

1 **Species delimitation of eight ascidian (Tunicata) species from the North Eastern**  
2 **Mediterranean**

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30 **Abstract**

31 Members of the tunicates, a subphylum of marine filter-feeder chordates, inhabit all marine  
32 and oceanic habitats from the subtidal to the abyssal. Considered as the closest relatives to the  
33 vertebrates, the tunicates are widely used as model organisms for evo-devo, allorecognition,  
34 senescence, and whole-body regeneration studies. Yet, species boundaries are poorly  
35 understood due to high morphological and genetic plasticity that characterize many tunicates  
36 taxa. Here we study taxonomy and the distribution of eight tunicate species (*Botrylloides niger*  
37 *Herdman, 1886/ aff. leachii*, *Botrylloides israeliense* Brunetti, 2009, *Botrylloides sp.*,  
38 *Botrylloides anceps* (Herdman, 1891), *Botryllus schlosseri* (Pallas, 1766), *Didemnum*  
39 *perlucidum* Monniot F., 1983, *Symplegma brakenhielmi* (Michaelsen, 1904) and *Polyclinum*  
40 *constellatum* Savigny, 1816) sampled from six Turkish North Eastern Mediterranean Sea sites  
41 and employed the mitochondrial barcoding marker (COI) for evaluating the relationships  
42 among geographically restricted and widely spread ascidian species. Species delimitation  
43 analysis was conducted using NCBI and the present study sequences. Morphological  
44 examinations were first done in the field and then, styelide colonies were cultured in the  
45 laboratory and studied using stereo and light microscopes. A putative new *Botrylloides* species  
46 (*Botrylloides sp.*) from the Antalya region was revealed, with 99% matching on the COI gene  
47 from Saudi Arabia, further awaiting for detailed traditional taxonomy.

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49 **Keywords:** Botryllid ascidians, *Didemnum*; *Polyclinum*; *Symplegma*, COI, species  
50 delimitation; Turkiye, Mediterranean Sea.

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## 61 **Introduction**

62 The ascidians (Phylum: Chordata, Subphylum; Tunicata) are a class of marine filter feeder  
63 organisms with ca. 3000 described species (<https://www.marinespecies.org/ascidiacea>) that  
64 inhabit all marine and oceanic habitats from the subtidal to the abyssal zone. As the closest  
65 relatives of the vertebrates, the tunicates are widely used as model organisms for evo-devo  
66 research analyses, for elucidating the evolution of immunity, senescence and ageing processes,  
67 for whole-body regeneration phenomena, stem cell biology, and more. Yet, species boundaries  
68 for many clades are poorly understood due to high genetic and morphological plasticity,  
69 revealing high cases of cryptic diversity (Burnet, 1971; Rinkevich *et al.*, 1993; Denoëud *et al.*,  
70 2010; Reem *et al.*, 2017, 2022; Viard *et al.*, 2019). Further, ascidians' classical classification  
71 requires experienced taxonomists (Rubinstein *et al.*, 2013), a vanishing scientific discipline, in  
72 addition to the difficulties in assigning differentiating taxonomic characteristics between  
73 closely related species (Rocha *et al.*, 2012). Nowadays, in addition to classical taxonomy,  
74 researchers use a wide range of molecular tools to elucidate biodiversity and to solve emerging  
75 species delineation issues (Karahan *et al.*, 2016; Brunetti *et al.*, 2017; Viard *et al.*, 2019; Reem  
76 *et al.*, 2017, 2022). The cytochrome oxidase subunit 1 (COI) gene is one of the most commonly  
77 used markers for this purpose (Hebert *et al.*, 2003).

78 Studies on the taxonomy and distribution of Mediterranean Sea ascidians, now almost  
79 bicentennial old (Schlosser and Ellis, 1756; Spallanzani & Chiereghin, 1784; Savigny, 1816)  
80 were concentrated primarily on western European coasts and seas, neglecting the eastern basin  
81 areas (Berrill, 1950; Rinkevich *et al.*, 1993). One example is the Levant, an area experiencing  
82 a continuous flow of non-indigenous species (NIS), including new exotic tropical ascidian  
83 species, entering the Mediterranean Sea through the Suez Canal (López-Legentil *et al.*, 2015;  
84 Zenetos *et al.*, 2017; Galil *et al.*, 2018). Another less studied area is the Turkish Mediterranean  
85 coastline, where the knowledge on ascidians has improved considerably in the past vicenary.  
86 So far, about 50 ascidian species (native and NIS), consisting primarily of solitary species,  
87 have been recorded from all over the Turkish coastlines (Uysal, 1976; Çınar *et al.*, 2006; Okuş  
88 *et al.*, 2007; Çınar, 2014), in comparison to 45 ascidian species reported from the Levantine  
89 basin, along the shores of Israel, Egypt, and the Gulf of Suez (Koukouras *et al.*, 1995; Halim  
90 & Abdel Messeih, 2016; Reem *et al.*, 2017). For updating some of the less studied ascidian  
91 species in the Turkish Mediterranean coasts, this study aimed in clarifying the taxonomy, and

92 further apprising the distributions of eight ascidian species by employing COI analyses and  
93 major morphological features.

94

## 95 **Materials and Methods**

### 96 ***Sampling and general morphological examinations***

97 Specimens were collected from patchy stony-rocky areas laying on sandy and shallow bottoms  
98 (underneath stones, <1 m depth) using razor blades in 6 sites along the Mediterranean coastline  
99 of Turkiye, placed, each, in 1,5 mL tube containing 70% ethanol, and kept in room temperature  
100 until use (Table S1, Fig. 1). Other colonial fragments were kept in 4% formaldehyde solution  
101 at room temperature for morphological analyses. Specimens of all Styelidae species (genera  
102 *Botryllus*, *Botrylloides* and *Symplegma*) were cultured on microscope slides in the Institute of  
103 Marine Sciences-Middle East Technical University (IMS-METU) aquaculture room according  
104 to Karahan *et al.*, (2022) and used for morphological examination. Morphological  
105 examinations were carried out for major morphological features (zooid distributions, colonial  
106 structures, colors, spicule shape, oral tentacles, blastogenic life cycles), first by the naked eye,  
107 followed by stereo and light microscopes (Olympus SZX16 - UC30 camera; Olympus CX43-  
108 ToupTek camera).

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

110 Total DNA was extracted from colony fragments according to a modified phenol-chloroform  
111 method (Karahan *et al.*, 2022). Isolated DNA was quantified using a Nanodrop  
112 spectrophotometer and diluted, when needed, to 20 ng/μl. PCR were performed in 50 μL total  
113 volume with 0.5 μM forward and reverse primers and around 10-20 ng/μl of DNA in a ready-  
114 to-use PCR Master Mix (Thermo Scientific) on the mitochondrial cytochrome oxidase subunit  
115 I (COI) gene, using Reem *et al.* (2017) primers (F2-  
116 ‘AMWAATCATAAAGATATTRGWAC’-3 and R2-‘  
117 AARAARGAMGTRTTRAAATTHCGATC’-3). The PCR products were purified and  
118 sequenced for forward and reverse directions by Macrogen Inc. (Seoul, South Korea). Detailed  
119 data for voucher specimens’ DNA (held in the IMS-METU genetic laboratory) were uploaded  
120 to the Barcode of Life Data System (BOLD, <http://www.boldsystems.org>; Table S2).  
121 BLAST analysis was performed using the GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>)  
122 and BOLD engines. Sequences were translated and aligned via MAFFT v7 (Kato *et al.*, 2018)

123 and trimmed using Jalview (v 2.11.1.7, Waterhouse *et al.*, 2009). The best model for the  
124 MrBayes (Ronquist *et al.*, 2012) was chosen via PhyML-SMS v3 software (SMS: Smart Model  
125 Selection in PhyML; Lefort *et al.*, 2017). MrBayes were run according to the GTR+R model  
126 for 10.800.000 combined states (two independent runs), resulting in a high effective sample  
127 size value (ESS=1829, >100). In total, 45002 trees were sampled after discharging a burn-in  
128 fraction of 25% and verifying for LnL stationarity. As convergence diagnostic, we confirmed  
129 an average standard deviation of split frequencies below 0.01 (=0.0085), and PSRFs (Potential  
130 Scale Reduction Factors) close to 1.0 (Ronquist *et al.*, 2012). Final trees were visualized with  
131 FigTree v.1.4.4 (Rambaut, 2018, <http://tree.bio.ed.ac.uk/software/figtree>). MEGA11 (Tamura  
132 *et al.*, 2021) was used to calculate the Kimura 2-P distance model with 1000 bootstrap and  
133 Gamma Distributed rates (Kimura, 1980).

#### 134 ***Database sequences***

135 On total 123 ascidian sequences were mined from NCBI in August 2022 to use species  
136 delimitation analyses. Sequences were chosen following the criteria of: having the same genus  
137 name, given a voucher record, approved by a taxonomist, and having at least 500 bp length.  
138 Yet, few exceptions with sequence lengths between 450 to 500 bp were included in the analyses  
139 (like *Botryllus tyreus*-DQ365851- 455bp). The IDs of database sequences were given on Fig.  
140 2.

#### 141 ***Species delimitation analyses***

142 Analyses were carried out using the Automatic Barcode Gap Discovery method (ASAP;  
143 Puillandre *et al.*, 2021), a sequence similarity clustering method, and the Poisson Tree  
144 Processes (PTP; Zhang *et al.*, 2013), a tree-based coalescence method. The hypothetical species  
145 identified by these methods were assigned as Operational Taxonomic Units (OTUs). ASAP  
146 analyses were performed on the web-based interface  
147 (<https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html>; accessed date: August 2022). Two  
148 metric options provided by ASAP were used for the pairwise distance calculations; Jukes-  
149 Cantor (JC69; Jukes and Cantor, 1969) and Kimura 2 parameter (K80; Kimura, 1980). PTP  
150 analyses were performed using the Bayesian implementation (bPTP), available on the web-  
151 based interface (<http://species.h-its.org/ptp/>, access date: August 2022). MrBayes output (tree)  
152 were used in the analyses by performing the default parameter values.

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154 **Results**

155 In total, 27 ascidian sequences from Turkiye were delineated, together with 123 NCBI-mined  
156 sequences. According to the common results of ASAP and PTP analyses, in total, 30 OTUs  
157 were defined for the genera *Botryllus*, *Botrylloides* and *Symplegma* (bOTU), 1 OTU for  
158 *Polyclinum* (pOTU) and 39 OTUs for *Didemnum* samples (dOTU, Fig. 2 a-c). Present study  
159 samples were located in the OTUs of 7 known species, and one sample was positioned with  
160 two Saudi Arabia samples as a new *Botrylloides* species (Fig. 2 a,b,c). The morphological and  
161 genetic analysis results of each species are detailed below and the ASAP, PTP and LnL scores  
162 are found in Fig. S1-S3. Whereas there were common results for most of the OTUs, ASAP  
163 assigned less OTU than the PTP in some groups. According to PTP analysis, almost all the *D.*  
164 *perlucidum* samples were assigned to different OTUs, same results were recorded for the *B.*  
165 *schlosseri* samples. The latest OTUs were decided according to all the analysis common  
166 results.

167 ***Botrylloides niger/aff. leachii* (Asciacea: Stolidobranchia: Styelidae: Botrylloides).** In  
168 total, 221 colony fragments were collected from 6 sampling sites (Table S1), and the sequence  
169 of the common haplotype (H1) was used for species delimitation analysis (Temiz *et al.*, 2022).  
170 Colonies revealed a wide range of color morphs, from a very light color (creamy) to total black  
171 (Table S2). The zooids were arranged in ‘leachii type’ systems (Brunetti, 2009), each ca. 1.8  
172 mm long with globular stomachs and same-plane common cloacal opening, where brachial  
173 sacs were occasionally seen through it (Fig. 3a<sub>1</sub>-a<sub>4</sub>). Two long (numbers 2-6), six middle  
174 (numbers 1, 3, 4, 5, 7 and 8), and many short tentacles containing numerous blood cells fringed  
175 the oral siphons (Fig. 3a<sub>4</sub>). The present study samples were assigned in bOTU-1 together with  
176 *B. niger/nigrum* and *B. aff. leachii*, 19% distance from the closest OTUs (bOTU-6 *B. leachii*,  
177 Fig. 2a, Table S3).

178 ***Botrylloides israeliense*.** In total, 36 colony fragments with light to dark color morphs (Table  
179 S2) were collected from only Mersin sites (Mezitli, Tisan, and Kızkalesi, Table S1), and the  
180 sequences of the one common (Kızkalesi sample) and one unique haplotype (Tisan sample)  
181 were used for species delimitation analysis. Zooids' lengths were ca. 2.3 mm, all arranged in a  
182 ‘leachii type’ system (Fig. 3b<sub>1</sub>). The stomach had a globular shape continuing after a long  
183 esophagus (Fig. 3b<sub>2</sub>). The zooids were positioned vertically to ampullas (Fig. 3b<sub>1</sub>, b<sub>3</sub>). Four

184 long (numbers 2, 4, 6, and 8) and four medium tentacles (numbers 1, 3, 5, and 7) fringed the  
185 oral siphons (Fig. 3b<sub>4</sub>).

186 The sequences of two haplotype (Tisan and Kızkalesi) were used for species delimitation  
187 analysis, then were assigned to bOTU-13 with *Botrylloides israeliense* sample from the  
188 Mediterranean coast of Israel (NCBI no:MG009580; Reem *et al.*, 2017) and two *Botrylloides*  
189 *sp.* samples (MN076468, MK978805, Viard *et al.*, 2019) from the English Channel and the  
190 Mediterranean Sea, respectively (Fig. 2a). The intra-bOTU-13 distances were 2 to 8%, and the  
191 closest OTUs distanced 16 to 19% (bOTU-12, *Botrylloides sp.*) (Fig. 2a, Table S4).

192 ***Botryllus schlosseri***. Four colonies collected under two rocks from the Hatay-Konacık region  
193 (Table S1), revealed three haplotypes (L12, L13 and L14; Fig. 2a). Haplotype L12 was further  
194 cultured in the IMS-METU mariculture system (38-40 ppt salinity and 24-26 °C), subcloned  
195 and colonial ramets survived under *in situ* conditions around three years. The colony's zooids  
196 lengths were ca. 1.2 mm revealing a brown color morph with yellow stripes (Fig. 4a<sub>1</sub>, 4a<sub>2</sub>). In  
197 time the pigmentation increased, and the blastogenic cycles became irregular.

198 All three Turkish haplotypes were assigned into bOTU-16 as Clade A colonies together with a  
199 database sample (MK575752). Other *B. schlosseri* samples mined from GeneBank were  
200 assigned to four different OTUs; bOTU-17, -18, -19, and -20 (Table S5). The intra-bOTU-16  
201 distance varied between 2 and 5%, and the highest distance was noted between our L14 sample  
202 and Clade E (bOTU-20) at 19.5 % (Fig. 2a, Table S5).

203 ***Botrylloides anceps***. In total 27 colonial samples were collected from Hatay-Konacık, Mezitli-  
204 Mersin, and Alanya-Antalya regions (Fig. 1, Table S1) and the sequence of the one common  
205 haplotype was used for species delimitation analysis. Zooids (between 1.5-2 mm long, Fig. 4  
206 and S4) were organized in 'leachii type' systems and color morphs vary from very light pink  
207 to dark brown, and creamy to purple (Fig. 4b<sub>1</sub>, b<sub>3</sub>). Two long, four middle, and six short size  
208 tentacles fringed the oral siphons (Fig. 4b<sub>2</sub>). The common cloacal opening is quite large,  
209 serving around 14 zooids (Fig. 4b<sub>4</sub>). Stomachs with globular shapes (Fig. 4b<sub>5</sub>). Laboratory  
210 follow-up observations revealed sexual reproduction under 38-40 ppt salinity and 26-28 °C,  
211 where eggs were recorded in zooids and primary buds, and testes on zooids (Fig. 4b<sub>6</sub>). While  
212 most progenies died at the oozoid stage, some survived 2-3 years under the IMS-METU  
213 mariculture conditions (16 to 32 °C), while young colonies grew, without signs for  
214 morphological hibernation (*sensu* Hyams *et al.*, 2017, 2022), and 2-3 years old colonies



215 showed exaggerated, yet condensed ampullae and irregular blastogenic cycles (all resembling  
216 onset of hibernation), a few months before their death (Fig. S5).

217 All 27 *B. anceps* samples belong to a single haplotype, and the Hatay region sample sequence  
218 was assigned to bOTU-21 together with the Israeli *B. anceps* (MG009581, Reem *et al.*, 2017)  
219 and *Botrylloides sp.* from Puerto Rico (MT637977; Streit *et al.*, 2021), differentiating with less  
220 than 0.01%. *Botrylloides cf. anceps* (MT873573, Salonna *et al.*, 2021) was assigned to another  
221 OTU (bOTU-22), ca. 18 % distance from bOTU-21 (Fig. 2a, Table S6).

222 ***Botrylloides sp.*** A single colony (brownish with a yellow stripe around the oral siphon and  
223 dorsal site) of ‘leachii type’ systems (Fig. 5a<sub>1</sub>) was collected from the Antalya-Kemer region  
224 (Table S1). A colony fragment (25 zooids) was cultured for one month, revealing 1.7 mm long  
225 zooids (Fig. 5a<sub>2</sub>, a<sub>3</sub>) and blastogenic cycles of five days long under 26°C and 38-40 ppt salinity  
226 (Fig. S6).

227 This colony was assigned to bOTU-8 together with two database samples from Saudi Arabia,  
228 Yanbu (*Botrylloides sp.*; ON053355-ON053356). The closest OTU (bOTU-9, *Botrylloides cf.*  
229 *pannosum*, MT873575; Bari, Italy; Salonna *et al.*, 2021) is of a 14% distance (Fig 2a, Table  
230 S7).

231 ***Symplegma brakenhielmi* (Asciidiacea; Stolidobranchia; Styelidae; Symplegma):** A single  
232 red colony was collected from the Mersin-Mezitli region under a rock (Fig. 1, Table S1). A  
233 ramet cultured in the aquaculture room (38-40 ppt, 27-28 °C; Fig. 5b<sub>1</sub>) revealed transparent  
234 tunic, with dispersed zooids arranged with no apparent systems. Both palleal and vascular  
235 budding were recorded, with the latter more prominent, 1 cm long ampullae and 1-3 cm long  
236 adult zooids (Fig. 5b<sub>1</sub>-b<sub>2</sub>).

237 This specimen was assigned to bOTU-30 as *S. brakenhielmi* together with a Panama sample  
238 (MT232734) at a 3% distance (Table S8, Fig. 2a) and two *Botrylloides chevalense* samples  
239 (KX650765 and KX650764) from India, yet with a 4-5% distance (Table S8, Fig. 2a). Other *S.*  
240 *brakenhielmi* samples, four Indian (KU360802, MH729325, MH729324, KT276228) and one  
241 from Italy (Bari; LS992554) samples were assigned to a different OTU (bOTU-27), together  
242 with *S. rubra* samples, ca. 28% distance to bOTU-30 (Fig. 2a, Table S8).

243 ***Polyclinum constellatum* (Asciidiacea: Aplousobranchia: Polyclinidae: Polyclinum):** Sixteen  
244 colonies (light brown, reddish, and gray with a beige system of zooids) were collected only  
245 Kızkalesi site in 2014 (Fig. 1, Fig. 5c<sub>1</sub>).



246 These samples belonged to two haplotypes and were assigned into a single OTU (pOTU-1)  
247 together with three database samples of *P. constellatum* from Italy and Florida (OL597608;  
248 MT873559; MW285146), and with *P. indicum* and *Polyclinum sp.* (KX650781; KX650778;  
249 KX138512) from India, with 6% intra- pOTU-1 distance (Table S9, Fig. 2b).

250 ***Didemnum perlucidum* (Asciacea: Aplousobranchia: Didemnidae: *Didemnum*):** This  
251 species was commonly observed on hard substrates of all sites (white, orange, light and dark  
252 creamy, and brownish color morphs). Colonies were less than 1mm thickness (Fig. 1; Table  
253 S1; Fig. 5d<sub>1</sub>, Table S2) and with star-shaped spicules (Fig. 5d<sub>2</sub>).

254 COI sequences from two colonies (from Alanya) were used for species delimitation analysis,  
255 together with the GeneBank data. In total, 39 OTUs were assigned for all *Didemnum* species  
256 (Fig. 2c). According to ASAP score present study samples were clustered under the dOTU-1  
257 together with *D. perlucidum* (Fig2c, Table S10) and *D. etiolum* (KY741541) samples from  
258 India, Australia and Puerto Rico with between the 0% and 5.7% distances (Table S10).

259

## 260 Discussion

261 This study reveals the existence and spatial distribution of eight colonial ascidian species from  
262 the shallow Turkish North Eastern Mediterranean coastlines. With the current sampling effort  
263 (focused on the very shallow waters), there is a high possibility that not all colonial species  
264 were sampled due to seasonality or deeper ascidian habitats. In total, five botryllid ascidians  
265 and one *Symplesma*, one *Polyclinum*, and one *Didemnum* species were recorded from six sites  
266 between Hatay and Antalya coastlines. Distance matrix, Bayesian tree, and PTPs and ASAP  
267 analyses scores were used for species assignment. Whereas more OTUs were recorded in PTP  
268 than the ASAP, common OTUs were chosen and differences were connected to data set size  
269 that was also experienced in the previous study (Goulpeau *et al.*, 2022).

270 *Botrylloides niger/aff. leachii* represents one of the emerged ambiguities in the taxonomic  
271 assignment and species delineation in botryllid ascidians (Reem *et al.*, 2018). The first record  
272 of *Botrylloides niger* Herdman, 1886 for the Mediterranean Sea was given by Peres (1958),  
273 and its wide distribution was reported from the Atlantic, Pacific, and Indian Ocean,  
274 Mediterranean Sea, and the Red Sea  
275 (<https://www.marinespecies.org/aphia.php?p=taxdetails&id=252289>). Based on mtCOI  
276 sequences, recent studies (Goutman *et al.*, 2020; Temiz *et al.*, 2022) reassigned the dwarf form

277 of *Botrylloides leachii* from the Levant (Brunetti, 2009) as *Botrylloides nigrum*. A study (Reem  
278 *et al.*, 2018) employing of three additional markers (18S, 28S, H3) contradicted literature  
279 suggestions, revealing minute distances between *Botrylloides leachii* and the ‘dwarf  
280 *Botrylloides leachii*’. As this issue has not yet been solved, we assigned this species here as  
281 *Botrylloides niger/aff. leachi*. Colonies of this species were collected from all the sampling  
282 sites, representing diverse color morphotypes previously recorded in the Mediterranean Sea  
283 (Rinkevich *et al.*, 1993; Brunetti, 2009), the Pacific and Atlantic oceans (Goodbody, 2000,  
284 2003; Monniot & Monniot 1994).

285 *Botrylloides israeliense* is a newly identified colonial ascidian (Brunetti, 2009) from the  
286 Mediterranean coast of Israel (Bay of Àkko and Mikhmoret) that was already studied in the  
287 past for some ecological and biological characteristics (Rinkevich *et al.*, 1993,1994).The  
288 genetic distances between the Turkish and the Israeli samples were between 3 to 5%, a value  
289 that is above the general eukaryotic species threshold (<2%, Hebert *et al.*, 2004) but still lower  
290 than some other cases (~20% Resch *et al.*, 2014; Reem *et al.*, 2022).

291 *B. schlosseri* is a highly invasive colonial ascidian distributed worldwide primarily in  
292 temperate shallow waters. Whereas the presence of the species on the Turkish coasts was  
293 reported before (Çınar, 2014; Kayış, 2011), no DNA sequence data was provided. So far, five  
294 clades have been assigned (A-E) worldwide (Reem *et al.*, 2022), and the present study samples  
295 clustered into the most common clade (A) with up to a 5.2% intra-clade distance as compared  
296 to the worldwide within clade A distance up to 6.3%. Further, according to ASAP and pPTP  
297 results clades B, C, D, and E were assigned as different species with min.11.2% and max.  
298 17.6% distance scores, a result that casts a query about the validity of current delimitation  
299 analyses.

300 *Botrylloides anceps* is also believed to be a NIS in the Mediterranean Sea. The first record was  
301 given from the Israeli coasts by Brunetti (2009). We recorded the species for the first time in  
302 2018 at Hatay, Mezitli, and Alanya sites. A previous visit to the sites had been done in 2012  
303 and no single *B. anceps* individuals were recorded, suggesting a recent introduction by the low  
304 (0.7%) distance between the Israeli and the Turkish samples. One important morphological  
305 characteristic of this study is the variable sizes assigned to *B. anceps* zooids, a morphometric  
306 quality that is changed according to the colony size, adding difficulties to this simple taxonomic  
307 characteristic for ascidian species identifications.

308 Colonies of presumably a new species *Botrylloides sp.* were recorded from only Antalya-  
309 Kemer sampling locations. This species matched 99% the NCBI samples uploaded from Saudi  
310 Arabia and the distance between the closest *Botrylloides* relatives was assigned at 14%  
311 (*Botrylloides cf. pannosum*; Australia). While this new botryllid ascidian species may have  
312 originated from the Red Sea, it has not been recorded from the Mediterranean coasts of Israel  
313 and the east sites of the Turkiye (Hatay and Mersin), thus making its origin fuzzy.

314 The first record of *Symplegma brakenhielmi* in the Mediterranean Sea was from the Lebanese  
315 coast, then from the Turkish Levantine coast (Bitar & Kouli-Bitar, 2001; Çınar *et al.*, 2006),  
316 based on only morphological characteristics. Other records included the central part of the  
317 Mediterranean Sea (Ulman *et al.*, 2017; Mastrototaro *et al.*, 2019). Our study on the  
318 Northeastern Mediterranean Sea reveals, for the first time, a species delimitation analysis that  
319 further indicated a possible misidentification and sequencing errors in the database, as the  
320 Turkish colonies were clustered with Panamian *S. brakenhielmi* and Indian *B. chevalense*  
321 samples, and were distinct from the Mediterranean (Bari, Italy) *S. brakenhielmi* cluster.  
322 Moreover, the present study sample is located as the most ancestral lineage on the tree with  
323 100% bootstrap support which may indicate polyphyly for the genus. On the other hand, like  
324 previous study (Mastrototaro *et al.*, 2019) *S. brakenhielmi* and *S. rubra* clustered together under  
325 the same OTU (bOTU-27), which is refer to the necessity of further morphological and genetic  
326 analysis for the whole genus.

327 With its wide range distribution (Western Atlantic, Western Indian Ocean, Indo-Pacific,  
328 Mediterranean and Aegean), *Polyclinum constellatum* has been considered a cryptogenic  
329 species (Dias *et al.*, 2013; Halim and Messeih, 2016; Aydın-Önen, 2018; Montesanto *et al.*,  
330 2022). Recording another name (*P. indicum*) under the *P. constellatum* OTU (pOTU1) was  
331 referred to as an error or synonymy (Montesanto *et al.*, 2022). Present study samples were  
332 highly matched (99-100%) with database specimens uploaded from various locations all over  
333 the world (Italy, Florida, India, and the Ionian Sea), a result indicating low intraspecific  
334 distance in a widely spread species and also monophyly.

335 *Didemnum perlucidum* is also a widespread species (Atlantic, Pacific, Indian Oceans, and  
336 Mediterranean Sea), and its native range is unknown (Lambert, 2002; Muñoz *et al.*, 2015;  
337 Novak & Shenkar, 2020). Here we provide the first barcode data for the Northeastern  
338 Mediterranean Sea populations and a high-resolution phylogenetic tree, revealing genetic

339 distances (0 to 6%) between the widely disturbed populations (Turkiye, India, Australia, Porto-  
340 Rico). Further, whereas the Turkish samples are clustered in a single OTU together with other  
341 *Didemnum perlucidum* samples, other valid species were assigned in the same OTU (like  
342 dOTU4, *Didemnum vexillum*, and *Didemnum granulatum*), while samples of other inclusive  
343 species i (e.g., *Didemnum psammatoide*) were further clustered under different OTUs (like  
344 dOTU 5, 7, and 8). All above highlighted the needs for taxonomic revision.

345 In conclusion, we provide here the descriptions for eight colonial ascidians from the Turkish  
346 North Eastern Mediterranean Sea shallow waters, including barcode data and species  
347 delimitation, supported with major morphological features of studied species. Besides high  
348 variation in morphotypes (*B. niger/aff. leachii* and *B. israeliense*), high (*B. schlosseri*) and low  
349 (*Didemnum perlucidum* and *P. constellatum*) genetic diversities were also recorded. A possible  
350 new *Botrylloides* species (*Botrylloides sp.*) from the Antalya region was revealed. It is also  
351 understood that the zooid length cannot always be used as an informative taxonomic feature  
352 for *B. anceps*. From a phylogenetic perspective, *Polyclinum* members were found closer to the  
353 Styelidae members than Didemnidae and a possible polyphyly was recorded for the *Symplegma*  
354 genus and Didemnidae family.

355

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363

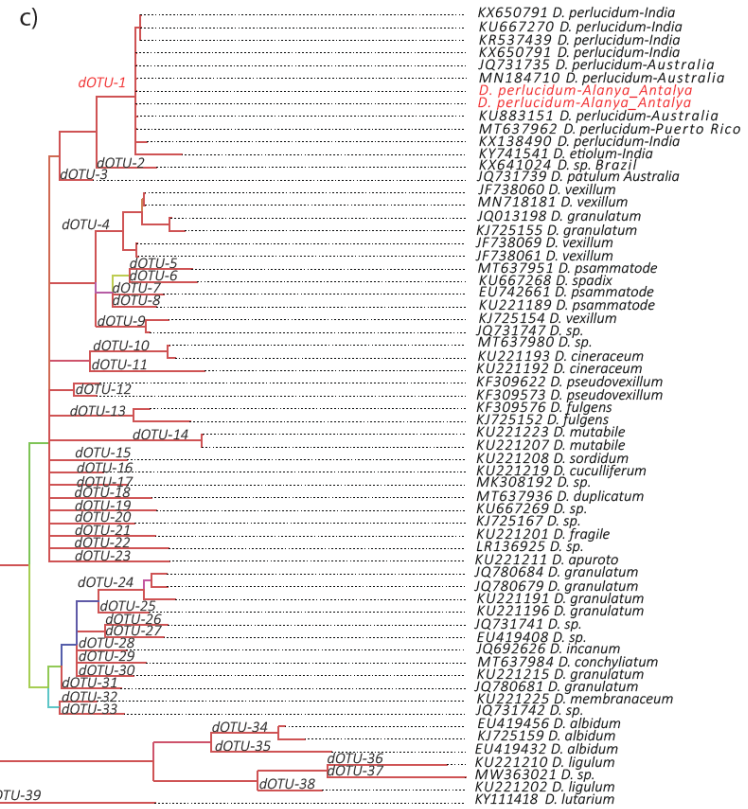
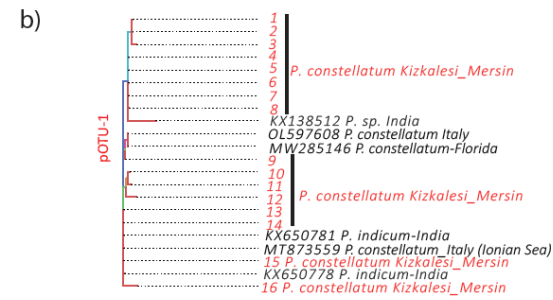
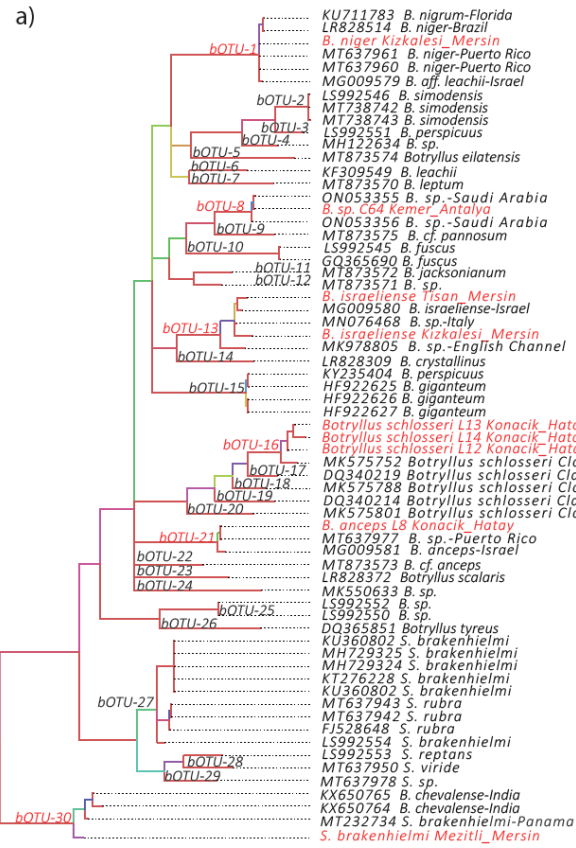
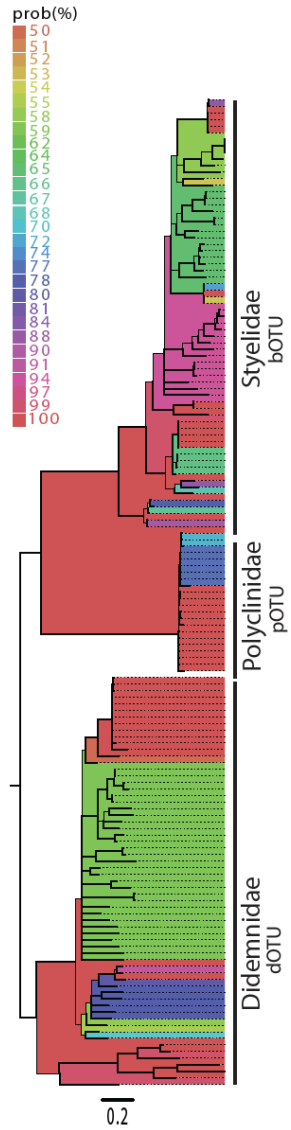
### 364 **Figures and legends**



365

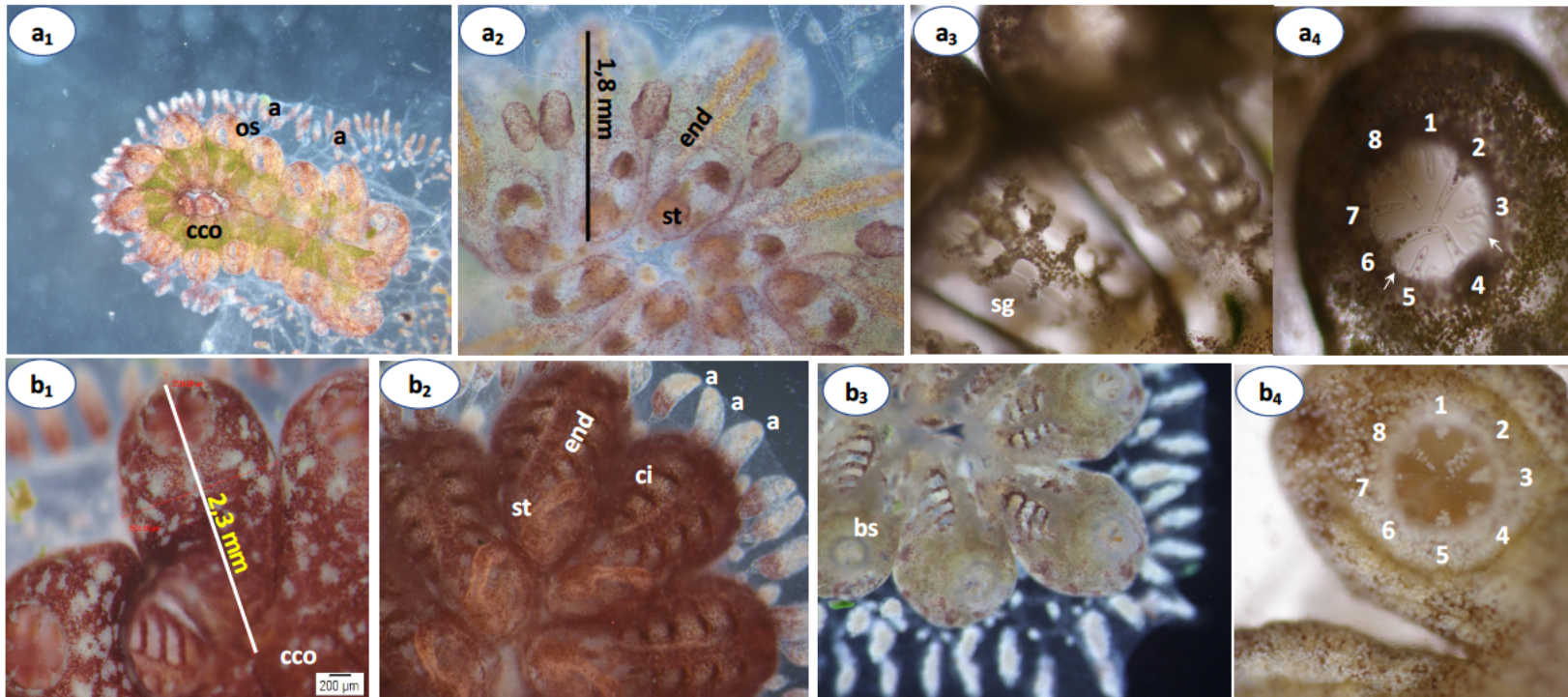
366 **Figure 1.** Map and Google Earth captures of the sampling sites. Credits; d-maps.com.



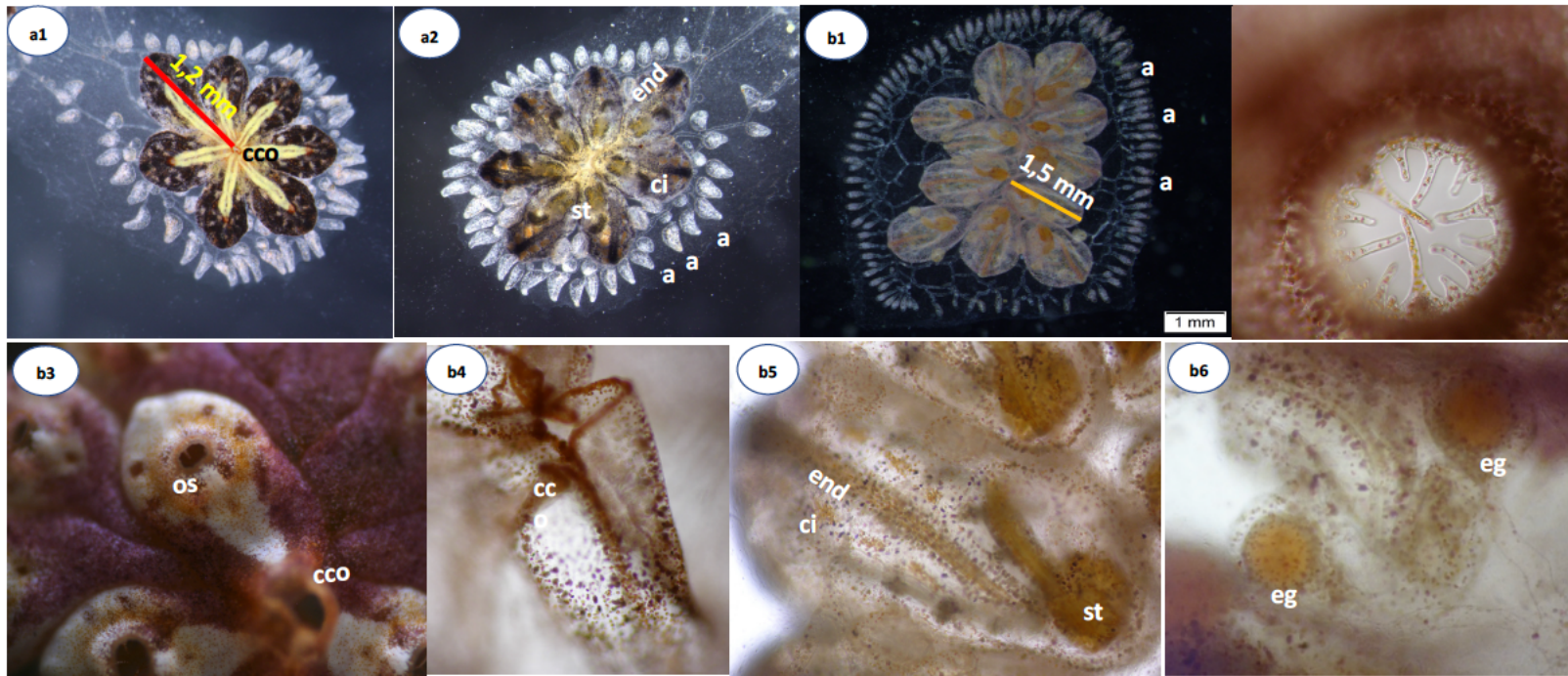


**Figure 2.** Bayesian majority rule consensus tree reconstructed from the ~ 600 bp COI sequence alignment. a) Styelidae specimens; b) *Polyclinum* specimens; c) Didemnidae specimens. The distance scale is given under the bootstrap probability colored tree, the NCBI IDs are provided next to the species name. OTU numbers are added on the line, according to common results of ASAP and bPTP analyses. Colors of Prob (%) scale, the main tree and a), b) and c) threes' branches represent probability values. The red colored letters are indicating the present study samples and OTUs. Abbreviations; B.: *Botrylloides*, bOTU: Styelidae family OTUs, pOTU: Polyclinidae family OTUs, dOTU: Didemnidae family OTUs.



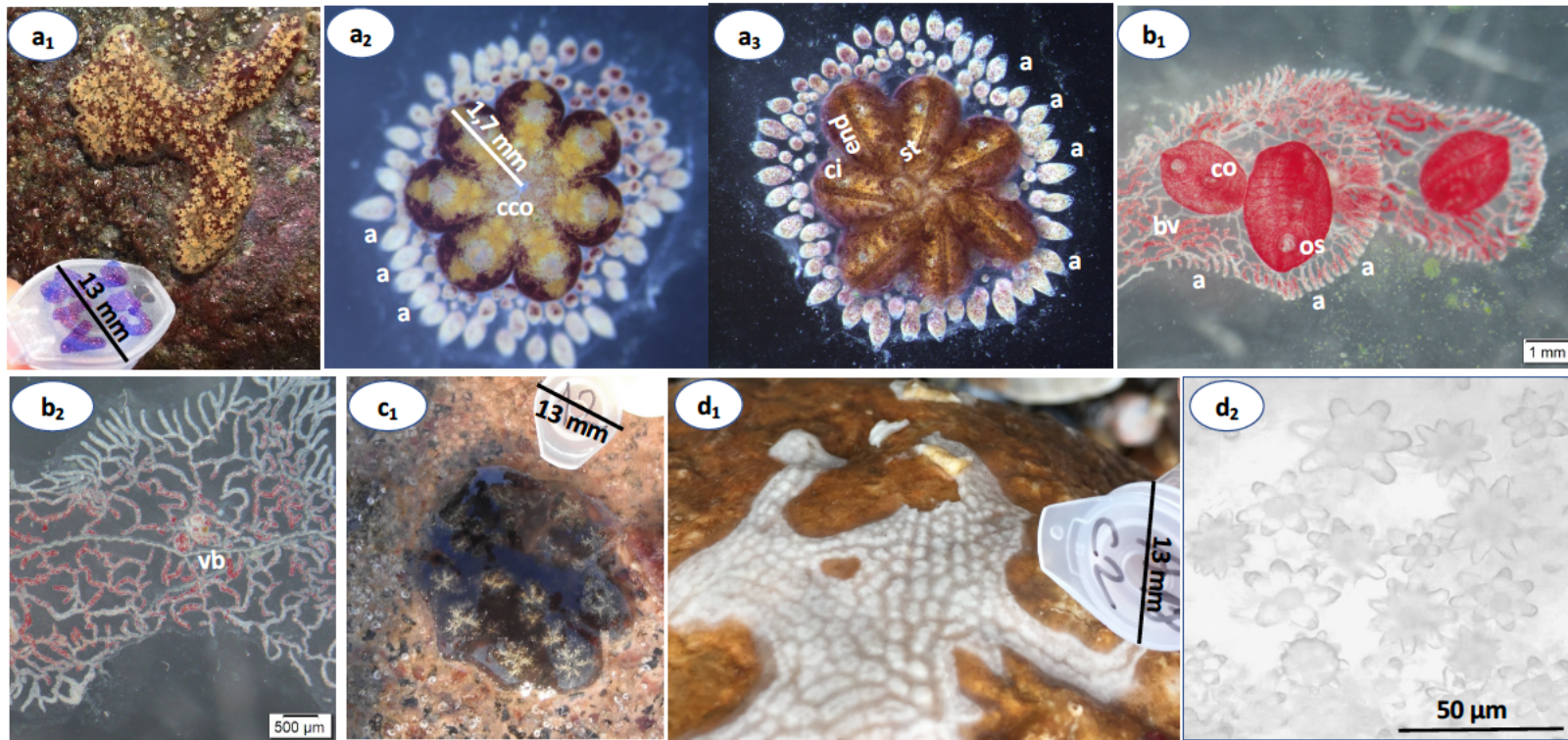


**Figure 3.** a<sub>1</sub>-a<sub>4</sub>) *Botrylloides niger/aff. leachii*, a<sub>1</sub>) Dorsal and, a<sub>2</sub>) Ventral view of yellow-green color morph of the colony, a<sub>3</sub>) View of brachial sacs through the common cloacal opening, a<sub>4</sub>) Oral siphon and tentacles; 2 long (2 and 6) and 6 middle lengths are numbered, and other short and random tentacles are indicated by arrows. b<sub>1</sub>- b<sub>4</sub>) *Botrylloides israeliense* b<sub>1</sub>) Dorsal view of a zooid, b<sub>2</sub>) Ventral view of the zooids, b<sub>3</sub>) Dorsal view of zooids with brachial sacs (white color morph), b<sub>4</sub>) Numbered oral tentacles (4 long and 4 medium lengths). Abbreviations: end; endostyle, ci: cell island, st; stomach, cco; common cloacal opening, a: ampullas, os: oral siphon



**Figure 4.** a<sub>1</sub>- a<sub>2</sub>) *Botryllus schlosseri* a<sub>1</sub>) Dorsal and a<sub>2</sub>) Ventral view of a cultured colony. b<sub>1</sub>-b<sub>6</sub>) *Botrylloides anceps*: b<sub>1</sub>) Ventral view of a pink color morph b<sub>2</sub>) Oral siphon and tentacles (6 long, 6 short and few very short), b<sub>3</sub>) Dorsal view of a purple color morph zooid b<sub>4</sub>) Common cloacal opening (cco), b<sub>5</sub>) Single zooid ventral view, b<sub>6</sub>) Primary bud with eggs. Abbreviations: end; endostyle, ci: cell island, st; stomach, eg; egg, cco; common cloacal opening, a: ampullas, bv; blood vessels, vb; vascular bud, os: oral siphon, co; cloacal opening.





**Figure 5.** a<sub>1</sub>- a<sub>3</sub>) *Botrylloides* sp. a<sub>1</sub>) field view a<sub>2</sub>) cultured colony's dorsal and a<sub>3</sub>) ventral view. b<sub>1</sub>-b<sub>2</sub>) *Symplegma brakenhielmi*; b<sub>1</sub>) Dorsal and ventral views of a colony, b<sub>2</sub>) Blood vessels and palleal budding, c<sub>1</sub>) *Polyclinum constellatum* dorsal view, d<sub>1</sub>-d<sub>2</sub>) *Didemnum perlucidum*; d<sub>1</sub>) Dorsal view of a colony, d<sub>2</sub>) the spicules of the colony. Abbreviations: end; endostyle, ci: cell island, st; stomach, eg; egg, cco; common cloacal opening, a: ampullas, bv; blood vessels, vb; vascular bud, os: oral siphon, co; cloacal opening.

## Supplementary Materials

Table S1. Sampling sites and date details

Table S2. General information of BoLD uploaded and other specimens

Table S3. Kimura-2 Parameter distance for bOTU-1 and -7.

Table S4. Kimura-2 Parameter distance for bOTU-13 and -12.

Table S5. Kimura-2 Parameter distance for bOTU-16, -17, -18, -19 and -20.

Table S6. Kimura-2 Parameter distance for bOTU-21 and -22

Table S7. Kimura-2 Parameter distance for bOTU-8 and -9.

Table S8. Kimura-2 Parameter distance for bOTU-27 and -30.

Table S9. Kimura-2 Parameter distance for pOTU-1

Table S10. Kimura-2 Parameter distance for dOTU-1

Fig. S1. ASAP score; Colors represent different OTUs. Number in line of the OTUs presents the total samples assigned to the same OTU. The first number line above the OTUs' columns presents the total OTU numbers, values at the second line indicate ASAP-scores (the lowest the score the better is the partition, Puillandre et al. 2021).

Fig. S2. PTP score; Blue lines present different OTUs, red lines present same OTUs

Fig. S3. LnL value of MrBayes analysis.

Fig. S4. When the zooid size of the brown morphotypes of *B. anceps* reached up to 2 mm.

Fig. S5. Large, retracted and condensed ampullas of *Botrylloides anceps*

Fig. S6. 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). Abbreviations: end; endostyle, st; stomach, cco; common cloacal opening, sg: stigmata, a: ampullas.

**Supplementary Materials:** Ten supplementary tables and six supplementary figures are provided as supporting information.

**Author Contributions:** Conceptualization, A.K.; Data curation, A.K., B.T., E.Ö., J.D., and B.R.; Methodology, A.K., B.T., and E.Ö.; Writing-original draft, A.K., B.T., E.Ö., J.D., and B.R. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** Sequences, trace files, image files, and the primers information for each COI haplotype were uploaded to the Barcode of Life Data System (Ratnasingham & Hebert, 2007). All the data will be public upon acceptance.

**Conflicts of Interest:** The authors declare no conflict of interest.

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