchia: Polyclinidae: Polyclinum). In 2012, a total of nineteen colonies were collected exclusively from the Kızkalesi site, out of which eight colonies were utilized for the present study (Fig. 1, Table S1, S2). Apart from recording the colony color morphotypes in the field, no other morphological observations were conducted on the *Polyclinum* specimens. A field image of the sample M20 is provided in the Figure 3.

For species delimitation, high-quality sequences from

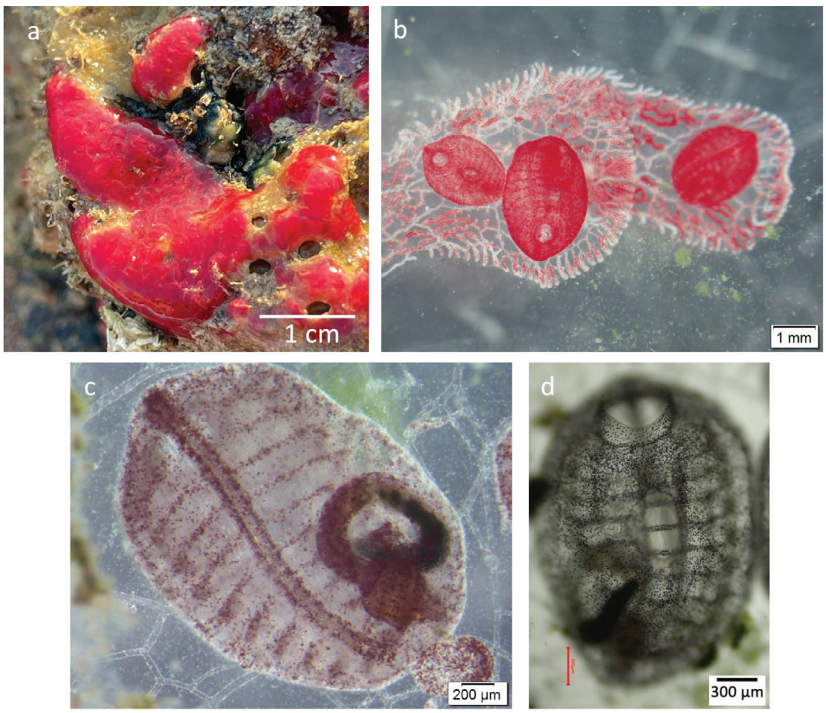

Fig. 7: *Symplegma brakenhielmi* (sample M2_23 a) a colony growing under a stone in the field, b) a colony growing on slide in the culture room; c-d) enlargement of zooids growing on slides.

eight specimens were used, along with 15 database samples of *P. constellatum* and *P. indicum*, as well as 24 other Polyclinidae specimens (Fig. 3). Based on both ASAP and PTP scores, all the samples from the present study were assigned to a single OTU alongside the 15 database *Polyclinum* samples, displaying a maximum intra-specific distance of 4% (Fig. 3, Fig. S2, 5, Table S9). The remaining Polyclinidae specimens were assigned to different OTUs (Fig. 3, Fig. S2, 5). According to NCBI Blast analysis, the coverage between the *P. constellatum* samples from the present study and the database samples of both *P. indicum and P. constellatum* ranged between 97-100% (Table S3).

***Didemnum perlucidum* (Ascidiacea: Aplousobranchia: Didemnidae: *Didemnum*).** The species was commonly found on hard substrates at all sites, displaying diverse colors, such as white, orange, light and dark creamy, and brownish (Fig. 1, Table S1). Two white samples collected from Antalya were included in the present study (Fig. 4, Table S2). The colonies had a thickness of less than 1mm and featured star-shaped spicules (Fig. 4).

Based on the ASAP analysis, the present study samples were grouped within a single OTU alongside nine database specimens of *D. perlucidum* from India, Australia, and Puerto Rico, with a maximum genetic distance of 2% (Fig. 4, Fig. S3, S6). In the PTP analysis, an additional specimen identified as *Didemnum etiolum* from India was assigned to the same OTU where all database *D. perlucidum* and present study specimens clustered together, with a maximum distance of 6% (Table S10). Other didemnid species were assigned to distinct OTUs (Fig. 4). The samples from the present study showed a perfect match (100%) with the database *D. perlucidum* samples in the NCBI Blast analysis (Table S3).

Discussion

The findings of this study disclose the occurrence and distribution patterns of seven colonial ascidian species along the coastline of the Northeastern Mediterranean region in Türkiye. Furthermore, a separate publication by Temiz *et al.* (2023) presents information on *B. niger*, an additional species obtained from the sampling sites of this study, which exhibits a wide range of color morphs. It should be noted that due to the focus on shallow waters in the sampling process, not all colonial species may have been included, possibly because of factors like seasonal variations or the existence of deeper ascidian habitats. The identification of species was determined by considering Blast results, genetic distance, PTPs, and ASAP analysis scores.

B. schlosseri is a globally distributed colonial ascidian species found in temperate shallow waters, known for its remarkable ability to invade and colonize new areas (Ben-Hamo & Rinkevich, 2021). Before this study, *B. schlosseri* had not been documented along the Turkish Mediterranean coast, apart from the Black Sea coast of Türkiye (Kayış, 2011). This study marks the first identification of DNA barcodes for *B. schlosseri* on the Turkish Mediterranean coast. Previous studies have recognized five global clades (A-E; Bock *et al.,* 2012; Reem *et al.,* 2022), and the samples in this study belonged to clade A, the most prevalent clade.

Botrylloides israeliense was initially named in 2009 by Brunetti (2009), although certain ecological and biological characteristics of the species had been previously studied by Rinkevich *et al.* (1993, 1994). In October 2012, we first encountered this species in the Kızkalesi region along the Turkish Mediterranean coastline, initially unaware of its status as a native or introduced species

in the area. The absence of recorded individuals in Hatay or Antalya sites, with sightings limited to Mersin sites, raised doubts about the species' origin. Based on the ASAP score, all the samples analyzed in the present study were determined to belong to a single operational taxonomic unit (OTU). However, the PTP analysis suggests the possibility of a distinct species in the Kızkalesi region. Previous studies have reported highlighted significant genetic differences (>10%) within botryllid ascidian species (Rocha *et al.,* 2019; Palomino-Alvarez, 2022; Reem *et al.,* 2022), underscoring the necessity for thorough morphological examinations to arrive at definitive conclusions regarding the species' classification. Until further examination is conducted, the possibility of two sister species should be considered; *B. israeliense* from the Tisan region and *Botrylloides* sp. from the Kızkalesi region.

Colonies belonging to a potentially new species, referred to here as *Botrylloides sp*., were exclusively observed in the Antalya-Kemer site. These colonies exhibited a 99% genetic similarity to a sample obtained from Saudi Arabia and its COI archived in the NCBI database. While it is plausible that this newly discovered botryllid ascidian species originated from the Red Sea, the absence of its presence along the Mediterranean coasts of Israel and the eastern sites of Türkiye (Hatay and Mersin) introduces uncertainty to its origin. To ascertain the species' classification, a comprehensive morphological examination is needed.

In the present study, *B. humilis* emerges as another intriguing species. While all the samples analyzed in this study were classified within a single OTU, along with the database specimens of *B. humilis* and *B. anceps*, there remains uncertainty surrounding the species name. The species was initially documented as *B. humilis* by Monniot (1988) from New Caledonia, but later Brunetti (2009) described it as *B. anceps* from the Mediterranean coast of Israel. Recently, Palomino-Alvarez *et al.* (2022) recorded the species along the Mexican coast, and provided a morphological description that is consistent with both Monniot (1988) and Brunetti (2009), suggesting that the species may have been misidentified as *B. anceps* (Herdman, 1891). This idea is further supported by referencing Brunetti's (2009) article on the *B. humilis* WoRMs page (https://www.marinespecies.org/aphia.php?p=taxdetails&id=250095). Following this preliminary study, our future plans involve preparing a comprehensive publication that includes all the color morphotypes, haplotypes, analysis of other gene regions, and a detailed morphological examination of the species.

In 2018, we made the first recorded observations of this species at the Hatay, Mezitli, and Alanya sites. During a previous visit to these sites in 2012, no *B. humilis* individuals were seen, indicating that the species must have arrived on the Turkish coastline between 2012 and 2018. Although the origin of the species remains unknown (Palomino-Alvarez *et al.,* 2022), it is believed to be an introduced species in the Mediterranean Sea (Brunetti, 2009). It is notable to note that despite observing only one haplotype and observing the species coexisting with other botryllid species along the Turkish coastline, it does not exhibit noticeable invasive characteristics in the area. Another interesting finding of our study is the presence of the species in both the eastern and western stations of the Kızkalesi region, while it is not found in the Kızkalesi sites, where primarily *B. israeliense* (or *Botrylloides* sp.) and *B. niger* species were recorded.

According to Palomino-Alvarez *et al.* (2022), *B. anceps* typically possesses 16 oral tentacles, whereas *B. humilis* has 12. In our samples, we also observed 12 oral tentacles and the Blast result showed a high similarity (99-100%) with the *B. humilis* samples of Palomino-Alvarez *et al.* (2022) and the *B. anceps* samples of Reem *et al.* (2017a). Additionally, our samples exhibited an enlarged pigment base at the two longest oral tentacles (7 and 11), which is consistent with the description by Palomino-Alvarez *et al.* (2022). However, the number of pigmented spots on the atrial languet and oral siphons considered species-specific features (Palomino-Alvarez *et al.* 2022), exhibited variations between our field and aquacultured samples. Furthermore, although Palomino-Alvarez *et al.* (2022) noted that *B. humilis* typically displays pale colors, we observed many distinct color morphs, most of which were not pale. Additionally, in the present study, we documented different zooid lengths for the same colony under consistent conditions over varying periods. This suggests that zooid length should not be considered a definitive taxonomic feature in botryllid ascidians.

Symplegma brakenhielmi was initially recorded in the Mediterranean Sea from the Lebanese coast, then from the Turkish Levantine coast (Bitar & Kouli-Bitar, 2001; Çınar *et al.,* 2006), based on only general morphology. Additional records include the central region of the Mediterranean Sea (Ulman *et al.,* 2017; Mastrototaro *et al.,* 2019). In this study, we obtained the first DNA barcode for this species from the Northeastern Mediterranean Sea and conducted species delimitation. Based on the ASAP analysis and considering the low genetic distance, we assigned the name *S. brakenhielmi* to our specimen. However, considering the PTP results and the lack of detailed morphological identification in a previous report (Çınar *et al.,* 2006) of the species from the Levant coast, it is plausible to consider the presence of a different *Symplegma* species in the area. Further morphological identification is needed to confirm or refute this proposition.

Polyclinum constellatum Savigny, 1816, was originally reported from the Indian Ocean (South Africa). Due to its extensive dispersion range, *P. constellatum* has been considered a cryptogenic species in various regions, including the Tropical Western Atlantic, Western Indian Ocean, Indo-pacific region, Mediterranean and Aegean Sea (Dias *et al., 2013*; Halim & Messeih, 2016; Aydın-Önen, 2018; Montesanto *et al*., 2022). The inclusion of another name (*P. indicum)* within the *P. constellatum* OTU has been regarded as an error or a synonym (Montesanto *et al.,* 2022). In our study, the samples collected exhibited a high degree of similarity (99-100%) to database specimens uploaded from various global locations (Italy, Florida, India, and the Ionian Sea), a result suggesting a low level of genetic divergence within a widely distribut-

ed species. Importantly, our study presents the first COI sequences of this species from the North Eastern Mediterranean region.

Didemnum perlucidum is a widespread species occurring in the Atlantic, Pacific, Indian Oceans, and Mediterranean Sea; however, its native range remains unknown (Dias *et al.,* 2016). Here, we present the first barcode data for the white color morph samples of *D. perlucidum* from the Northeastern Mediterranean Sea. Through species delimitation analysis, we found that the samples from our samples clustered with India, Australia, and Porto-Rico, showing a genetic distance of 2%. Due to the limited number of samples analyzed, we cannot make definitive assumptions on the origin of species. However, we can confidently state that *D. perlucidum* is the most commonly encountered ascidian species along the North Eastern Mediterranean coastlines, and similar to other ascidians, it was only observed under stones.

In this study, we present comprehensive records of seven colonial ascidians found in the shallow waters of the Turkish North Eastern Mediterranean Sea. Our results included barcode data and species delimitation, supported by morphological and life cycle features. Among the species examined, we observed high morphological variation in *B. israeliense and B. humilis*, while *B. schlosseri* exhibited high genetic diversity and *P. constellatum* displayed low genetic diversities. Additionally, we identified a possible new *Botrylloides* species (referred to as *Botrylloides* sp.) from the Antalya region. To our knowledge, this study provides the first presence records and DNA barcodes of *B. israeliense, B. humilis, B. schlosseri*, *D. perlucidum* and *P. constellatum*, *Botrylloides* sp. and also the first DNA barcode for *S. brakenhielmi* in the area.

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Supplementary Data

The following supplementary information is available online for the article:

Suppl. Movie-1: Gonads of *B. humilis* colonies (L6) under the light microscope.

Table S1. Sampling sites' coordinates, date and salinity information.

Table S2. Present study samples' details. AN: accession number.

Table S3. NCBI Blast results.

Table S4. The Kimura-2 Parameter distance results of *Botryllus schlosseri* samples.

Table S5. The Kimura-2 Parameter distance results of *Botrylloides israeliense.*

Table S6. The Kimura-2 Parameter distance results of *Botrylloides* sp. and its possible closest relatives.

Table S7. The Kimura-2 Parameter distance results of *Botryllus humilis.*

Table S8. The Kimura-2 Parameter distance results of *Symplegma brakenhielmi.*

Table S9. The Kimura-2 Parameter distance results of *Polyclinum constellatum.*

Table S10. The Kimura-2 Parameter distance results of *Didemnum perlucidum.*

Fig. S1: The figure displays the ASAP scores of Styelidae specimens. 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.

Fig. S2: The figure displays the ASAP scores of Polyclinidae specimens. 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.

Fig. S3: The figure displays the ASAP scores of didemnid specimens. 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.

Fig. S4: PTP score of Styelidae; Blue lines represent different OTUs, red lines represent the same OTUs. Red letter represents the present study samples.

Fig. S5: PTP score of Polyclinidae; Blue lines represent different OTUs, red lines represent the same OTUs. Red letter represents the present study samples**.**

Fig. S6: PTP score of Didemnidae; Blue lines represent different OTUs, red lines represent the same OTUs. Red letter represents the present study samples.

Fig. S7: LnL values of MrBayes analyses, a) Styeliadae, b) Aplousobranchia c) Didemnidae.

Fig. S8: Blastogenic cycle of *Botrylloides* sp. Dorsal and ventral view of the Blastogenic stages; a-b) stage-D (Jun 07, 2018), c-d) stage-A (Jun 08, 2018), e-f) stage-B (Jun 09, 2018), g) Dorsal-stage C (Jun 10, 2018), h) Dorsal – stage D (Jun 11, 2018).

Fig. S9: Color morphotypes of *B. humilis*, L3, L4 L6 from Hatay (Konacık), C6 Antalya (Alanya), M2_16 Mersin (Mezitli).

Fig. S10: When the zooid size of *B. humilis* reached up to 2 mm (the photographs were captured via a cell phone).

Fig. S11: Ovary and testes of *B. humilis* (L6). a) The yellow frame represents the heart border, the green frame represents the testis, and the blue frame represents the ovary. b) The picture was captured by focusing on the testes using only the overhead light option.

Fig. S12: Large, condensed ampullas and irregular, small zooids of 3-year-old *B. humilis* colony.