MITOCHONDRIAL AND NUCLEAR DNA PHYLOGEOGRAPHIES FOR TWO BOTRYLLID ASCIDIAN SPECIES FROM THE NORTH-EASTERN MEDITERRANEAN SEA

A THESIS SUBMITTED TO
INSTITUTE OF MARINE SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ESRA ÖZTÜRK

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
MARINE BIOLOGY AND FISHERIES

SEPTEMBER 2020

Approval of the thesis:

THESIS TITLE

submitted by **ESRA ÖZTÜRK** in partial fulfillment of the requirements for the degree of **Master of Science in Marine Biology and Fisheries, Middle East Technical University** by,

Assoc. Dr. Barış Salihoğlu
Dean, Graduate School of **Institute of Marine Sciences** _____

Prof. Dr. Zahit Uysal
Head of the Department, **Marine Biology and Fisheries** _____

Assoc. Prof. Dr. Arzu Karahan
Supervisor, **Marine Biology and Fisheries, METU** _____

Examining Committee Members:

Prof. Dr. İrfan Kandemir
Biology, Ankara University _____

Assoc. Dr. Arzu Karahan
Marine Biology and Fisheries, METU _____

Prof. Dr. Ahmet Erkan Kıdeyş
Marine Biology and Fisheries, METU _____

Date: 23.09.2020

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Esra ÖZTÜRK

Signature:

ABSTRACT

MITOCHONDRIAL AND NUCLEAR DNA PHYLOGEOGRAPHIES FOR TWO BOTRYLLID ASCIDIAN SPECIES FROM THE NORTHEASTERN MEDITERRANEAN SEA

Öztürk, Esra
Master of Science, Marine Biology and Fisheries
Supervisor: Assoc. Dr. Arzu Karahan

September 2020, 105 pages

With approximately 3000 species, ascidians are the largest tunicate group in the world seas. *Botrylloides* belongs to the genera of the worldwide spread Botryllid ascidians. In the Eastern Mediterranean coasts, a total of six Botryllid ascidians have been observed up to date. However, there is no study considering the distribution and phylogeographic analysis of ascidian species along the Mediterranean Sea's Turkish coasts.

Within this study's scope, specimens were collected from the coastal area of Hatay (Konacık), Mersin (Mezitli, Kızkalesi, Tisan) and Antalya (Alanya) regions comprising approximately 550 km of coastline between 2012 and 2019. Mitochondrial (COI, CytB) and nuclear (18s, H3) gene regions were amplified for the analysis. For COI, 100% amplification success was obtained for 62 samples; however, the success rate varied from zero to 100% for the other markers according to sampling location. The intra- and inter-specific distances were calculated between the haplotypes/locations. The Median-joining network analysis of haplotypes was

done, and the phylogenetic trees were constructed for the samples. Neutrality tests and mismatch distribution analyses were applied to detect any demographic change. *B. anceps*, *Botrylloides sp.* and *Botrylloides israeliense* were recorded on the Turkish coasts of Levantine Sea for the first time. The phylogeographic analysis was done only for *B. anceps* and *Botrylloides sp.,* which could be sampled at Konacık, Mezitli, Kızkalesi, and Alanya sampling stations, in contrast to *B. israeliense,* for which only one specimen could be encountered in Tisan during sampling.

No genetic variation was detected for the COI and CytB regions of *B. anceps* and the 18S gene regions of *Botrylloides israeliense.* H3 gene showed low variation for *B. anceps,* thus resulted in an insignificant neutrality test and Raggedness statistics. Therefore, *B. anceps* was found to have low invasive capacity due to low genetic variation related to a single introduction event that happened via anthropogenic ways and colony age. For *Botrylloides sp.,* both mitochondrial markers have shown variations; however, not enough for a significant P-value. Thus, population size change or population expansion could not be detected for *B. anceps* and *Botrylloides sp.* species.

On the other hand, Mezitli and Kızkalesi samples of *Botrylloides sp.* have shown strong genetic differentiation; F_{ST}=0.556^{COI} and 0.361^{CytB}, is an outcome of the limited dispersal capacity of the larva. A chimera formation, a single colony, consisting of at least two colonies with distinct genotypes, recorded between *B. israeliense* and *Botrylloides sp.* The blastogenesis, weekly asexual reproduction, of the species was monitored for the first time in this study. In this study, the distribution of three ascidian species on Turkey's Mediterranean coasts was revealed, and their populations were compared for phylogeographic analysis. Populations were found to be in a constant size, and the need for the use of combined markers was emphasized.

Keywords: *Botryllid Ascidians, Mitochondrial and Nuclear DNA, Phylogeography, North-East Mediterranean*

ÖZ

KUZEY-DOĞU AKDENİZ'DEN İKİ BOTRYLLID ASİDİYAN TÜRÜNÜN MİTOKONDRİYEL VE NÜKLEER DNA FİLOCOĞRAFYASI

Öztürk, Esra
Yüksek Lisans, Deniz Biyolojisi ve Balıkçılık
Tez Yöneticisi: Doç. Dr. Arzu Karahan

Eylül 2020, 105 sayfa

Tüm dünya denizlerinde yaklaşık 3000 kadar türü bulunan asidiyanlar tulumlular içindeki en büyük gruptur. *Botrylloides*, dünyada yayılım gösteren botryllid tulumluları grubuna ait cinstir. Doğu Akdeniz'de total 6 Botryllid türü tespit edilmiştir. Fakat Türkiye'nin Akdeniz kıyılarındaki asidiyan dağılımları ve filocoğrafyası ile ilgili herhangi bir çalışma yapılmamıştır.

Bu çalışma kapsamında, 2012 ve 2019 yılları arasında örnekler Hatay (Konacık), Mersin (Mezitli, Kızkalesi, Tisan) va Antalya (Alanya)'dan yaklaşık 550 km uzunluğu kapsayan kıyısal bölgeden toplanmıştır. Toplanan 62 örneğin COI bölgesi %100 başarı ile çoğaltılabilmiş fakat diğer belirteçler için amplifikasyon başarı oranı örnekleme istasyonlarına göre sıfır ile %100 arasında değişmiştir. Mitokondriyal (COI, CytB) ve nükleer (18s, H3) gen bölgeleri çoğaltılmıştır. Haplotipler/lokasyonlar için tür içi ve türler arası uzaklıklar hesaplanarak, median-joining network analizleri yapılmış ve filogenetik ağaçlar oluşturulmuştur. Neutrality test ve mismatch dağılım analizi herhangi bir demografik değişikliği belirlemek amacıyla yapılmıştır. *Botrylloides anceps*, *Botrylloides sp.* ve

Botrylloides israeliense türleri ilk defa Levanten Denizi'nde Türkiye kıyılarında kayıt edilmiştir. Filocoğrafik analiz Tisan'dan tek bir örnekleme yapılan *B. israeliense*'nin tersine Konacık, Mezitli, Kızkalesi ve Alanya istasyonlarından birden fazlaörnekleme yapılabildiği için *B. anceps* ve *Botrylloides sp*. türleri için yapılabilmiştir.

COI ve CytB gen bölgeleri *B. anceps* için, 18S gen bölgesi de *Botrylloides sp.* için varyasyon göstermemiştir. H3 geni *B. anceps* türü için varyasyon göstermişse de neutrality test ve Raggedness indeks istatistiki olarak önemli değerler vermemiştir. *B. anceps* türünün genetik varyasyonunun az olmasından dolayı istila edebilme kapasitesi düşük bulunmuştur ve bu durum bölgeye antropojenik yollarla tek girişin olması ve koloni yaşıyla ilişkilendirilmiştir. *Botrylloides sp.* türü için kullanılan iki mitokondriyal bölgede de varyasyon gözlenmiştir fakat P-değeri önemli olacak kadar değildir. Dolayısıyla, hem *B. anceps* hem de *Botrylloides sp*. türü için populasyonda büyüme gözlenmemiştir. Fakat Kızkalesi ve Mezitli'den toplanan *Botryllodies sp.* örnekleri arasında larvanın kısıtlı yayılım kapasitesinin sonucu olan güçlü genetik farklılaşma (F_{ST}=0.556$_{COI,}$ 0.361$_{CytB}$) iki mitokondriyal bölge için de gözlenmiştir. *B. israeliense* ve *Botrylloides sp.* arasında farklı genotiplerde birden fazla koloni içeren kimerik oluşum gözlenmiştir. Çalışma ile türlerin blastogenik döngüleri, haftalık eşeysiz üreme, ilk kez tanımlanmıştır. Bu çalışmada, Türkiye'nin Akdeniz kıyılarında üç asidiyan türünün dağılımı verilmiştir ve populasyonlar filocoğrafik olarak karşılaştırılmıştır. Populasyonların sabit büyüklükte olduğu gözlemlenmiş ve belirteçlerin birlikte kullanılması gerektiği vurgulanmıştır.

Anahtar Kelimeler: *Botryllid Asidiyanlar, Mitokondriyal and Nükleer DNA, Filocoğrafya, Kuzey-Doğu Akdeniz*

To my family

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Arzu Karahan for her encouragement, patience and help during my study. She was always open to my ideas and suggestions which made me feel like a colleague rather than a student. I am really thankfull for that.

I also would like to thank committee members Dr. İrfan Kandemir and Dr. Ahmet Erkan Kıdeyş for their valuable comments and suggestions for my thesis.

I would like to thank the Institute of Marine Sciences in general.

Last but not the least, I would like to thank my family for their endless love and support.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

INTRODUCTION

1.1 General Features of Ascidians

Tunicates have bilateral symmetry, and their name comes from a polysaccharide (tunicin) matrix, which is the animal imbedded in (Matthysse et al., 2004). Ascidians are marine filter-feeding invertebrates and classified under the sub-phylum Tunicata, which belongs to the phylum Chordata. Whereas the ascidians are grouped under the Chordata, based on the characteristics of larvae; notochord, nerve cord, and a muscular tail (Jeffrey and Swalla, 1997; Zeng and Swalla, 2005; Swalla, 2006), when they attach to a substratum, the tail with notochord is absorbed, so the adults lose their vertebrate characteristics (Swalla et al., 2000). On the other hand, as a primitive chordate, tunicates are the closest sister group to the vertebrates (Braun et al., 2019).

Tunicates consist of three classes; Ascidiacea, Appendicularia, and Thaliacea. Class Ascidiacea are grouped into three sub-orders; Aplousobranchia, Phlebobranchia, and Stolidobranchia (Zeng and Swalla, 2005, Figure 1). Stolidobranchia has a particular phylogenetic position because of its complex and variable morphological traits (Pérez-Portela et al., 2009). Styelidae family grouped under the Stolidobranchia sub-order consists of both colonial and solitary ascidian forms (Zeng et al., 2006). *Botryllus* and *Botrylloides* (Saito et al., 2001) are two of the family's colonial genera. *Botryllus* genus is described by the characteristics such as; star-like systems, ovary residing anterior or dorsal to the testis, and embryo development peribranchial space. On the other hand, *Botrylloides* genus has such features; ladder shape growing system, ovary posterior to the testis, embryo development in a brooding pouch (Van Name, 1945; Berrill, 1950; Saito et al., 2001; Figure 2). Brooding pouch formation occurs from either peribranchial- or branchial-epithelium, and brooding sac formation plays a strategic role in the sexual selection in botryllids (Saito et al., 2001).

Oikopleura dioicaon from the Tunicata subphylum was reported to have the smallest chordate genome size (around 60 Mb, Seo et al., 2001). On the other hand, *Ciona intestinalis* estimated genome size was reported as 160 Mb (Simmen, 1998); it was the first ascidian and seventh animal whose whole-genome was sequenced (Dehal et al., 2002). At the base of this information, Holland et al. (1994) and Makabe et al., (2001) postulated that having the base chordate genome indicate no genome duplication in the taxa, compared to two genome duplication events happened in the phylum Chordata during the evolution of vertebrate. On the other hand, the genome size of *Botryllus schlosseri,* the first botryllid ascidians whose whole-genome was sequenced, was reported as 580 Mb by Voskoboynik et al. (2013). The draft genome of the *Botryllodies leachii* was also presented as 159 Mb by Blanchoud et al. (2018) recently.

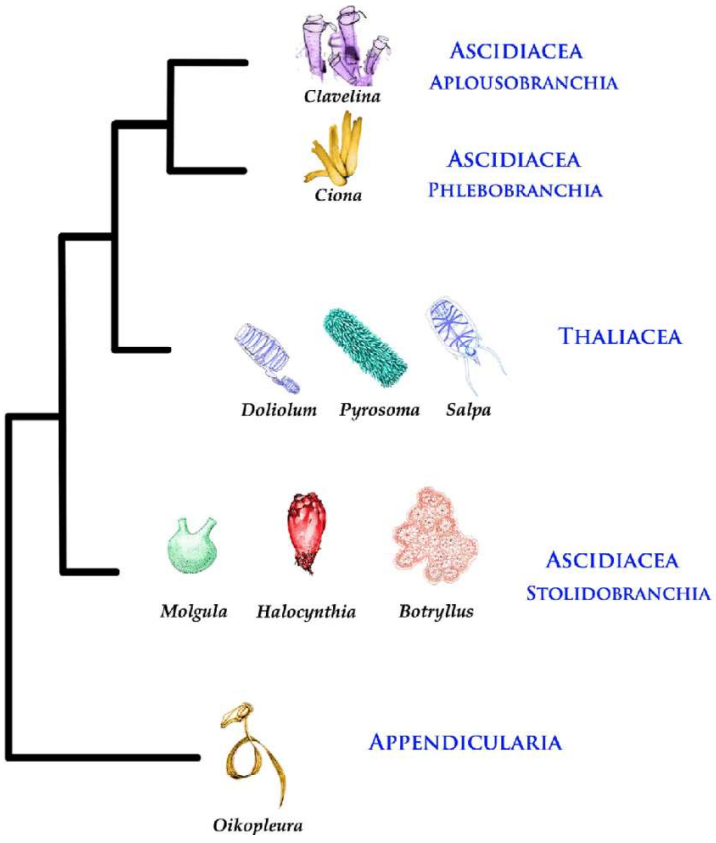


Figure 1. Phylogenetic tree of Tunicates (Franchi & Ballarin, 2017)

Beside of that, there is a debate on the ancestry of colonial features; it is declared that colonial features evolved a few times in the ascidians' evolutionary history; thus, it is uncertain whether the ancestral form was solitary or colonial (Wada et al., 1992; Swalla et al., 2000).

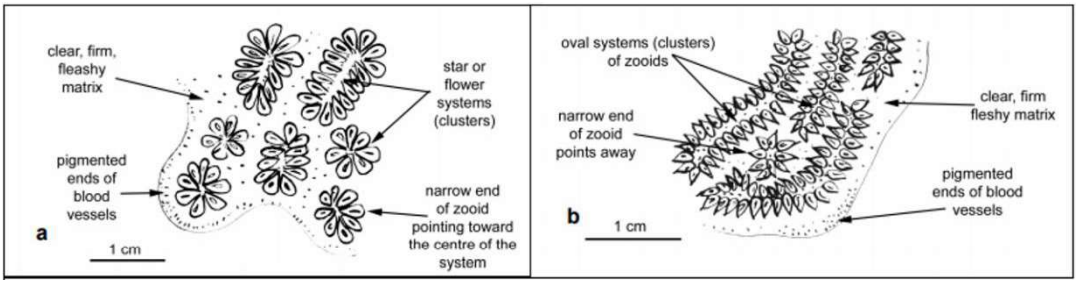


Figure 2. Schematic view of a) star-shaped system of *Botryllus* and b) ladder type system of *Botrylloides* (Carver et al., 2006)

1.2 Reproduction in Ascidians

As sessile organisms, ascidians, either colonial or solitary, are hermaphrodite. On the other hand, colonial ascidians are capable of both sexual and asexual reproduction, which the latter is related to colony formation and growth, and solitary ascidians only reproduce sexually (Berrill, 1975; Nakauchi, 1982; Davidson et al., 2004). Most of the colonial ascidians are viviparous giving birth the young which developed inside the mother body or ovoviviparous producing the young by means of eggs inside the mother body (egg size; 420-720 μm); *Botryllus* genus species are ovoviviparous (the egg size of *Botryllus schlosseri* is 300 μm), and *Botrylloides* genus species either viviparous or ovoviviparous (egg size *of Botrylloides leachii* is 260 μm) (Berrill, 1975; Saito et al., 2001; Rodriguez et al., 2014). The solitary species of ascidians are reported as oviparous laying eggs with no development inside the mother body (egg size; 100-150 μm), which play a main role in the further dispersion of embryo rather than the tadpole larvae (Berrill, 1950; Berrill, 1975).

Because of their hermaphroditic nature, ascidians have both self-fertility and self-sterility to a degree (Berrill, 1975). Self- and cross-fertilization have been reported in *Botryllus schlosseri* (Sabbadin, 1971; Berrill, 1975). Having both

fertilization systems has resulted in increased heterogeneity, in terms of colorfulness within the same species (Watterson, 1945; Berrill, 1975). A colony reproduces sexually along its life span until the zooids become old enough for sexual production (Kott, 1952, 1969; Haven, 1971).

1.2.1 Asexual Breeding; Budding

The colonial ascidians have two different buddings (growth) systems; palleal and vascular budding. Budding in ascidians is grouped into two categories in terms of functionality; propagative budding which assures the colony growth and survival budding known also as hibernation (*Clavelina, Diazona*) and aestivation (*Botryllus, Botrylloides, Aplidium*) which assures the survival of the colony when the environmental conditions are harsh (Nakauchi, 1981; 1982).

The palleal budding is observed in the Botryllinae subfamily and represents the buds that grow laterally from the zooid body wall's epithelium. It repeats around a weekly basis and called as 'blastogenetic cycle' or 'blastogenesis'. In this process, a colony harbor three generations at the same time; mature zooids, primary buds, and secondary buds (budlets) (Manni et al., 2006). The staging method, which blastogenic cycle was divided into four stages (A-D) were first proposed by Watanabe (1953). A new cycle begins following the take-over by the opening of the zooids' siphons, and bud-budlet growth can be observed gradually where primary buds become zooids, and budlets become primary buds in the forthcoming process (Manni et al., 2006).

The vascular budding is observed in the *Botrylloides* and *Botryllus* genus, and occurs when all zooids and their palleal buds are not present; only the vascular system and the compact blood vessels remain (Oka and Watanabe, 1957, 1959; Berrill, 1975, Figure 3). New buds shape from the blood vessels and ampullas.

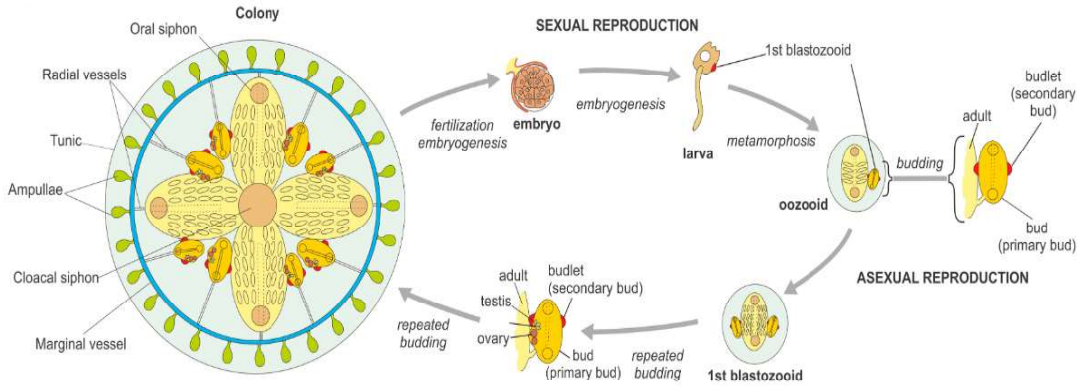


Figure 3. Schematic representation of sexual and asexual reproduction in ascidians (Manni et al., 2018)

1.2.2 Sexual Breeding in Ascidians

Although the variances in the latitudes on particular places affect the sexual breeding season, the primary determinant of the season is temperature (Berrill, 1975). It can be affirmed that the period between August and September is the most ordinary encountered breeding season, according to the most observed ascidians. However, it must be noted that it can take time from May to October or moreover, throughout the year for some species depending on the environment's annual temperature changes (Berrill, 1975; Svane and Young, 1989).

During the period blastozooids produce egg and give rise to the larva, which metamorphize to oozoids (Manni and Burighel, 2006, Figure 4). In the colonial ascidians; oozoids contribute to the formation of the new juvenile colonies after the growth of the post-attachment larvae. When the colonies are established with enough blastozooids, the system reaches sexual maturity (Berrill, 1975, Figure 3).

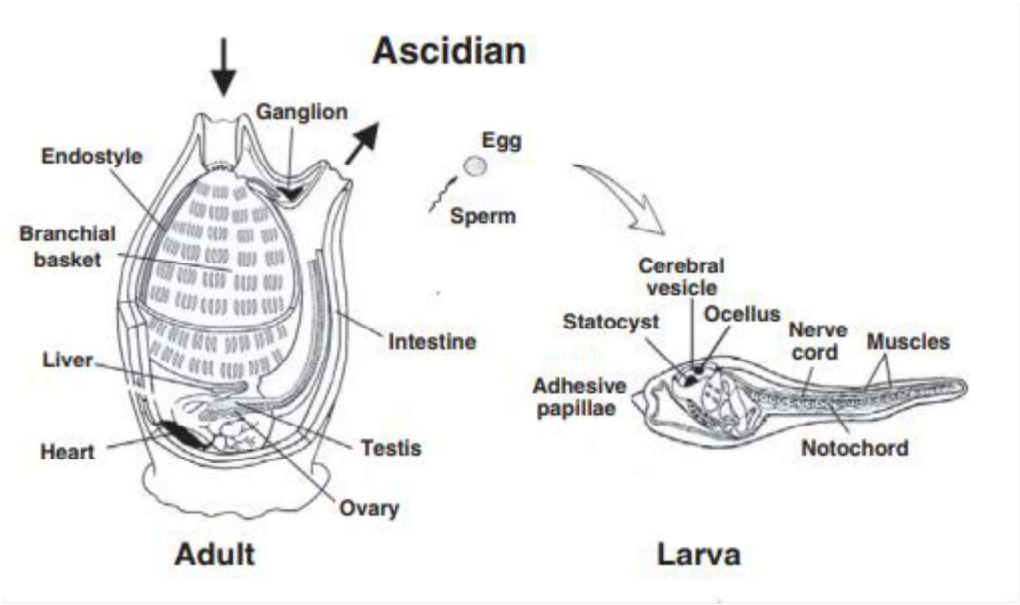


Figure 4. Schematic representation of adult ascidian and tadpole larvae (Holland, 2016)

1.2.3 Hatching and Tadpole Activity

There are three types of tadpole larvae of ascidians; the first type is the ones developing within few days depending on the temperature while drifting in the sea which is from small eggs of oviparous species and they swim freely 12-36 hours before the attachment; the second type is the one from larger eggs of oviparous species having longer swimming time; the last ones from larger eggs of viviparous species swimming freely from few minutes to 2-3 hours because this kind of diversity is associated with settlement area (Berrill, 1975). Ascidians mostly prefer firm structures such as rocks on the shallow waters for attachment and development because these areas are protected from inter-tides and sub-tides (Satoh et al., 1990). The attachment of the tadpole larvae to a settlement area (rigid and clear) results with a vertical movement; from the upper part (high light intensity) to the lower parts (low light intensity) (Berrill, 1975). In the upper parts, the behavior of tadpole larvae for locomotion is driven by light positively and gravity negatively; however, the behavior is driven by gravity positively and light negatively at the lower parts (Crisp and Ghobashy, 1971). The overall locomotive activity is resulted by the response to

both gravity and light and the attachment of the larvae to a favorable area by the anterior part (Grave and Woodbridge, 1924; Grave and Nicoll, 1936).

1.2.4 Metamorphosis and Development

The larvae of ascidians have two main purposes; 1) spreading as possible as far from the mother colony and 2) selecting a suitable habitat for the settlement (Svane and Young, 1989). For the initiation of the metamorphosis, the tadpole larvae require to attach to a suitable surface (Lynch, 1961; Berrill, 1975). The attachment surface for ascidians in the coastal zone varies from natural (rocks, weeds, mangrove roots) to artificial (rope, pile, dock, ship hulls) structures (Carman et al., 2007; Simkanin et al., 2016). By the metamorphosis, non-feeding motile larvae turn to be a sessile filter-feeding adult (Cloney, 1982).

The tadpole larvae of *Botrylloides* and *Botryllus* can swim freely several minutes before attachment, which limits the dispersal along with the viviparity, sometimes that can extend to 2-5 hours utmost (Berrill, 1975). The eggs produced by viviparous species are larger than the oviparous ones, which correlates with the larger tadpole larvae which in turn cause an increase in the swimming velocity and higher swimming rate is vital for tadpole larvae to overcome the tides during the attachment period (Berrill, 1975; Svane and Young, 1989).

According to Degnan et al. (1997), metamorphosis in the ascidians is catalyzed by a variety of ions, such as copper, potassium. On the other hand, Davidson and Swalla (2002) reported that metamorphosis is induced by osmotic changes, outnumbering, and shock. The first event of metamorphosis is the secretion of adhesives from the papillae and retraction of papillae followed by losing the tail, mesenchymal cell migration that happens through the whole body and tunic, the outer layer of the larval tunic lose, rotation of internal organs occurs, ampullae grow and extent, cerebral complex absorbs and as a final, development of the adult organs complete (Cloney, 1982; Davidson and Swalla, 2002). During the metamorphosis,

ascidians lose the nerve cord and notochord, acquired in the embryogenesis while forming gill slits and endostyle (Eri et al., 1999).

After the metamorphosis, colonial ascidians will get into a repetitive budding process, which will form genetically identical zooids (blastozooids) by asexual reproduction (blastogenesis) and continue for the lifetime of the colony (Tiozzo et al., 2008).

1.3 Chimerism and Allorecognition in Ascidians

Chimerism is defined by the presence of at least two genomes from different origins in one organism and directed by the self-nonself recognition system called allorecognition (Rinkevich, 2005; Voskoboynik et al., 2018). Allorecognition in ascidians results in two ways, after the colonies the same species touch each other's either fusion or rejection happens. This fusion/rejection processes are controlled by a highly polymorphic locus known as fusion/histocompatibility complex (Oka and Watanabe, 1957; Tanaka and Watanabe, 1973; Katow and Watanabe, 1980; Scofield and Nagashima, 1982; Watanabe and Tanade, 1982; Saito et al., 1994, Figure 5). If the colonies share at least one common allele for the FuHC locus, the colonies fuse and become one forming a chimera or rejection happens when the colonies don't share at least one FuHC allele (Oka and Watanabe, 1957; Saito et al., 1994; Voskoboynik, 2009). Also, somatic and germline replacements between the partners of chimera were observed in *Botryllus* colonies (Sabbadin and Zaniolo, 1979) resulting in the combined genotypes in somatic and germ cell of both chimeric partners (Pancer et al., 1995; Stoner and Weissman, 1996; Stoner et al., 1999; Voskoboynik, 2009). When the rejection occurs, zooids of one of the colonies are absorbed by the other (Rinkevich and Weissman, 1992). Saito et al. (1994) state that it may be driven for the competition of more space and it is called as 'stem cell parasitism'.

Chimeric entities in a colony have increased the development and survival rate, proliferation and competition success, and better adaptation to the environmental conditions and increased heterogeneity (Rinkevich, 2011; Casso et al., 2019). Natural chimerism in colonial marine invertebrates is restricted mainly to kin. It was hypothesized that chimerism through fusion increases the colonization success and makes the chimeric entity more resistant to environmental conditions (Voskonoynik, 2009; Casso et al., 2019). According to the study done by Casso et al. (2019), 44% of the colonies in the study of invasive ascidian *Didemnum vexillum* were chimeric which could be the main reason for the species invasion success.

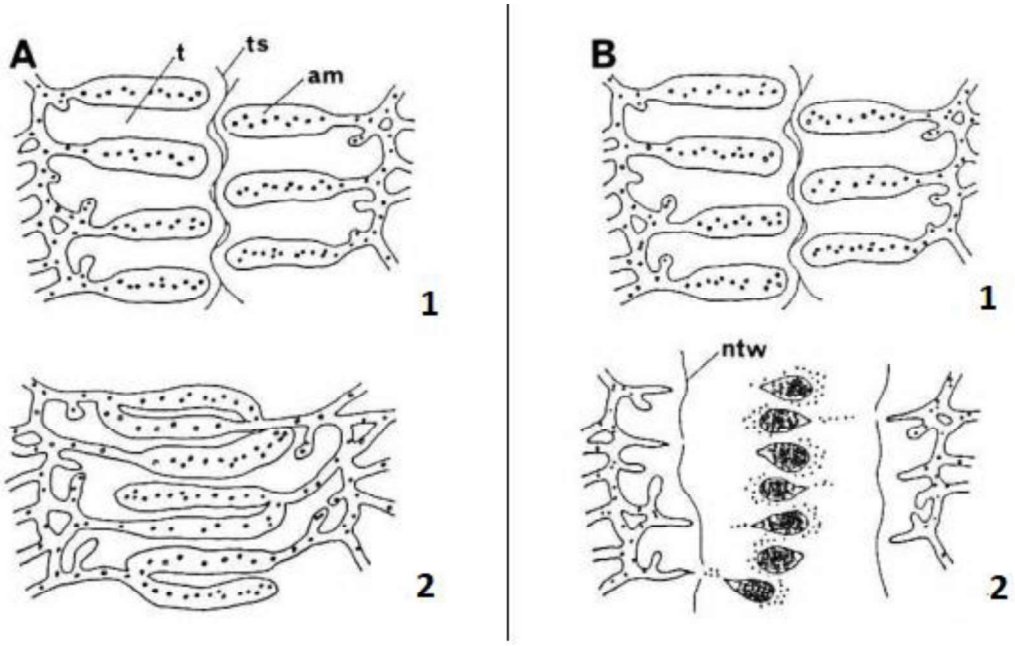


Figure 5. Schematic representation of fusion (A) and rejection (B); 1: first stage, 2: last stage, t: tunic, am: ampulla, ts: tunic surface, ntw: new tunicate wall (Saito et al., 1994)

1.4 Fouling and Invasive Nature of Ascidians

There has been a rapid increase in the number of non-indigenous ascidians all around the world seas (Coles et al., 1999; Lambert, 2001). This increase can directly be associated with the shipping activities and ascidians fouling nature (Lambert and Lambert, 1998). They can be transported via attaching boats, crafts,

trays (Carlton and Geller, 1993; Lambert, 2001) and spread by shells or external surfaces of the other species, such as oysters and mussels (Lambert, 2001). They also prefer the areas where strong tides and flows protect the settlement (Lambert, 2007; Tracy and Reyns, 2014). Non-indigenous species firstly colonize on artificial structures such as buoys, and these constructions provide a particular area for the invaders in which endemic species cannot thrive on it. And these human-made structures create a distinct environment, thus causes an increase in biodiversity in ports (Schoener, 1982; Lambert and Lambert, 1998; Connell, 2000; Lambert, 2001). Bak et al. (1998) stated that high bacterial communities originated from anthropogenic sources caused an increase in the ascidian's numbers. Considering the filter-feeding features of ascidians, bacterial populations as a nutritional resource can explain the persistence of the ascidians in ports.

Invasive ascidians show a strong dominance compared to other fouling species because of their competitive behavior for space and tolerance to more extensive ranges of environmental conditions (Sims, 1984; Naranjo et al., 1996; Nomaguchi et al.,1997; Lambert, 2001). In addition to this, some ascidian species (e.g., *Ciona intestinalis*) was found to cause a change/decrease in the structure/species numbers of the fouling community (Lambert and Lambert, 1998, 2003; Ranasinghe et al., 2005; Tracy and Reyns, 2014; Blum et al., 2017).

1.5 Botryllid Ascidians in the Mediterranean Sea

Sixty-four ascidians species out of around 3000 are non-indigenous in all the seas (Shenkar and Swalla, 2011). Whereas the ascidian species number for the Mediterranean Sea was given as 132 by Peres (1958, 1967), the number rose to 229 by 2011 (Coll et al., 2010; Shenkar and Swalla, 2011). On the other hand, the records from the Western Mediterranean comprise 165 ascidians species; 86 records were given for the Eastern Mediterranean part and 45 for the Levantine site (Koukouras et al., 1995). According to Koukouras et al. (1995), 36 ascidians from the Eastern Mediterranean are endemic species, and two of them are Lessepsian.

There are botryllid ascidians that are worldwide spread; species numbers were reported as 32 and 19 for *Botryllus* and *Botrylloides* genus, respectively (Shenkar et al., 2019). *Botryllus schlosseri*, *Botrylloides violaceus,* and *Botrylloides diegensis* are invasive species of these two genera, and they have a wide distribution in all world seas (Shenkar and Swalla, 2011). *Botrylloides pizoni* was recently reported from Italy by Brunetti and Mastrototaro (2012). It was considered as introduced species because it only grows on human-made structures in a limited area (Rocha et al., 2019). On the other hand, six botryllid ascidians species have been reported for the Eastern Mediterranean Sea (Israel, Egypt, and Gulf of Suez shores); *Botrylloides aff. leachii, Botrylloides niger, Botrylloides anceps, Botrylloides israliense, Botryllus schlosseri,* and *Botryllus rosaceus* (Halim & Abdel Messeih, 2016; Reem et al., 2017).

Botrylloides anceps, a native of the Indo-Pacific region, was recorded for the first time in Australia by Herdman (1891) as *Sarcobotrylloides anceps.* Another record was then given by Michaelsen (Hartmeyer & Michaelsen, 1928) from New Zealand under the name of *Botryllus anceps*. Due to being examined when it is in the juvenile stage, *Botryllus gracilis* was identified as different species although it is an only a younger form of *Botrylloides anceps,* and *Botryllus gracilis* was reported from Australia (Hartmeyer & Michaelsen, 1928; Hastings, 1931; Millar, 1966) and New Caledonia (Tokioka, 1961; Monniot, 1988). Lastly, *Botryllus humilis* was reported from New Caledonia by Monniot (1988) as *Botrylloides anceps* (Brunetti, 2009). The detailed taxonomy of *B. anceps* was done by Brunetti (2009) for the samples from Israeli coasts, and the first barcode (MG009581.1) of *B. anceps* has been given from Mediterranean coasts (Reem et al., 2017).

B. israeliense was recorded for the first time from the Mediterranean Sea's Israel coasts and identified by Brunetti (2009; Reem et al., 2017). According to Reem and Rinckevich (2014), it is probably originated from the Red Sea.

1.6 Ascidians in the Turkish Coasts of the Mediterranean Sea

Ascidians of the Mediterranean coasts of Turkey are poorly known, and mostly the contribution was made by Uysal (1976). He has recorded 16 ascidians species (*Diazona violacea, Rhopalaea neapolitana, Ciona intestinalis, Ascidia virginea, Phallusia mammillata, Polycarpa pomaria, Distomus variolosus, Botryllus schlosseri, Pyura squamulosa, Microcosmus sulcatus, Halocynthia papillosa*) from the Turkish waters of the Mediterranean Sea (Aslan, 2006). Later on, three non-indigenous ascidians species (*Phallusia nigra, Herdmania momus, Symplegma brakenhielmi*) were recorded by Çınar et al. (2006) in the Levantine coast of Turkey. *Halocyntia papillosa* and *Microcosmus sabatieri* have been observed in Datça-Bozburun protected area, which resides at the intersection between the Aegean Sea and Mediterranean Sea (Okuş et al., 2004). Six ascidian species (*Botryllus schlosseri, Clavelina lepadiformis, Microcosmus sabatieri, Microcosmus sulcatus, Phallusia mammillata ve Pycnoclavella nana*) were reported from Fethiye Bay by Okuş et al., (2007). Lately, a revision has been done by Çınar (2014) according to this 50 ascidian species were reported for all the Turkish Seas, 32 of them inhabit the Mediterranean coasts of Turkey. From those, nine (*Pyura dura, Microcosmus polymorphus, Microcosmus exasperates, Polyclinella azemai, Aplidium turbinatum, Ciona rulei, Phallusia fumigate, Ascidia virginea and Ascidia mentula*) were a new record, *Botryllus schlosseri, Botryllus renierii,* and *Botrylloides leachii* were the only botryllid ascidians represented by Çınar (2014) for the Mediterranean coast of Turkey. Six ascidian species were recorded along the Levantine coasts of Turkey by Karahan (YÖP-701-2018-2666, 2020); *Botryllus schlosseri* (Konacık-new locality record), *Botrylloides aff. leachii* (new record), *Botryllus sp*. (Konacık-new record-possibly new species), *Polyclinum indicum* (new record), *Didemnum perlucidum* (new record), *Symplegma brakenhielmi* (Mezitli-new locality record).

1.7 Genetic Tools on Ascidian Studies

Despite their strong dispersal capacity, populations of marine species can show high genetic diversity. The physical barriers were addressed to understand the reasons for this divergence. On the other hand, cryptic species refer to the species that contain individuals who are morphologically identical to each other but different species which recently diverged and identifying cryptic species is quite problematic processes. The use of molecular tools helped overcome this problem (Helberg, 1994, 1996; Palumbi et al., 1997; Knowlton, 2000; Feral, 2002; Palumbi, 2004). Mitochondrial DNA has been widely used to understand population structure and evolutionary history (Avise et al., 1987; Avise, 2000; Palumbi et al., 1997; Pérez - Portela and Turon, 2008). Molecular tools such as COI, 18S, CytB were widely used for population genetics, biogeography, phylogeography, phylogeny, phylogenetics, DNA barcoding and invasion history of ascidians (e.g. *Cystodytes dellechiajei, Phallusia nigra, Botrylloides violaceus, Ciona intestinalis, Didemnum vexillum, Microcosmus squamiger, Styela clava, Halocynthia roretzi, Botryllus schlosseri*) (Wada et al., 1992; 2011; Lopez-Legentil and Turon, 2006; Kim et al., 2012; Vandepas et al., 2015; Zhan et al.,2015; Ananthan & Murugan, 2016; Reem et al., 2017).

Genetic markers were also used to investigate possible genetic variation and any speciation between the different color morphs of some ascidians such as *Didemnum molle*, *Pseudodistoma crucigaster, Ecteinascidia turbinata* (Tarjuelo, 2004; Lopez-Legentil and Turon, 2007; Hirose, et al., 2008). Although mitochondrial DNA is quite compatible for the phylogenetic, phylogeographic and population differentiation level analysis, study of lineages over a single genetic marker may reflect the evolutionary history of only that locus rather than the whole-genome (Maddison, 1997; Nichols, 2001, Nydam and Harrison, 2010). For interpretations with strong confidence, phylogenetic relationships need to be evaluated by more than one genetic marker. Thus, to achieve a better resolution of

spatial and demographic patterns, mitochondrial and nuclear data must be used together (Ballard and Whitlock, 2004; Pérez-Portela and Turon, 2008).

1.8 Ascidians as Model Organisms for Aging, Regeneration and Cancer Research

Invertebrates have been used as medicinal purposes for 4,000 years, and they have been used model organisms since the 1890s (Wilson-Sanders, 2011). A tunicate *Ciona intestinalis* was one of the first invertebrates model utilized by researchers according to a study published in 1896 by Castle (Wilson-Sanders, 2011). Tunicates were studied to understand the various fields from development to aging processes. The solitary ascidian *C. istestinalis* was also under investigation for endocrine function and metabolism (Sherwood et al., 2006). Moreover, the species was introduced as a model organism to study Alzheimer's disease pathogenesis (Virata and Zeller, 2010). Colonial ascidians are used as a model organism in the studies as well. Botryllid ascidian *Botryllus schlosseri* is used to reveal molecular basis of allorecognition by Ben-Shlomo (2008). The usefulness of *B. schlosseri* for aging, stem cell, and regeneration studies were presented by Voskoboynik and Weissman (2015). Senescence in *B. schlosseri* is also studied for possible tumor cell treatments considering the senescence acts tumor suppressor (Saretzki, 2010; Rinkevich, 2017). Another botryllid ascidian *Botrylloides leachii* is also used as a model organism for the whole-body regeneration studies (Blanchoud, 2018). Ascidians has also been used in pharmacology to new drug discovery; *Polyclinum indicum* extract has been shown to have anti-cancer activity against cervical cancer cell inducing apoptosis (Pusphabai Rajesh et al., 2010).

1.9 Objectives of the Study

Ascidians have high invasive species within the taxa and they have ecological, and economic impacts. Thus, their presence and their distribution must be unveiled to control the possible invasions. Phylogeographic research is a fundamental approach to sorting organisms' spread rate and locational distribution (Crisp et al., 2011; Donoghue and Edwards, 2014). This study aimed to reveal the botryllid ascidian species and their distribution along Turkey's Mediterranean coasts and do a phylogeographic analysis between the populations using mitochondrial (cytochrome oxidase I and cytochrome B) and nuclear markers (histone 3 and 18S). On the other hand, when the pharmaceutical potential of these species is considered, and as a model organism for aging, regeneration cancer research, the study also aimed to identify the botryllid species through Turkey's northeastern Mediterranean coast.

MATERIALS AND METHODS

2.1 Sampling

Sampling was performed between 2012 and 2019 from seven shores along the Mediterranean coasts of Turkey, comprising nearly 550 km of coastline. Samples were collected from Hatay (Konacık), Mersin (Mezitli, Kızkalesi, Tisan) and Antalya (Alanya, Side, Kemer) regions (Figure 6). In total, 62 colonial fragments of specimens were removed from their substrates by razor blade, consisting of 34 *Botrylloides sp.* specimens from Kızkalesi and Tisan; 27 *Botrylloides anceps* specimens from Konacık, Mezitli and, Alanya; 1 *Botrylloides israeliense* specimens from Tisan. None of these species were found at Side and Kemer sampling locations.

Figure 6. Sampling locations in the north-eastern Mediterranean: Konacık (Hatay), Mezitli (Mersin), Kızkalesi (Mersin), Tisan (Mersin), Alanya (Antalya), Side (Antalya), Kemer (Antalya) (credit; Google Earth, d-maps)

Samples were collected from the coastal zones up to 0.5 m below the sea surface at seven stations. The details about coordinates, salinity, and PH values of the stations were given in Table 1. In October, the highest salinity was measured in Tisan (40.2 ppt) and the lowest in Mezitli (39.2) stations.

Table 1. Sampling Stations.

					N: number of samples collected		
Sampling areas	**Date**	**Coordinates**	**Salinity (ppt)**	**pH**	**Ba**	**Bs**	**Bi**
Konacık (Hatay)	Sep. 2012	36°21' 38.79"N 35°49' 16.90"E	40	7.9	-	-	-
	26.09.18				8	-	-
Alanya (Antalya)	Sep. 2012	36°33' 33.85"N 31°57' 07.41"E	39.8	8.0	-	-	-
	24.10.18				9	-	-
Mezitli (Mersin)	Sep. 2012	36°43' 58.76"N 34°31' 18.53"E	39.2	8.13	-	-	-
	06.09.18				8	-	-
	14.10.19				2	5	-
Kızkalesi (Mersin)	Sep.July 2014	36°27' 27.52"N 34°08' 38.17"E	39-40	~8.0	-	12	-
	01.11.17				-	2	-
	07.06.18				-	4	-
	05.07.18				-	5	-
	03.08.18				-	3	-
	07.10.18				-	3	-
Tisan (Mersin)	03.10.18	36°09' 27.95"N 33°40' 59.90"E	40.2	8.3	-	-	1
Side (Antalya)	24.10.18	36°46' 00.78"N 31°23' 07.62"E	37.5	7.8	-	-	-
Kemer (Antalya)	25.10.18	36°35' 59.55"N 30°34' 30.66"E	40.1	7.9	-	-	-

Botrylloides anceps=Ba; *Botrylloides sp.*=Bs; *Botrylloides israeliense*=Bi

2.2 DNA Extraction and Amplification

The fragments of colonies were homogenized in 120-240 ml of lysis buffer (0.25 M Trisborat pH 8.2, 0.1 M EDTA, 2% SDS, 0.1 M NaCl and 0.5 M $NaClO_4$). An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed by vortex for 10 min and centrifuged for 10 min 14,000g, 4 ºC. The aqueous phase was further extracted with chloroform/ isoamyl alcohol (24:1). The DNA was precipitated with absolute ethanol, washed with 70% ethanol and resuspended in water (Paz et al. 2003). If necessary, DNA diluted with molecular grade water to adjust the concentrations around 5-20 ng/µl. Polymerase chain reactions (PCR) were run for the mitochondrial cytochrome oxidase subunit I (COI), Histone 3 (H3), 18S, and Cytochrome B (CytB) gene regions. Primer details were given in Table 2 and PCR condition in Table 3. PCR prepared in 20 µl total volume with 0.5 µM forward and reverse primers and around 10 ng/µl of DNA in a ready to use PCR Master Mix (0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM MgCl2, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP).

Table 2. The details of forward and reverse primers

Primer Name	Primer sequence	Length-bp	Reference
DEG COI F2	5-‘AMWAATCATAAAGATATTRGWAC-3	700	Reem at al., 2017
DEG COI R2	5-‘AARAARGAMGTRTTRAAATTHCGATC-3		
H3 F1	5-‘ATGGCTCGTACCAAGCAGACVGC-3	300	Reem at al., 2017
H3 R1	5-‘ATATCCTTRGGCATRATRGTGAC-3		
18S A	5-‘AACCTGGTTGATCCTGCCAGT-3	2500	Reem at al., 2017
18S B	5-‘GATCCTTCTGCAGGTTCACCTAG-3		
CytB-F	5-‘TGRGGNCARATGWSNTTYTG-3	400	Atsumi & Saito, 2011
CytB-R	5-‘GCRAANARRAARTAYCAYTC-3		

PCR was performed with an initial denaturing step at 95°C for 15 min, followed by 30-35 amplification cycles; denaturation at 95°C for 30 s; annealing at 43-60°C for 45s; elongation at 72°C for 60 s, and a final elongation step at 72°C for

10 minutes (Table 3). The PCR products were screened on 1.5% agarose gel, and Macrogen Inc did sequencing, South Korea, for both forward and reverse directions.

Table 3. PCR conditions for each primer, COI; Cytochrome oxidase sub-unit I, H3; Histone 3, 18S, Cytochrome oxidase B; cytB

Primer	Denaturation	Annealing	Elongation	Final	Cycle	Soak
COI	95°C /30 s	45°C /45 s	72°C /60 s	72°C /10	35	12°C /∞
H3	95°C /30 s	55°C /45 s	72°C /60 s	72°C /10	35	12°C /∞
18S	95°C /30 s	60°C /45 s	72°C /60 s	72°C /10	35	12°C /∞
CytB	95°C /30 s	43°C /45 s	72°C /60 s	72°C /10	30	12°C /∞

2.3 Data Analysis

Assemble of two strands were done by using DNA Baser Sequence Assembler (version 5.15.0, Heracle Biosoft, 2017) and CodenCode Aligner software (version 9.0.1, CodonCode Corporation, Dedham, Massachusetts, 2019). Sequences were aligned and trimmed by BioEdit software (Hall, 1999). The species assignments were done by BLAST analysis of the Genebank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BOLDsystem (https://www.boldsystems.org/index.php/IDS_OpenIdEngine).

The COI gene region was used to specify the organisms, and the other markers were used for deep phylogeography. Nucleotide and haplotype diversity (Nei, 1987; Lynch and Crease, 1990) in/between populations were calculated in DNAsp version 5.0 software (Librado & Rozas, 2009). The numbers of haplotypes (Nh) and polymorphic sites (Np), total numbers of mutations (Eta), G+C composition of haplotypes, and parsimony-informative sites were calculated in DNAsp software (v.5.0, Librado & Rozas, 2009). Raggedness indexes based on mismatch distribution (Harpending, 1994) were calculated to detect population size changes (v.5.0, Librado & Rozas, 2009). Tajima's D (Tajima, 1989), Fu's Fs test (1997), Fu and Li's F (Fu & Li, 1993), Fu and Li's D (Fu & Li, 1993) and R_2 test (Ramos-Onsins & Rozas, 2002) were calculated to comprehend if there is a

population expansion or not (DNAsp software, v.5.0, Librado & Rozas, 2009). Genetic differentiation (F_{ST}, Reynolds et al., 1983) and gene flow (Nm) calculations were performed to detect genetic differentiation and gene flow between the populations (v.5.0, Librado & Rozas, 2009).

The intra- and inter-specific distances among the sequences and distance among the haplotypes were calculated using Molecular Evolutionary Genetics Analysis (MEGA **7,** Kumar et al., 2016) software for each marker. The Bayesian tree construction was done using MrBayes 3.2.6 implemented in Geneious Prime using the GTR substitution model (Nst:6), inv gamma rate variation with 1.000.000 iterations (Huelsenbeck & Ronquist, 2011) and it was visualized via FigTree 1.4.3. (Rambaut, 2016). The phylogeographic relationships between the haplotypes were drawn in the Network (v.4.6.1.2) software using the median-joining algorithm (Bandelt et al., 1999).

Besides the present study samples, some database samples from Israel loaded by Israel Oceanographic and Limnological Research Institute were used during the analysis to increase the phylogeographic resolution; *Botrylloides anceps* (MG009581.1; MG009586.1; MG009594.1), *Botrylloides israeliense* (MG009585.1; MG009585.1), *Botrylloides aff. leachii* (MG009593.1), *Botrylloides schlosseri* (MG009595.1).

2.4 Morphological Analysis

Colonies were attached to glass microscope slides (Corning®, 75 x 50mm) during sampling using fine threads removed after adhesion the colonies. Colonies were kept at constant culturing conditions of 22°C and 40 ppt salinity of seawater inside the 5 liters' aquarium. *Nannochloropsis sp*. mixture was used to feed colonies, and clean seawater replaced the present one every two days. After the colonies' attachment, they were cleaned in a weekly period with razor blades and brushes (Karahan et al., 2020).

The blastogenic cycles *of B. anceps* and *Botrylloides sp.* were monitored and recorded between 07.11.2018 - 18.11.2018. The branchial siphon (for water uptake), cloaca (for excretion), stomach, an endostyle, and ampullae (condensed ends of the circulatory system) of the colonies were monitored under the Olympus S16 microscope and photographed using the Olympus DP26 camera. The tentacles located at the brachial siphon were pictured under the Olympus SZX2.

Watanabe's (1953) staging method was followed to determine the blastogenic stages. Under the constant conditions of the aquaculture room, four stages of the cycle were designated according to the following criteria;

Stage A; The adult zooids have an open siphon and common cloaca,

Stage B; Heartbeat of the primary buds which reside at both sides of an adult zooid is visible,

Stage C; Secondary bud formation happens on both sides of primary buds and pigmentation of the primary bud increases,

Stage D; Siphons and common cloaca are closed, and all the adult zooids are absorbed, and the primary buds become new adult zooids, secondary buds become primary buds.

RESULTS

In total, 62 samples were collected from five sampling stations; Konacık (Hatay), Mezitli (Mersin), Kızkalesi (Mersin), Tisan (Mersin), and Alanya (Antalya). Three species of *Botrylloides* genus from these sampling locations were encountered; *Botrylloides anceps* (27 samples), *Botrylloides sp.* (34 samples), and *Botrylloides israeliense* (1 sample). *Botrylloides anceps* samples were collected from Konacık (L1, L3, L4, L5, L6, L7, L8, L10), Mezitli (M2-1, M2-3, M2-4, M2-5, M2-11, M2-16, M2-18 M2-19, P3, P6), and Alanya (C4, C5, C6, C7, C8, C9, C13, C14, C15), *Botrylloides sp.* from Mezitli (F2, F3, F5, F6, F7) and Kızkalesi (BS7, BS9, BSS1, BSS2, BSS3, BSS4, J18-BS5, J18-BS6, J18-BS10, J18-BS11, J18-BS12, Z7, Z17, Z18, GK23, GK28, GK42, 1MBDES, Bides3, Bides4, Bides5, Botryllus1, Botryllus2, 3M, 8M, M4, M5, M6, M7), and *Botrylloides israeliense* from Tisan (G3) (Appendix A.). The details of number of collected samples of species have been given in Table 1.

Table 4. The details about the samples number and PCR amplification success

Species	**Marker/Station**		**COI**		**CytB**		**18S**		**H3**	
	Station		**N**	**SR %**	**N**	**SR %**	**N**	**SR %**	**N**	**SR %**
B. anceps	**T**	**27**	**27**	**100**	**23**	**85**	**16**	**60**	**8**	**30**
	Konacık	8	8	100	7	88	8	100	6	75
	Mezitli	10	10	100	8	80	5	50	1	10
	Alanya	9	9	100	8	89	3	33	1	11
Botrylloides sp.	**T**	**34**	**34**	**100**	**22**	**65**	**17**	**50**	**0**	**0**
	Mezitli	5	5	100	4	80	3	60	0	0
	Kızkalesi	29	29	100	18	62	14	49	0	0
B. israeliense	**T**	**1**	**1**	**100**	**1**	**100**	**1**	**100**	**0**	**0**
	Tisan	1	1	100	1	100	1	100	0	0
	All Total	**62**	62		46		34		8	

T: Total number of the samples collected from each and all stations; N: number of the samples amplified for each marker; SR: PCR amplification Success Rate of each marker for each station and total samples

3.1 Molecular Results

Four genetic markers were used for the molecular analysis of *Botrylloides anceps*, *Botrylloides sp. and B. israeliense* species; Cytochrome oxidase-I (COI), cytochrome B (CytB), 18S, and Histone (H3). The PCR amplification successes of COI for the all three species were recorded as 100%. The H3 amplification was succeed on only for the *B. anceps* species. On the other hand, the ratio of the other markers' changes between zero and 100% (Table 4). Nucleotide composition, haplotypes and PCR product lengths information for the markers were given in Table 5.

Table 5. The details of haplotypes and nucleotide compositions (%) for COI, CytB, 18S and H3.

Species	Markers	Haplotypes	T	C	A	G	bp
Botrylloides anceps	COI	H_{COI-I}	46.41	10.31	25.41	17.86	543
	CytB	H_{CytB-I}	51.35	10.47	21.28	16.89	296
	18S	H_{18S-I}	23.76	22.88	24.17	29.20	1709
	H3	H_{H3-I}	15.45	32.02	22.75	29.78	356
		H_{H3-II}	15.45	31.46	23.32	29.78	356
		H_{H3-III}	15,45	31,74	23,03	29,78	356
Botrylloides sp..	COI	H_{COI-I}	44.85	13.05	20.22	21.88	544
		H_{COI-II}	45.04	12.87	20.22	21.88	544
		$H_{COI-III}$	45.04	12.87	20.40	21.69	544
	CytB	H_{CytB-I}	47.13	13.97	20.70	18.21	401
		$H_{CytB-II}$	47.13	13.97	20.70	18.21	401
		$H_{CytB-III}$	46.88	14.22	20.70	18.21	401
		$H_{CytB-IV}$	47.38	13.72	20.70	18.21	401
	18S	H_{18S-I}	24.18	22.31	24.53	28.98	1708
Botrylloides israeliense	COI	H_{COI-I}	44.85	13.24	19.85	22.06	544
	CytB	H_{CytB-I}	45.14	14.96	20.95	18.95	401
	18S	H_{18S-I}	24.18	22.31	24.53	28.98	1708

bp: base pairs, A: Adenine, G: Guanine; T: Thymine, C: Cytosine

For the phylogeographic analysis; the number of haplotypes, haplotype diversity, the total number of mutations, nucleotide diversity, the number of polymorphic sites, parsimony informative sites, Tajima's D, Fu's Fs statistics, Fu and Li's F, Fu and Li's D, raggedness statistics, Ramos-Onsins and Rozas statistics

were counted for the all sample and each location separately for *B. anceps* (Table 6) and *Botrylloides sp.* (Table 7).

Table 6. Population parameters of North-Eastern Mediterranean populations of *B. anceps* based on the COI, CytB, 18S and H3 gene regions.

B. anceps	**Locations**	**COI**	**cytB**	**18s**	**H3**
Nh	Total	1	1	1	3
	Konacık	1	1	1	3
	Mezitli	1	1	1	-
	Alanya	1	1	1	-
Hd (Sd)	Total	0	0	0	0.607 (0.164)
	Konacık	0	0	0	0.733 (0.155)
	Mezitli	0	0	0	-
	Alanya	0	0	0	-
Eta	Total	0	0	0	2
	Konacık	0	0	0	2
	Mezitli	0	0	0	-
	Alanya	0	0	0	-
Pi (Sd)	Total	0	0	0	0.002 (0.001)
	Konacık	0	0	0	0.003 (0.001)
	Mezitli	0	0	0	-
	Alanya	0	0	0	-
G+C content	Total	0.282	0.274	0.521	0.617
	Konacık	0.282	0.274	0.521	0.616
	Mezitli	0.282	0.274	0.521	-
	Alanya	0.282	0.274	0.521	-
S	Total	0	0	0	2
	Konacık	0	0	0	2
	Mezitli	0	0	0	0
	Alanya	0	0	0	0
Parsimony informative sites	Total	0	0	0	1
	Konacık	0	0	0	1
	Mezitli	0	0	0	-
	Alanya	0	0	0	-
Tajima's D	Total	-	-	-	0.069^{NS}
	Konacık	-	-	-	-0.612^{NS}
	Mezitli	-	-	-	-
	Alanya	-	-	-	-
Fu's Fs statistics	Total	-	-	-	-0.224^{NS}
	Konacık	-	-	-	0.172^{NS}
	Mezitli	-	-	-	-
	Alanya	-	-	-	-

Table 6 (cont'd)

Fu and Li' D	Total	-	-	-	-0.149^{NS}
	Konacık	-	-	-	-0.612^{NS}
	Mezitli	-	-	-	-
	Alanya	-	-	-	-
Fu and Li' F	Total	-	-	-	-0.108^{NS}
	Konacık	-	-	-	-0.479^{NS}
	Mezitli	-	-	-	-
	Alanya	-	-	-	-
r	Total	-	-	-	0.096^{NS}
	Konacık	-	-	-	0.222^{NS}
	Mezitli	-	-	-	-
	Alanya	-	-	-	-
R2 statistic	Total	-	-	-	0.213^{NS}
	Konacık	-	-	-	0.239^{NS}
	Mezitli	-	-	-	-
	Alanya	-	-	-	-

Number of polymorphic sites (S), number of haplotypes (Nh), total number of mutations (Eta), nucleotide diversity (Pi), haplotype diversity (Hd), raggedness statistics (r), Ramos-Onsins and Rozas statistics (R^2), standard deviation (Sd), statistically not important (^{NS}).

The North eastern Mediterranean costs *Botrylloides sp.* samples were represented by only one haplotype (H_{18S-I}) of 18S marker; thus, Tajima's D, Fu's Fs statistics, Fu and Li's D, Fu and Li's F statistics, Raggedness statistics, R2 statistics, genetic differentiation (F_{ST}) and gene flow (Nm) calculations between the sampling areas could not be assessed for the total, Mezitli, and Kızkalesi samples (Table 7).

Table 7. Population parameters of North-Eastern populations of *Botrylloides sp.* based on the COI, CytB and 18S gene regions.

Botrylloides sp.	**Locations**	**COI**	**CytB**	**18s**
Nh	Total	3	4	1
	Mezitli	3	2	1
	Kızkalesi	2	3	1
Hd (Sd)	Total	0.266 (0.092)	0.680 (0.073)	0
	Mezitli	0.700 (0.218)	0.667 (0.204)	0
	Kızkalesi	0.069 (0.063)	0.647 (0.069)	0
Eta	Total	2	3	0
	Mezitli	2	1	0
	Kızkalesi	1	2	0
Pi (Sd)	Total	0.001(0)	0.002 (0)	0
	Mezitli	0.002 (0.001)	0.002 (0.001)	0
	Kızkalesi	0	0.002 (0)	0

Table 7 (cont'd)

G+C content	Total	0.349	0.322	0.513
	Mezitli	0.347	0.320	0.513
	Kızkalesi	0.349	0.323	0.513
S	Total	2	3	0
	Mezitli	2	1	0
	Kızkalesi	1	2	0
Parsimony informative sites	Total	1	3	0
	Mezitli	0	1	0
	Kızkalesi	0	2	0
Tajima's D	Total	-0.694^{NS}	0.325^{NS}	-
	Mezitli	-0.973^{NS}	1.633^{NS}	-
	Kızkalesi	-1.149^{NS}	1.016^{NS}	-
Fu's Fs statistics	Total	-0.725^{NS}	-0.085^{NS}	-
	Mezitli	-0.829^{NS}	0.540^{NS}	-
	Kızkalesi	-1.183^{NS}	0.691^{NS}	-
Fu and Li' D	Total	-0.778^{NS}	0.992^{NS}	-
	Mezitli	-0.973^{NS}	1.633^{NS}	-
	Kızkalesi	-1.671^{NS}	0.885^{NS}	-
Fu and Li'F	Total	-0.872^{NS}	0.930^{NS}	-
	Mezitli	-0.954^{NS}	1.277^{NS}	-
	Kızkalesi	-1.757^{NS}	1.053^{NS}	-
r	Total	0.300^{NS}	0.118^{NS}	-
	Mezitli	0.350^{NS}	0.556^{NS}	-
	Kızkalesi	0.748^{NS}	0.131^{NS}	-
R^2 statistics	Total	0.106^{NS}	0.154^{NS}	-
	Mezitli	0.245^{NS}	0.333^{NS}	-
	Kızkalesi	0.183^{NS}	0.206^{NS}	-
Fst	Kızkalesi-Mezitli	0.556***	0.361*	-
Nm	Kızkalesi-Mezitli	0.400	0.880	-

Number of polymorphic sites (S), number of haplotypes (Nh), total number of mutations (Eta), nucleotide diversity (Pi), haplotype diversity (Hd), Raggedness statistics (r), Ramos-Onsins and Rozas statistics (r), genetic differentiation (Fst), gene flow (Nm) (If $Nm < 1$= very limited gene flow; If $Nm > 1$= enough gene flow to neglect the genetic drift; and if $Nm > 4$= randomly mating local populations), standard deviation (Sd), statistically not important (^{NS}), statiscally important (*); (P 0.01<P<0.05: *), (0.001<P < 0.01: **), (P < 0.001: ***).

3.1.1 Cytochrome Oxydase-I (COI)

3.1.1.1 COI- *Botrylloides anceps* (Herdman, 1891)

All of the *Botryllodies anceps* samples (27) have been sequenced for the COI gene region. After alignment and trimming, the total length was 543 base pairs. Only one haplotype (H_{COI-I}) was recorded for the marker. Nucleotide composition of H_{COI-I} was calculated as 46.41% (T); 10.31% (C); 25.41% (A); 17.86% (G) (Table 5).

The haplotype diversity was calculated as zero. There was no polymorphism for the COI gene region within/between Konacık, Mezitli, and Alanya populations, the number of mutations and the number of the polymorphic sites, parsimony informative sites and nucleotide diversity was also calculated as zero. The G+C content for the COI gene fragment was recorded as 0.282 (Table 6).

Because of having only one haplotype, the genetic distance between the populations (Konacık, Mezitli, and Alanya) was calculated as zero. The present sample sequences were also compared with a *B. anceps* record from the Israel coasts (MG009581.1). According to this analysis, five mutation steps differences were observed between the haplotypes/samples (Figure 7), and the genetic distance was calculated as 1.1%. All *B. anceps* records of the present study and Israeli record were clustered together in the Bayesian tree with a 100% bootstrap probability (Figure 8). According to the Bayesian tree, the present study haplotype was found more ancestral than the Israeli samples (Figure 8).

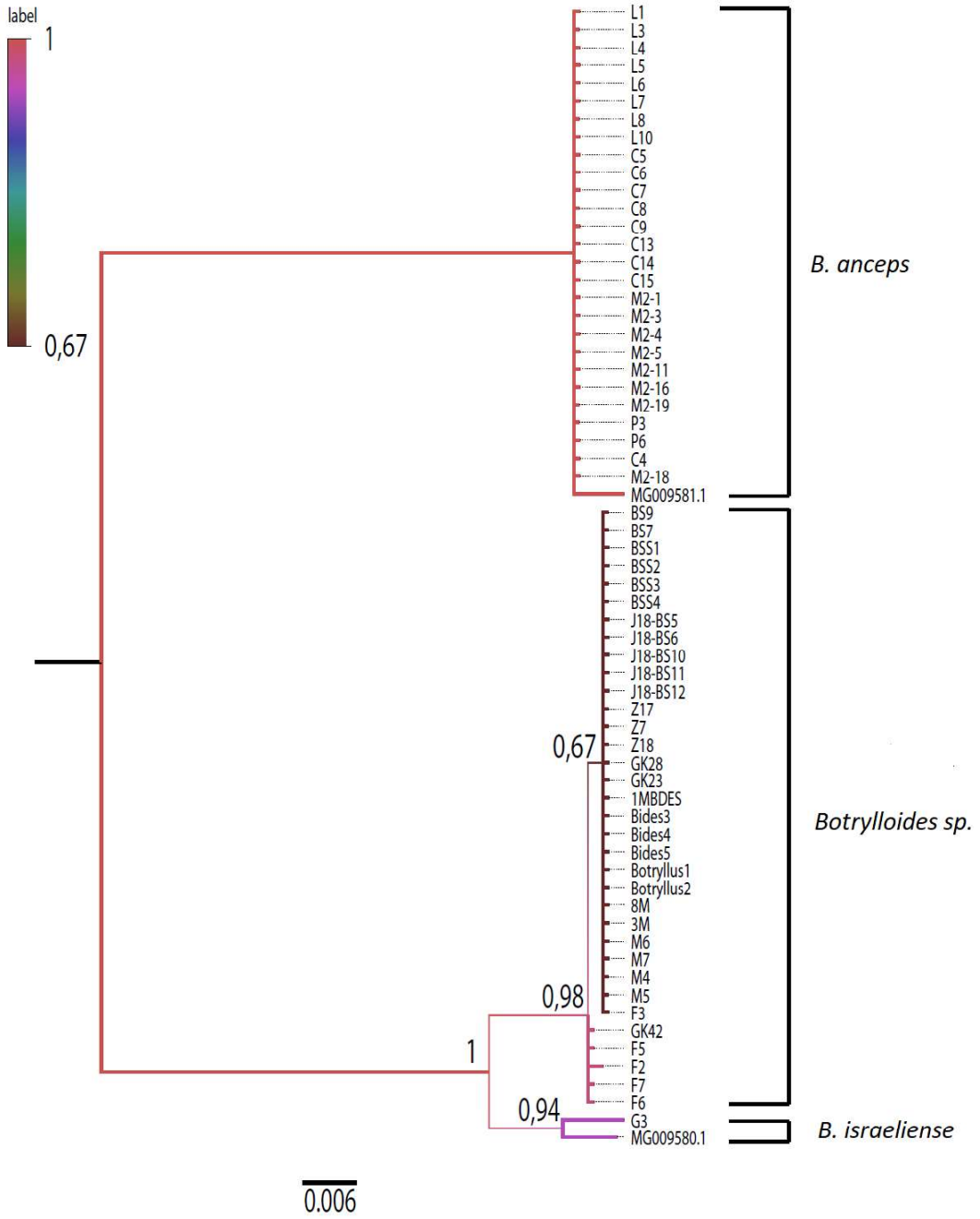


Figure 7. The Bayesian tree *of B. anceps*, *Botrylloides sp.* and *B. israeliense* species/haplotypes based on the COI gene fragment. 1.000.000 bootstrap replicates were run using the GTR susbsitution model and inv gamma rate variation. The scale bar represents the distance of 0.006 nucleotide substitution. Colors represent the bootstrap probability values. Bootstrap supports were given on the nodes. Accession numbers are given for the species obtained from database.

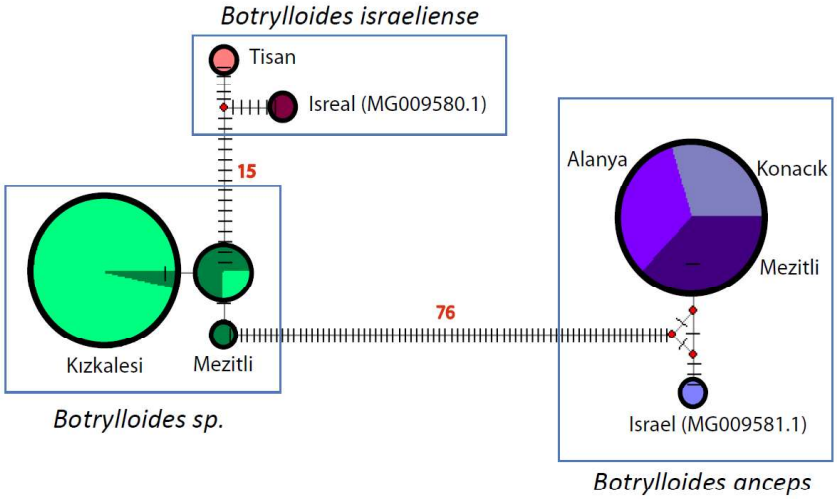


Figure 8. Median Joining Network analysis of *B. anceps, Botrylloides sp.* and *B. israeliense* species/haplotypes based on the COI gene fragment. Each circle represents a haplotype. Sample sizes are proportional to pie magnitudes. Colors represent different species and color tints represent the subpopulations. Purple: *B. anceps*; Green: *Botrylloides sp.*; Claret red: *B. israeliense*. Red dots are median vectors. The lines represent the mutation steps between haplotypes. Red numbers indicate numbers of mutation steps. The accession numbers of the database samples were written with the location.

3.1.1.2 COI- *Botrylloides sp.*

Amplification of the COI gene region was managed with 100% success, and the total length was 542 bp after trimming. In total, three haplotypes (H_{COI-I}, H_{COI-II}, $H_{COI-III}$) were recorded. Mean nucleotide compositions of the haplotypes were calculated as 44.98% (T); 12.93% (C); 20.28% (A); 21.82% (G) (Table 5).

The pairwise genetic distances between the haplotypes were calculated as; 0.002 (H_{COI-I}-H_{COI-II}); 0,004 (H_{COI-I}-$H_{COI-III}$); 0.002 (H_{COI-II}-$H_{COI-III}$). Haplotype diversities for total, Mezitli and Kızkalesi samples were calculated as 0.266; 0.700; 0.069, respectively. The number of haplotypes, total number of mutations and the number of polymorphic sites were calculated as 3 (Nh), 2 (Eta), 2 (S) for all samples and Mezitli samples and 2 (Nh), 1 (Eta), 1 (S) for Kızkalesi samples. Parsimony informative site was 1 in all samples and 0 in both Mezitli and Kızkalesi samples.

The mean G+C content of all haplotypes was calculated as 0.349. Nucleotide diversity for all, Mezitli and Kızkalesi samples were recorded as 0.001; 0.002; 0.0001, respectively. Tajima's D, Fu and Li's D, Fu and Li's F, Fu's Fs, R2 statistics values for total samples were calculated as -0.694, -0.778 -0.872, -0.725, 0.106 respectively and the values were no significant (Table 7). Tajima's D, Fu and Li's D, Fu and Li's F, Fu's Fs, R2 statistics values for Kızkalesi station were calculated as -1.149, -1.671, -1.757, -1.183, 0.183, respectively and the values were not found significant also (Table 9). Tajima's D, Fu and Li's D, Fu and Li's F, Fu's Fs, R2 statistics values for Mezitli samples were calculated as -0.973, -0.973, -0.954, -0.829, 0.245, respectively and with no significant probability (Table 7).

Only one and two mutation steps were recorded between the haplotypes (Figure 7). Both H_{COI-I} and H_{COI-II} haplotypes were encountered at Mezitli and Kızkalesi sampling stations while $H_{COI-III}$ was encountered only in Mezitli station (Figure 7). The Pairwise genetic distance between the Kızkalesi and Mezitli samples was found to be 0.2% (Table 8). According to the Bayesian tree *Botryllodes sp.* haplotypes were clustered on different branches with 67% (H_{COI-I}) and 98% (H_{COI-II} and $H_{COI-III}$) supports (Figure 8).

Table 8. The genetic distances (%) between *Botrylloides sp.* and *B. israeliense* for the COI, 18S and H3 marker based on Kimura-2 parameter distance model.

Marker/ Region/ Species		**COI**			**18S**			**CytB**	
		Botrylloides sp.		***B. israeliense***	***Botrylloides sp.***		***B. israeliense***	***Botrylloides sp.***	
		K	**M**	**T**	**K**	**M**	**T**	**K**	**M**
Botrylloides sp.	**M**	0.002	-	-	0.000	-	-	0.003	-
B. israeliense	**T**	0.046	0.045	-	0.000	0.000	-	0.062	0.062
B. israeliense	**I**	0.048	0.047	0.025	0.024	0.024	0.024	-	-

KK; Kızkalesi, M; Mezitli, T; Tisan, I; Israel

Genetic differentiation (F_{ST}) and gene flow (Nm) values between the Kızkalesi and Mezitli samples were calculated as 0.556 and 0.400 (Table 7) Raggedness statistics for populations size change were recorded to be 0.300, 0.748

and 0.350 for all, Kızkalesi, and Mezitli samples, respectively (Table 7). All the mismatch graphs of *Botrylloides sp.* for COI were unimodal, implying recent population expansion for all samples, Kızkalesi, and Mezitli. However, the null hypothesis of population expansion was rejected because of the higher raggedness values (r > 00.4) referring to no population expansion for *Botryllodies sp.* samples (Figure 9).

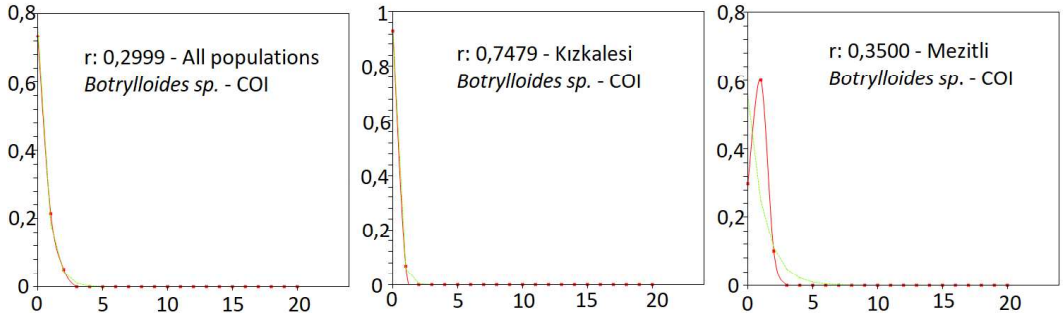


Figure 9. The mismatch distribution graphs of the COI for Kızkalesi, Mezitli and all samples of *Botrylloides sp.* r: Raggedness statistic values. The X-axis: the number of pairwise differences, while the Y-axis: frequency. The red line: observed frequency for stable population size, the green line: expected frequency.

3.1.1.3 COI- *Botrylloides israeliense* Brunetti, 2009

A total of 542 bp were used for the analysis after alignment and trimming for the COI. Nucleotide compositions were calculated as 44.85% (T); 13.24% (C); 19.85% (A); 22.06% (G) (Table 5).

Tisan sample gave a 97.59% match with the *Botrylloides israeliense* (MG009580.1) sample from Israel provided in the GenBank. The genetic distance between the Tisan and Israel samples was calculated as 2.47% in MEGA software (Table 8). The pairwise distances between *Botrylloides israeliense* sample (Tisan) and *Botrylloides sp.* haplotypes were calculated as 4.6% (H_{COI-I}), 4.4% (H_{COI-II}) and 4.6% ($H_{COI-III}$).

Inter-specific distances: In total, 78-80 mutation steps differences were recorded between the *B. anceps* and *Botrylloides sp.* haplotypes and around 100

mutation steps between the *B. anceps* and *Botryllodies israeliense* species of the present study (Figure 7). Twenty and twenty-one mutation steps were counted between the *Botrylloides sp.* haplotypes and *Botryllodies israeliense* sample from Tisan and Israel (MG009580.1) (Figure 7). *Botrylloides israeliense* (Tisan) gave 4.6% and 4.5% genetic distances, respectively with the Kızkalesi and Mezitli samples of *Botrylloides sp.* (Table 8). The genetic distance between the Kızkalesi-Mezitli *Botrylloides sp.* samples with B. *israeliense* (Israel) was recorded as 4.8% and 4.7%, respectively (Table 8).

3.1.2 Cytochrome Oxydase-B (CytB)

3.1.2.1 CytB- *Botrylloides anceps*

A total of 23 sequences were acquired for the CytB marker. The final length of the CytB gene fragments was 296 bp. One sample from Konacık, one sample from Alanya, and two samples from Mezitli areas failed to be amplified for the marker. Only one haplotype (H_{CytB-I}) was recorded. Nucleotide composition of H_{CytB-I} was calculated as 51.35% (T); 10.47% (C); 21.28% (A); 16.89% (G) (Table 5).

The haplotype diversity, total number of mutations, number of polymorphic sites, parsimony informative sites, and the nucleotide diversity was calculated as zero, because of the having only one haplotype from Konacık, Mezitli and, Alanya areas (Figure 10). As a result, no polymorphism and genetic distance could be recorded within/between sampling areas. Besides this, no record was able to mine from the databases; thus, no comparison could able to make at this perspective. The G+C content was calculated as 0.274 (Table 6). All the *B. anceps* samples of the present study were clustered in one pie chart at the Network (Figure 9) analysis and in one branch on the Bayesian tree with the support of 100% bootstrap probability (Figure 11).

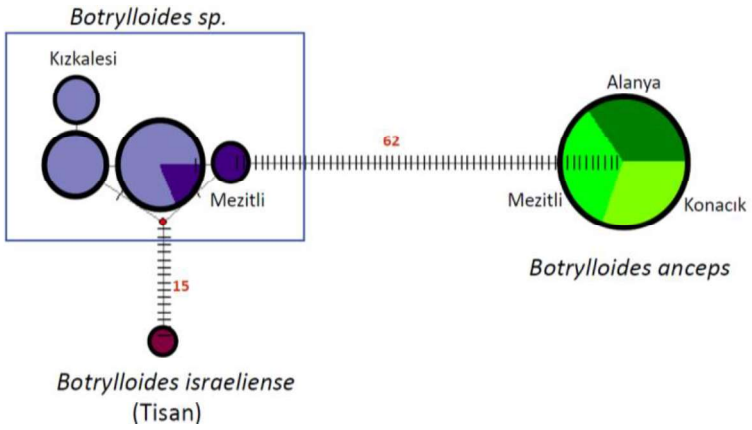


Figure 10. Median Joining Network analysis of *B. anceps, Botrylloides sp.* and *B. israeliense* species/haplotypes based on the CytB gene fragment. Each circle represents a haplotype. Sample sizes are proportional to pie magnitudes. Colors represent different species and color tints represent the subpopulations. Green: *B. anceps*; Purple: *Botrylloides sp.*; Claret red: *B. israeliense*. Red dots are median vectors. The lines represent mutation steps between haplotypes. Red numbers indicate numbers of mutation steps.

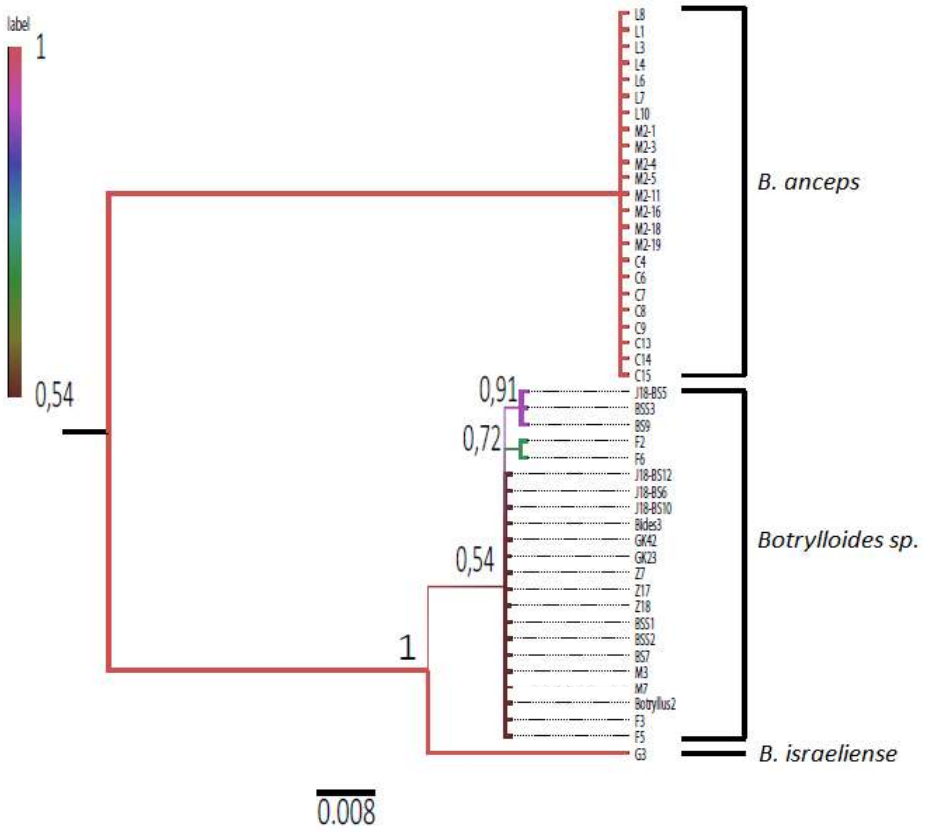


Figure 11. The Bayesian tree of *Botrylloides anceps, Botrylloides sp.* and *B. israeliense* species/haplotypes based on the CytB gene fragment. 1.000.000 bootstrap replicates were run using the GTR susbsitution model and inv gamma rate variation. The scale bar represents the distance of 0.008 nucleotide substitution. Colors represent the bootstrap probability values. Bootstrap supports were given on the nodes. Accession numbers are given for the species obtained from database.

3.1.2.2 CytB- *Botrylloides sp.*

In total, twenty-two sequences were acquired for the CytB marker. Eleven samples out of twenty-nine from the Kızkalesi region and one sample out of five from the Mezitli region failed to be amplified for the marker. After alignment and trimming, the total length of the CytB gene fragments were 401 bp, and four haplotypes (H_{CytB-I}, $H_{CytB-II}$, $H_{CytB-III,}$ $H_{CytB-IV}$) were observed. Mean nucleotide compositions of the haplotypes were calculated as 47.13% (T); 13.97% (C); 20.70% (A); 18.21% (G) (Table 5).

The pairwise genetic distances between the haplotypes were calculated as 0.005 (H_{CytB-I}-$H_{CytB-II,}$); 0.003 (H_{CytB-I}-$H_{CytB-III,}$); 0.003 (H_{CytB-I}-$H_{CytB-IV}$); 0.003 ($H_{CytB-II}$-$H_{CytB-III,}$); 0.008 ($H_{CytB-II}$-$H_{CytB-IV}$); 0.005 ($H_{CytB-III}$-$H_{CytB-IV}$). Haplotype diversity was calculated 0.680 for total samples and 0.667 for both Mezitli and Kızkalesi samples. There were 4 haplotypes in total samples, 2 haplotypes in Mezitli and 3 haplotypes in Kızkalesi (Table 7). Total number of mutations, the number of polymorphic sites and parsimony informative sites were calculated as 3 (Eta), 3 (S), and 3 for all the samples; 1 (Eta), 1 (S), and 1 for Mezitli samples and 2 (Eta), 2 (S), and 2 for the Kızkalesi samples, respectively. The mean G+C content of all haplotypes were calculated as 0.322. The nucleotide diversity was recorded as 0.002 for all the locations. Tajima's D, Fu and Li's D, Fu and Li's F, Fu's Fs, R2 statistics for all samples were calculated as 0.325, 0.992, 0.930, -0.085 and 0.154, respectively, with no significance P value. Tajima's D, Fu and Li's D, Fu and Li's F, Fu's Fs, R2 statistic values for the Kızkalesi samples were calculated as 1.016, 0.885, 1.053, 0.691 and 0.206 respectively and the values were not significant. Tajima's D, Fu and Li's D, Fu and Li's F, Fu's Fs, R2 statistic values for the Mezitli samples were calculated as 1.633, 1.633, 1.277, 0.540 and 0.333, respectively, without any significance P value (Table 7). One to four mutation steps between the CytB haplotypes of *Botrylloides sp.* were recorded (Figure 9). H_{CytB-I} haplotype was encountered in both Kızkalesi and Mezitli locations, $H_{CytB-II}$ and $H_{CytB-III}$ only in Kızkalesi, and $H_{CytB-IV}$ only in Mezitli (Figure 10) areas. The pairwise genetic

distance between the Kızkalesi and Mezitli samples were calculated as 0.3% Table 8.

According to the Bayesian tree three branches were formed for *Botryllodies sp.* sequences. The first branch was consisted of H_{CytB-I} and $H_{CytB-III}$ with support of 54% bootstrap probability. The second branch was formed with $H_{CytB-IV}$ with 73% bootstrap probability. The last branch with 91% support consisted of $H_{CytB-II}$ (Figure 11).

The genetic differentiation between Kızkalesi and Mezitli samples and gene flow were calculated; F_{ST} = 0.361, Nm = 0.880. Raggedness statistics (r) for population size change were found as 0.118, 0.132 and 0.556 for all, Kızkalesi and Mezitli samples, respectively for the CytB gene region (Table 7). All the graphs have shown unimodal mismatch distribution, which could be a sign of recent population expansion; however, raggedness values (r > 00.4) didn't support the population expansion of all, Kızkalesi and Konacık samples (Figure 12).

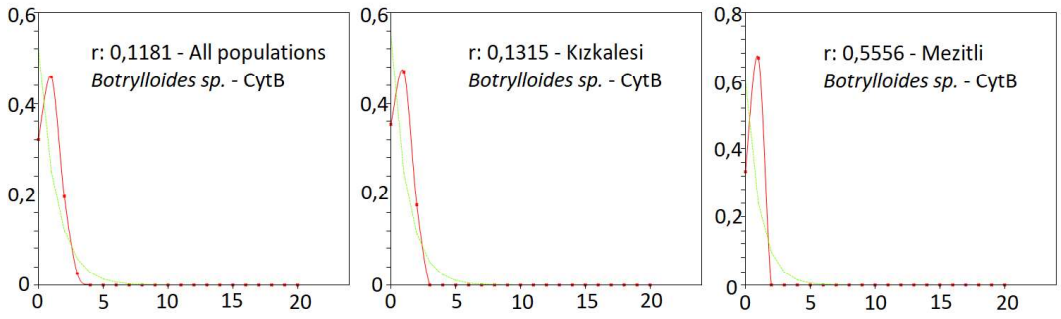


Figure 12. The mismatch distribution graphs of the CytB for Kızkalesi, Mezitli and all populations of *Botrylloides sp.* r: Raggedness statistic values. The X-axis: the number of pairwise differences, while the Y-axis: frequency. The red line: observed frequency for stable population size, the green line: expected frequency.

3.1.2.3 CytB- *Botrylloides israeliense*

The CytB gene fragments were aligned and trimmed to 401 bp. The nucleotide compositions were calculated as 45.14% (T); 13.24% (C); 19.85% (A); 22.06% (G) (Table 5). No data was mined from the databases for the species CytB region, because of that no analysis has been done at this perspective.

Inter-specific distances: In total, 62-66 mutations steps were counted the between *Botrylloides anceps* and *Botrylloides sp.* and 78 between the *Botrylloides anceps* and *Botrylloides israeliense* species (Figure 10). *Botrylloides sp.* haplotypes and *Botrylloides israeliense* (Tisan) separated with 16-17 mutation steps in the network analysis (Figure 9). The genetic distance between *Botrylloides sp.* and *B. israeliense* (Tisan) was 6.2% for both Kızkalesi and Mezitli (Table 8).

The pairwise genetic distance between *Botrylloides israeliense* (Tisan) and *Botrylloides sp.* haplotypes were calculated as 6.3% (HI); 6.3% (HII); 6.0% (HIII); 6.0 (HIV) for the CytB gene region. *Botrylloides israeliense* (Tisan) distanced 6.2% from *Botrylloides sp.* samples collected from Kızkalesi and Mezitli (Table 8).

3.1.3 18S

3.1.3.1 18S- *Botrylloides anceps*

In total, 16 *B. anceps* samples sequences were obtained from the amplification of the 18S gene region. After alignment and trimming, the total length was 1709 bp. All the Konacık samples have been amplified for the marker. However, only three samples out of nine were amplified from Alanya station, and only five samples out of ten were able to be amplified from Mezitli station. There was also one haplotype ($H_{18S\text{-}I}$) for the 18S marker. Nucleotide composition of $H_{18S\text{-}I}$ was calculated as 23.76% (T); 22.88% (C); 24.17% (A); 29.20% (G) (Table 5).

Haplotype diversity was calculated as zero because of detecting only one haplotype for the Konacık, Mezitli, and Alanya samples (Figure 11). There wasn't any polymorphism for the 18S gene region within/between sampling areas, as result of this; the total number of mutations, the number of polymorphic sites, parsimony informative sites, nucleotide diversity and the genetic distance was calculated as zero. The G+C content for the CytB gene fragment was recorded as 0.521 (Table 6).

No mutation was recorded between the Turkey and Israeli (MG009586.1) samples (Figure 13). Thus, Konacık, Mezitli, Alanya, and Israel samples were located in the same pie-chart at the Network and with 100% bootstrap support on the same branch at the Bayesian tree (Figure 13-14).

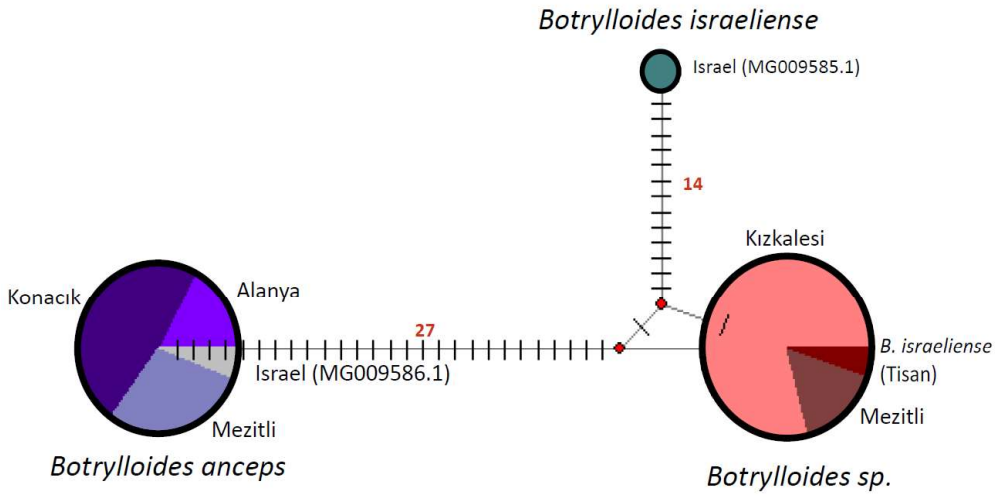


Figure 13. Median Joining Network analysis of *B. anceps, Botrylloides sp.* and *B. israeliense* species/haplotypes based on the 18S gene fragment. Each circle represents a haplotype. Sample sizes are proportional to pie magnitudes. Colors represent different species and color tints represent the subpopulations. Purple: *B. anceps*; Pink: *Botrylloides sp.*; Green: *B. israeliense*. Red dots are median vectors. The lines represent mutation steps between haplotypes. Red numbers indicate numbers of mutation steps. The accession numbers of the database samples were written with the location.

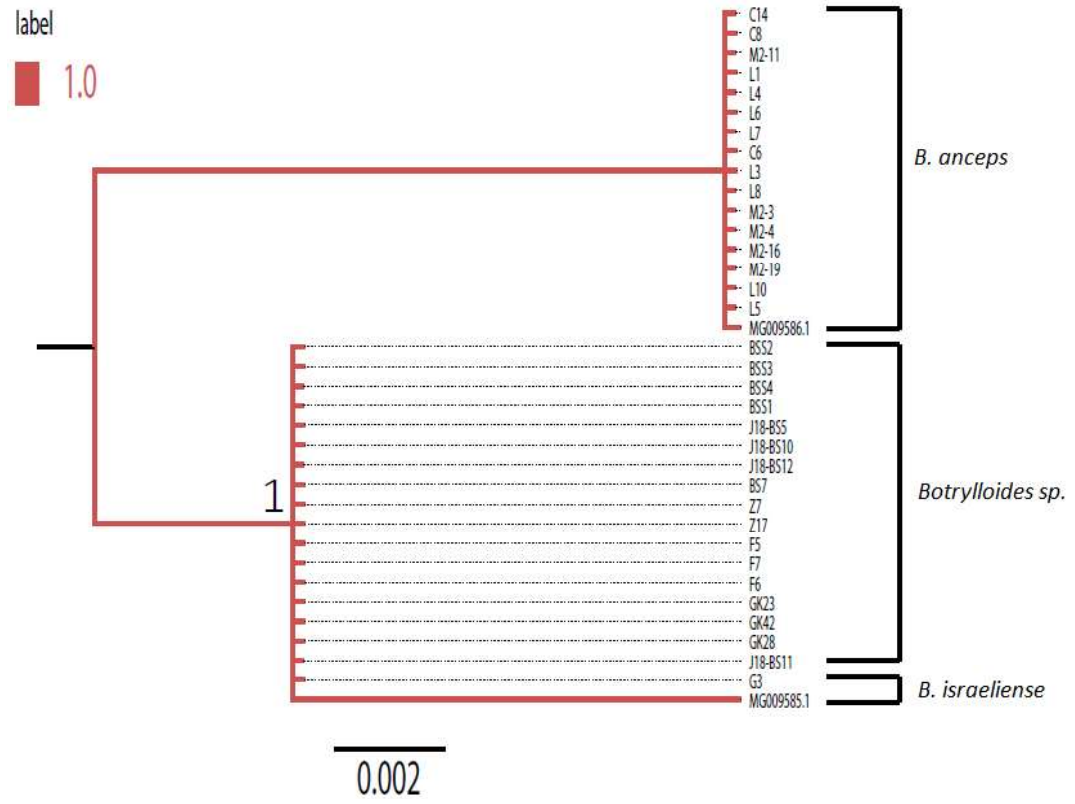


Figure 14. The Bayesian tree of *B. anceps, Botrylloides sp.* and *B. israeliense* species/haplotypes based on the 18S gene fragment. 1.000.000 bootstrap replicates were run using the GTR susbsitution model and inv gamma rate variation. The scale bar represents the distance of 0.002 nucleotide substitution. Colors represent the bootstrap probability values. Bootstrap supports were given on the nodes. Accession numbers are given for the species obtained from database.

3.1.3.2 18S- *Botrylloides sp.*

Seventeen sequences were obtained from the amplification of the 18S gene region in total. Fifteen samples out of twenty-nine from the Kızkalesi and two samples out of five from the Mezitli areas failed to be amplified for the marker. After alignment and trimming, the total length of the 18S gene fragments was counted as 1708 bp. Only one haplotype ($H_{18S\text{-}I}$) was recorded. Nucleotide compositions were calculated as 24.18% (T), 22.31% (C), 24.53% (A), 28.98% (G) (Table 5).

Haplotype diversity was calculated zero because there was only one haplotype observed (Table 7). No polymorphism was observed for the 18S gene region within/between Mezitli and Kızkalesi samples and total number of mutations,

the number of polymorphic sites and parsimony informative sites were zero (Table 7). Nucleotide diversity was calculated as zero. The G+C content for the 18S gene fragment was calculated as 0.513. According to the network analysis, Kızkalesi and Mezitli samples were not separated by any mutation and they consisted one haplotype ($H_{18S\text{-}I}$) (Figure 13). Thus, the genetic distance was calculated as zero between Kızkalesi and Mezitli samples of *Botrylloides sp.* for the 18S (Table 8).

All *Botrylloides sp.* sequences from Mezitli and Kızkalesi, *B. israeliense* (Tisan) and *B. israeliense* (Israel-MG009585.1) clustered together with 100% bootstrap probability (Figure 14). According to Bayesian tree, *Botrylloides sp.* was found more ancestral than *Botrylloides israeliense* (Israel).

3.1.3.3 18S- *Botrylloides israeliense*

After alignment and trimming, the 18S gene fragments were reduced to 1708 bp. Nucleotide compositions were calculated as 24.18% (T); 22.31% (C); 24.53% (A); 28.98% (G) (Table 5.). The genetic distance between *B. israeliense* (Tisan) and *B. israeliense* (Israel-MG009585.1) mined from GenBank were found to be 2.4% (Table 12), while no genetic distance (0%) were found between *Botrylloides israeliense* (Tisan) and *Botylloides sp.* samples collected from Kızkalesi and Mezitli for the 18S gene region and sequences of *Botrylloides sp*. and *B. israeliense* (Tisan) all belonged to the same haplotype.

Inter-specific distances: Mutation steps were calculated as 28 between *B. anceps* (Turkey) and *Botrylloides sp.* (Turkey), as 42 between *B. anceps* (Turkey) and *Botrylloides israeliense* (Israel-MG009585.1), and as 28 between the *B. anceps* and *Botrylloides israeliense* (Tisan) (Figure 13).

Also, mutation steps between *Botrylloides sp.* and *B. israeliense* samples of this study for the 18S marker was calculated as zero. On the other hand, 15 mutation steps were recorded between the Turkey coast *Botryllodies sp.* sample with Israeli

Botrylloides israeliense (MG009585.1) (Figure 13). The genetic distances between the Kızkalesi and Mezitli *Botrylloides sp.* samples' with Tisan *B. israeliense* were calculated as zero; on the other hand, it was recorded as 2.4% between the Kızkalesi and Mezitli *Botrylloides sp.* samples' with Israeli *B. israeliense,* for the marker (Table 8).

3.1.4 Histone 3 (H3)

3.1.4.1 H3- *Botrylloides anceps*

In total, 8 sequences were obtained from the amplification of the H3 gene region. Two samples (L5, L10) failed to be amplified from the Konacık station. On the other hand, only one sample (M2-19) out of ten from Mezitli, and one (C14) out of nine from Alanya stations have been amplified for the H3 marker. After alignment and trimming the final length was 356 bp. Three haplotypes ($H_{H3\text{-}I}$, $H_{H3\text{-}II}$, $H_{H3\text{-}III}$) were calculated for the marker. The mean nucleotide composition of these three haplotypes was calculated as 15.45% (T); 31.74% (C); 23.03% (A); 29.78% (G). The nucleotide compositions for each haplotype were presented in Table 5.

Out of all the markers used in this study, polymorphism was observed only in the H3 gene region for *Botrylloides anceps* species. Three haplotypes ($H_{H3\text{-}I}$, $H_{H3\text{-}II}$, $H_{H3\text{-}III}$) were recorded, with one and two mutation steps between, and the pairwise distances between the haplotypes were calculated as 0.006 ($H_{H3\text{-}I}$-$H_{H3\text{-}II}$), -0.003 ($H_{H3\text{-}I}$-$H_{H3\text{-}III}$), 0.003 ($H_{H3\text{-}II}$-$H_{H3\text{-}III}$). Whereas all the haplotypes ($H_{H3\text{-}I}$, $H_{H3\text{-}II}$, $H_{H3\text{-}III}$) were encountered in the Konacık region, only $H_{H3\text{-}I}$ was recorded in Mezitli and Alanya regions (Figure 15).

Haplotype diversity was calculated as 0.607 for all samples, and as 0.733 for the Konacık samples. The total number of mutations, the number of polymorphic sites, and parsimony-informative sites were calculated as 2 (Eta), 2 (S), and 1 respectively for both all and Konacık region samples. The mean G+C content of all

haplotypes was calculated as 0.617 for all samples. Nucleotide diversity has been recorded as 0.002 and 0.003 for the all and Konacık station samples, respectively.

Konacık was the only station with more than one H3 haplotypes; thus, statistics were calculated just for the Konacık samples. The Tajima's D, Fu and Li's D, Fu and Li's F, Fu's Fs, Raggedness and R2 statistics were calculated as -0.612; -0.612; -0.479; 0.172; 0.222; 0.239, respectively, and the values were not found significantly important. On the other hand, these values were calculated for the total samples as 0.069; -0.149; -0.108; -0.224; 0.096; 0.213, respectively, with also not significant statistical value (Table 6).

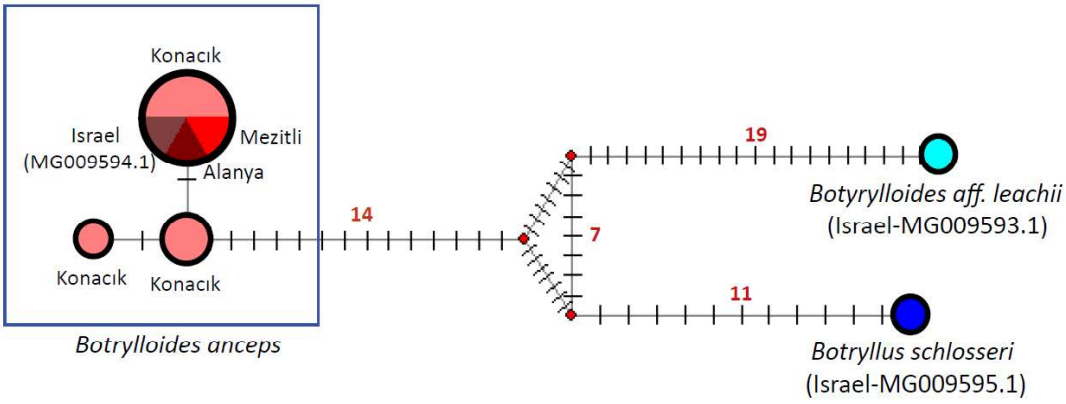


Figure 15. Median Joining Network analysis of *B. anceps* species/haplotypes based on the H3 gene fragment. Each circle represents a haplotype. Sample sizes are proportional to pie magnitudes. Colors represent different species and color tints represent the subpopulations. Pink: *B. anceps*; Blue: *B. schlosseri*; Mint: *B. aff. leachii*. Red dots are median vectors. The lines represent the mutation steps between haplotypes. Red numbers indicate numbers of mutation steps. The accession number of the database samples was written with the location.

The pairwise genetic distances between the Konacık and Mezitli-Alanya-Israel samples was calculated as 0.2% and zero between the Mezitli-Alanya, and Israel samples (Table 9).

Table 9. The pairwise genetic distances (%) of *B. anceps* for the H3 gene region based on the Kimura-2 parameter distance model.

Stations	**Mezitli**	**Konacık**	**Alanya**
Konacık	0,.02	-	-
Alanya	0.000	0.002	-
Israel-MG009594.1	0.000	0.002	0.000

Because of failing to amplify the H3 gene region for the *Botrylloides sp*. and *Botrylloides israeliense* samples, H3 records of *Botrylloides aff. leachii* and *Botryllus schlosseri* from Israel were used as outgroups to increase the resolution of Network and Bayesian tree (Figure 15). In the Network analysis, the Israel sample was clustered in the $H_{H3\text{-}I}$ (Figure 13) together with the other Turkey samples and two Konacık haplotypes ($H_{H3\text{-}II}$ and $H_{H3\text{-}IIII}$) clustered as private (Figure 15). In the Bayesian tree, *B. anceps* samples were clustered into two branches with 99% bootstrap support (Figure 16). The first cluster consisted of $H_{H3\text{-}II}$ (L3, L4) and $H_{H3\text{-}III}$ (L1) haplotypes from the Konacık and was supported with 57% bootstrap (Figure 16). The second cluster consisted of $H_{H3\text{-}I}$ from Konacık (L6, L7, L8), Mezitli (M2-19), Alanya (C14), and Israel (MG009594.1) samples, and bootstrap support was recorded as 52% (Figure 16).

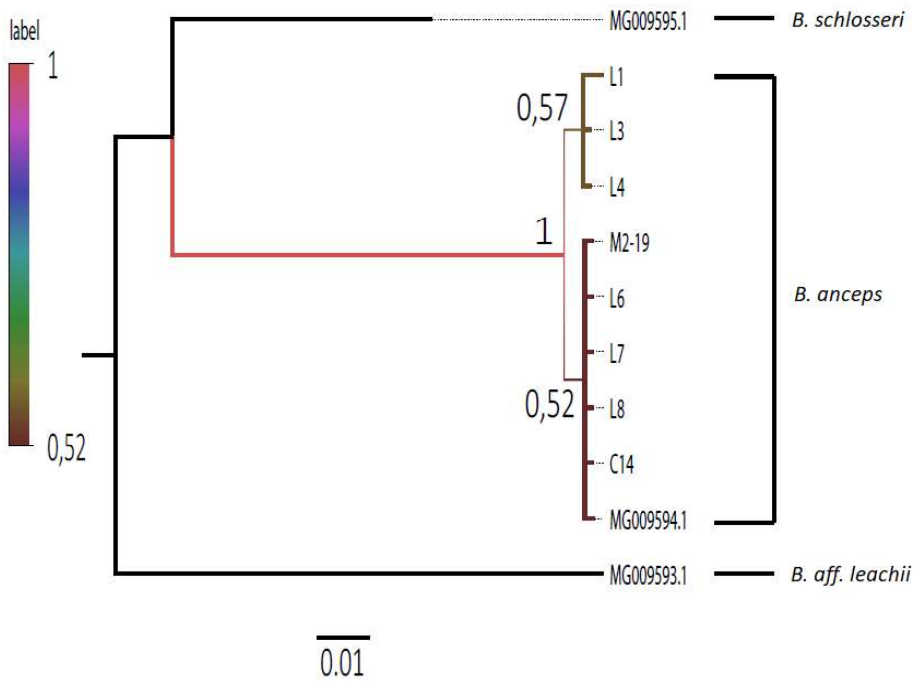


Figure 16. The Bayesian tree of *B. anceps* species/haplotypes based on the H3 gene fragment. 1.000.000 bootstrap replicates were run using the GTR susbsitution model and inv gamma rate variation. The scale bar represents the distance of 0.01 nucleotide substitution. Colors represent the bootstrap probability values. Bootstrap supports were given on the nodes. Accession numbers are given for the species obtained from database.

The gene flow (Nm) between the Konacık, Mezitli, and Alanya samples could not be calculated due to a lack of haplotype diversity. Raggedness statistics (r) for population size change were found to be 0.096 and 0.222 for the all and Konacık samples, respectively (Table 6). Both all samples and Konacık samples resulted in unimodal mismatch distribution which shows a recent population expansion; however, the raggedness values were above the threshold (00.4) value which rejected the null hypothesis of population expansion (Figure 17).

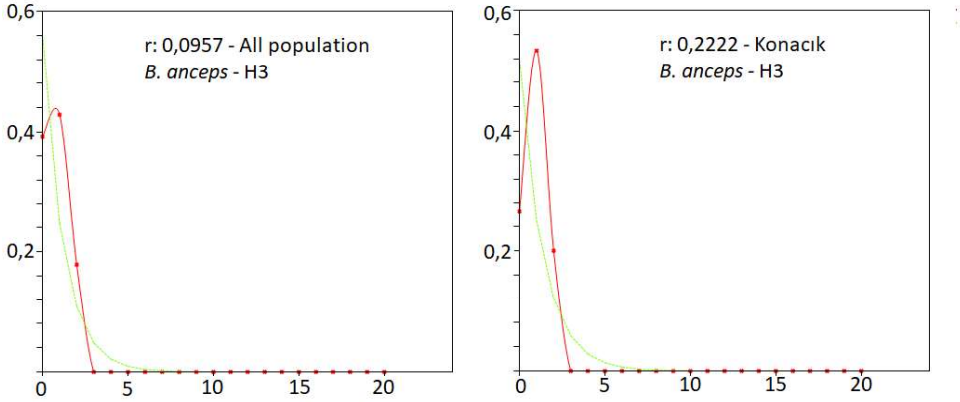


Figure 17. The mismatch distribution graphs of the H3 for Konacık and all *B. anceps* samples. r: Raggedness statistic values. The X-axis: the number of pairwise differences, while the Y-axis: frequency. The red line: observed frequency for stable population size, the green line: expected frequency.

3.2 Morphological Results

3.2.1 *Botrylloides anceps* (Herdman, 1891)

B. anceps samples were observed to possess leachii type morphology with light pink, purple, reddish-orange, brownish-red, dark brown, blackish-brown colors. Zooids were buried in a gelatinous matrix, which is called as tunicin. The branchial siphon for water uptake, cloaca for excretion, stomach, an endostyle, ampullae) were given in (Figure 18A, 18B). According to the comparison of different color morphs, 12 oral tentacles were recorded for the pink morph, 18 for the reddish-orange morph (Figure 18D, E). On the other hand, the pink morph tentacles showed six long and six short structures; the reddish-orange morph was irregular. The blastogenic cycle has lasted seven days under the laboratory conditions for *B. anceps* (Figure 18C), all of the stages were observed under the microscope; Stage A (Figure 18C, Day2), Stage B (Figure 18C, Day3), Stage C (Figure 18C, Day4), Stage D (Figure 18C, Day8). The cycle had continued all over again in the whole life span.

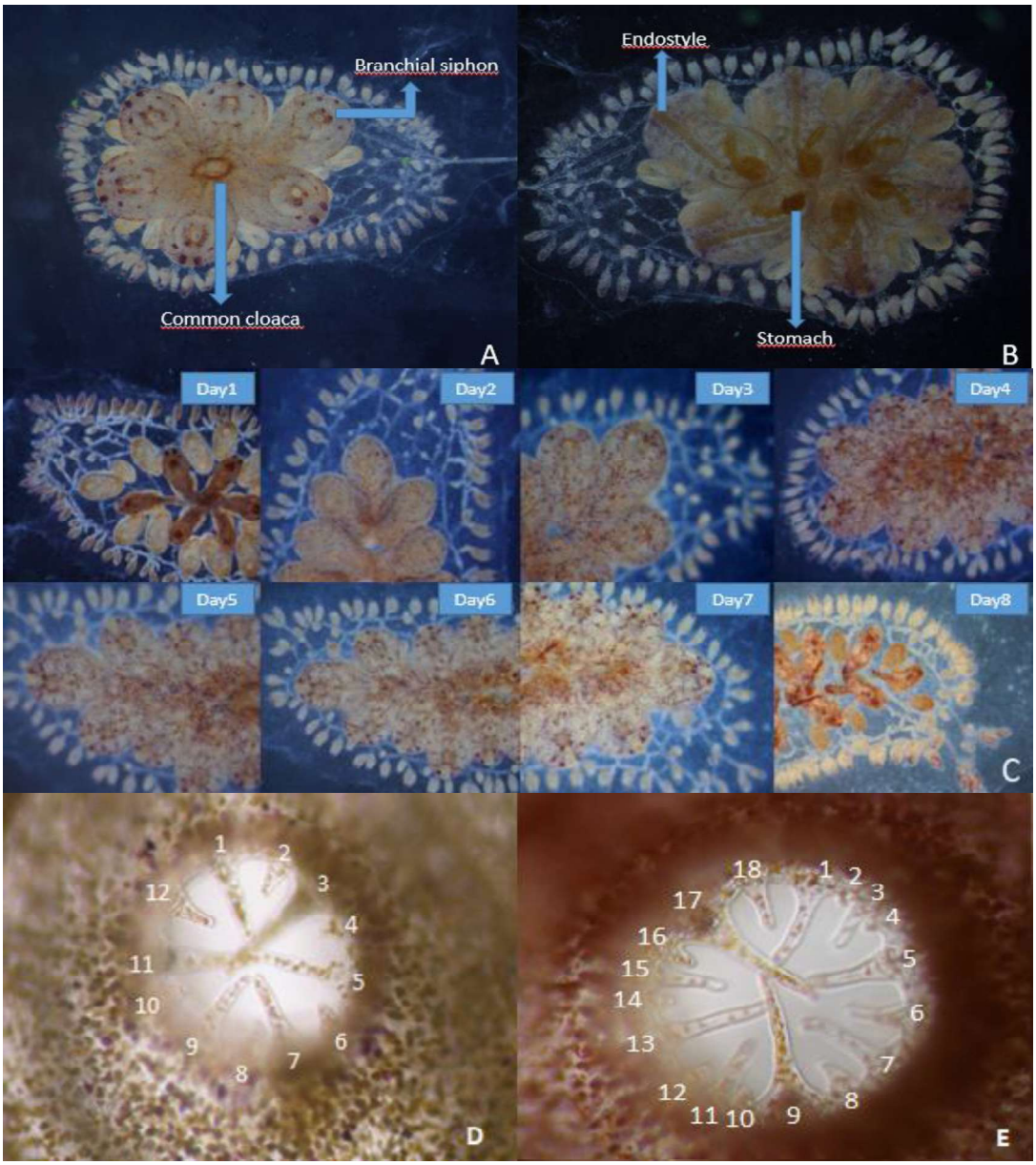


Figure 18. Morphological records of the of *B. anceps*; A) from ventral side, B) from dorsal side and C) the blastogenic cycle, D) the oral tentacles of the pink and E) the red morphs

3.2.2 Botrylloides sp.

Botrylloides sp. samples were recorded in their natural substrata possessing brown, reddish-brown, light brown, and black underground colors, and zooids were characterized by light pigments that extend through anterior of the zooids. Colonies were recorded having leachii type growth and ventral/dorsal side photographs in which branchial siphon, stomach, common cloaca, ampullae are visible were given in Figure 19A, 19B. Like in other Botryllids, zooids were buried in tunicin. The zooids of *Botrylloides sp.* were observed to possess eight oral tentacles which four long and four shorts (Figure 19C) ones. According to Mukai and Watanabe's (1953) staging method, *Botrylloides sp.* were determined to have eight days of the blastogenic cycle under the culturing conditions and all the stages could be monitored under the microscope (Figure 19); Stage A (Day1), Stage B (Day3), Stage C (Day6), Stage D (Day8). The cycles had repeated each other in all life.

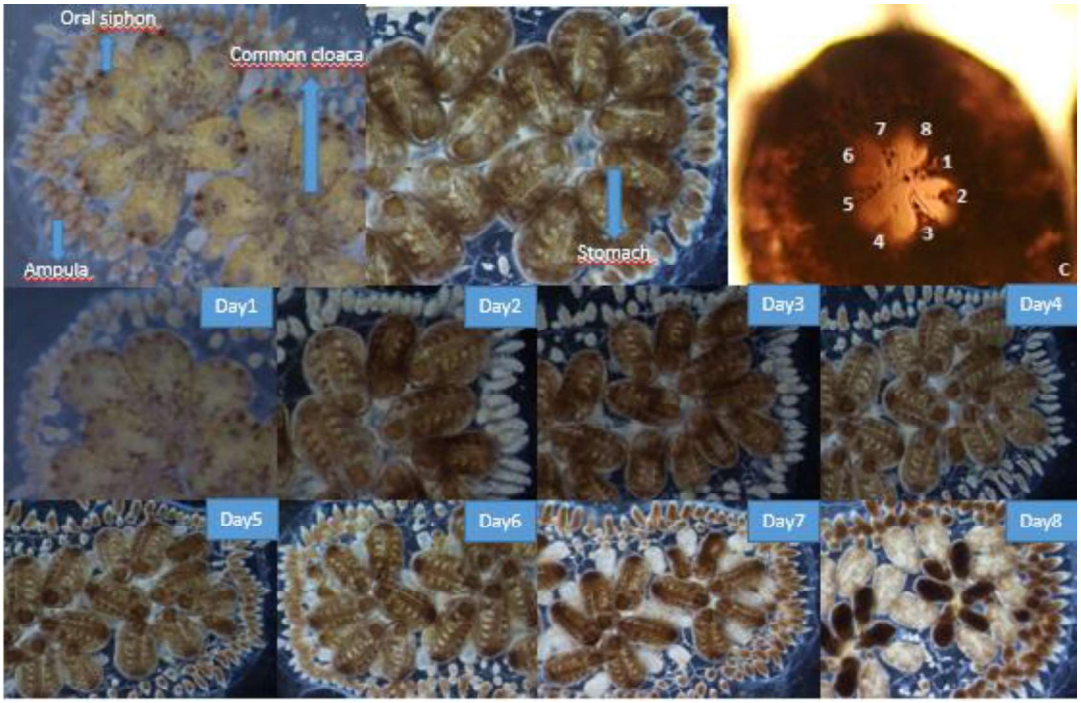


Figure 19. Morphologic photographs of *Botrylloides sp*. species A) from ventral side, B) from dorsal side C) the oral tentacles and D) the blastogenic cycle

3.2.3 *Botrylloides israeliense* Brunetti, 2009

Photograph of *B. israeliense* (Tisan) from its natural habitat was given in Figure 20. The colony was observed to have leachii type extension, and zooids were buried in the gel matrix, which is tunicin. The colonies have the brown colored background, and each zooid has cream/yellow strips which reside along the anterior side of zooids. Ventral and dorsal photographs and the blastogenic cycle of *Botrylloides israeliense* could not be monitored because the species could not be cultured in the laboratory environment.

Figure 20. Photograph of *B. israeliense* from its natural substrata

DISCUSSION

In the present study, besides the morphological records, two mitochondrial (COI, CytB) and two nuclear (18S, H3) markers were used for the phylogeographic analysis of North-eastern Mediterranean Botryllid ascidians. Whereas a 100% success rate was gained from the COI amplification, only a few *B. anceps* samples' H3 amplification was successful, mostly contaminant organism products were obtained, and also CytB and 18S success varied between 0 and 100% for the species.

4.1 Use of Different Gene Regions for Phylogeographic Studies

It was stated that the mutation rate of mitochondrial DNA to nuclear changes from 2 to 6 times for some invertebrates (Allio et al., 2017). On the other hand, Huang et al. (2007) reported slow evolving mitochondrial DNA at different invertebrate taxa as plesiomorphic features, an ancestral trait shared by some groups in the taxa. The accelerated mutation rate was not found proportional to nucleotide diversity in two different flowering plant species, and it was interpreted as a possible result of the effective size reduction (Sloan et al., 2012). Out of all the markers, polymorphism was recorded only at the H3 gene region for *Botrylloides anceps*. On the other hand, while two mitochondrial gene regions (COI, CytB) were found as polymorphic for the *Botrylloides sp.* species, there was no haplotype for the nuclear marker (18S). When the high mutation rate of the close taxa (*B. schlosseri*) is considered, it can be explained with a low effective population size (N_e); it is relevant with the total sample size of the present study (Avise et al., 1988). A high substitution rate was reported for the tunicates for nuclear and mitochondrial genes (Griggio et al., 2014). Griggio et al. (2014) studied a total of 28 mitogenomes of ascidians species, and they have reported a homogenous evolutionary rate at both intra- and inter-specific levels. Moreover, Grigio et al. (1994) stated that the COI gene has less power to resolve phylogenetic distances at lower taxa than whole-mitogenome. On

the other hand, the mitogenomes recognized high intraspecific divergence, which possibly corresponds to speciation.

Although mitochondrial DNA is widely used for phylogenetic, phylogeographic analysis, and speciation studies, due to its high mutation rate, it also causes ambiguities because of the high homoplasy, a character shared by different taxa but not by their common ancestor (Nabholz et al., 2009). This contradiction gives rise to the vitality of running other markers apart from mitochondrial markers such as COI for the delimitation of Botryllid species. Reem et al. (2017) reported the genetic distance between *Botrylloides anceps* (Israel) and *Botrylloides israeliense* (Israel) species as 0.186 and our results were compatible with since the interspecific distance between *B. anceps* (Turkey) and *B. israeliense* (Tisan) were calculated as 0.204 for the COI marker. Reem et al. (2017) also stated that the COI marker might cause incorrect species assignment by resulting lower genetic distances between the different species. For example, whereas *Botrylloides pizoni* and *Botrylloides perspicuous* are two different species based on the morphological features, a 100% match was recorded from blast analysis when their COI sequences compared. Moreover, a species from the *Botrylloides* genus differed with only 1.7% from a *Botryllus* genus species. Because of that Reem et al. (2017) used additional markers (18S, 28S, H3) in their study.

A comparison of *B. anceps* and *B. israeliense*'s H3 gene region is important because it was the only polymorphic marker for *B. anceps*. However, it could not be possible since the amplification of H3 failed for the *B. israeliense* both in the present and Reem et al. (2017) study. Instead, *B. anceps* and *B. aff. leachii* were compared for the H3 marker; it has resulted in 0.14 distance for both comparisons between the Turkey and Israel's *B. anceps* samples with Israeli *B. aff. leachii*. For the COI, the distance between *B. aff. leachii* (Israel) and *B. anceps* (Israel) species were given as 0.143 in the Reem et al. (2017), it was recorded as 0.154 in the present study. For 18S, the calculated distance between *B. aff. leachii* (Israel) and *B. anceps* (Turkey) were found 0.023 in the present study, and it was given as 0.029 by Reem et al. (2017).

Besides, very close distance results were gained from the comparison of *B. aff. leachii* (Israel) and *B. israeliense* (Israel-Tisan) species (0.171 Reem et al. 2017 and 0.173 in the present study). Our results are consistent with Reem's results because the distances for all markers gave more or less the same results. While the highest interspecific distances were noted for COI (0.140) and H3 (0.155) markers, the lowest was recorded from 18S (0.023). Moreover, *Botrylloides leachii* and *Botrylloides aff. leachii* (resulted in a 0.137-distance) are considered different species when the threshold value is taken into account (Reem et al.; 2017). However, they diverged by 0.009 distance for the 18S marker and 0.025 distance for the H3, and they grouped as sister clades in the phylogenetic tree with high support (74%^{18S} and 100%^{H3}) (Reem et al.; 2017). Thus, Reem et al. (2017) stated that besides the genetic markers, other traits, such as biological and ecological features, must be considered for taxonomic assignment and phylogeographic analysis of the botryllid ascidian. Whereas the polymorphism was recorded in the nuclear marker (H3) for *B. anceps*, the mitochondrial markers (COI, CytB) were polymorphic for *Botrylloides sp.* Moreover, chimeric formation between *Botrylloides israeliense*, and *Botrylloides sp*. could be detected using both mitochondrial and nuclear markers. These approve the necessity of using combined markers for precise interpretations.

4.2 Turkish Coastline Tunicates

Studies on ascidian fauna on the Turkish coasts (Mediterranean Sea, Aegean Sea, Marmara Sea, and the Black Sea) are not comprehensive in terms of biodiversity and phylogeography. They are mostly limited to the first records of indigenous and non-indigenous species from the Turkish coasts and checklists. Besides, the records are based on the morphology; no DNA barcode was submitted to any database for the Turkish ascidian fauna. Thus, there is no ascidian study, including the genetics of ascidians from the Turkish coast. *Microcosmus polymorphus* was recorded in Turkish Coasts of Aegean Sea for the first time (Cihangir et al., 2011). Some studies were done about their fouling or pollution indicator nature, while some were

investigated for the possible bioactive molecules (Koçak and Küçüksezgin, 2000; Konuklugil et al., 2018). Presence of *Ciona intestinalis, Didemnum sp., Botryllus schlosseri, Botrylloides sp*. in the Aegean Sea can be deducted from the studies done on the species by Koçak and Küçüksezgin (2000). Dinçaslan and Öber (2004) analyzed the morphology of seven Ascidiacea species from İzmir Bay: *Ciona intestinalis, Ascidiella aspersa, Ascidia mentula, Phallusia mammilata, Clavellina lepadiformis, Halocynhia papillosa* and *Molgula manhattensis*. According to Konukligil et al. (2018), *Microcosmus vulgaris* is present in the Aegean Sea. According to the latest update on non-indigenous species from the Mediterranean ports, *Clavelina oblonga* from Datça was given as a new record for Turkey; Fethiye and Finike was recorded as new locality record from the Mediterranean coasts of Turkey for the *Diplosoma listerianum*. Also, new locality record was provided for the *Microcosmus exasperatus* from Datça, and *Styela pricata* was recorded in Fethiye and Finike as widespread (Ulman et al.; 2017). Non-indigenous *Styela clava* was recorded in the Marmara Sea (İzmit Bay) by Çınar (2016). *Botryllus schlosseri* was recorded on a seahorse from The Turkish coasts of the Black Sea (Kayiş, 2011). According to sampling conducted by Yalçın (2018) to determine the ascidian fauna on the coastline of Ordu province from the Black Sea, five species were recorded; *Botryllus schlosseri, Botryllus sp., Ascidiella aspersa, Ciona intestinalis, Molgula manhattensis*.

According to Miroğlu and Yalçın (2017, conference publication), Turkish coasts consist of 50 ascidian species in total; 32 species from the Mediterranean Sea, six species from the Black Sea 28 species from Aegean and 30 species from the Marmara Sea were presented in the literature about ascidian fauna. The number of tunicate species in the Turkish coast was recorded as 62, including 51 species from the Ascidiacea class (Çınar, 2014; Aydin-Onen, 2018). Non-indigenous *Polyclinum constellatum* was reported for the first time in the Aegean Sea by Aydin-Onen (2018), which increased the number of recorded species from Ascidiacea to 52. With the sampling done by Karahan (YÖP 701 2018 266, 2020), the number of ascidian species rose to 56. In this study, 3 more ascidian species were added to the Turkish

ascidian fauna list. *Botrylloides anceps, Botrylloides sp.,* and *Botrylloides israeliense* were encountered in the Mediterranean Coasts of Turkey for the first time in this study, The Turkish ascidian fauna list is still incomplete; thus, a comprehensive study must be performed to understand biodiversity and phylogeography.

Table 10. Botryllid Ascidians on Turkish Coasts

Species	Location	Reference
Botryllodes schlosseri (Pallas, 1766)	BS, SM, AS, LS	1, 2, 3, 4, 5
Botrylloides leachii (Savigny, 1816)	LS, AS	3, 4
Botryllus renierii (Lamarck, 1815)	SM	1
Botrylloides aff. leachhi Herdman, 1886	LS	6
Botrylloides anceps (Herdman, 1891)	LS	7
Botrylloides israeliense Brunetti, 2009	LS	7
Botrylloides sp.	LS	7
Botryllus sp1.	BS	5
Botryllus sp2.	LS	6

Levantine Sea (LS); Aegean Sea (AS); Sea of Marmara (SM); Black Sea (BS); 1. Demir 1952; 2. Băcescu, 1961; 3. Pınar, 1974; 4. Aydın-Onen, 2018; 5. Yalçın, 2018; 6. Karahan, 2020; 7. This study.

4.3 Phylogenetics and Phylogeography Studies on Ascidians

Since ascidians are distributed worldwide, there are many phylogeographic studies concerning the ascidians from different places in the world seas. Pineda et al. (2011) studied the genetic structure and global phylogeography (Indian Ocean, Atlantic Ocean, Pacific Ocean, Mediterranean Sea) of a solitary ascidian *Styela plicata* indicating that many populations of the species are well structured and differentiated because of the recurrent introductions. Phylogeography of *Halocynthia roretzi* from Korea and Japan was studied by Kim et al. (2011), concluding that three populations of the species were divided into structured and differentiated two groups. Phylogeography and population genetics of *Botryllus schlosseri* in the Mediterranean Sea has been studied by sampling in several Mediterranean countries (Israel, Italy, Spain, France, Croatia, Greece) concluding substantial genetic diversity between Eastern and Western Mediterranean (Reem et al., 2017). Karahan et al. (2016) studied the influence of catastrophic events such as

floods on the genetic structure of *B. schlosseri* collected from Elkhorn Yacht Club (California) for twelve years between 1996-2008 using microsatellites and mitochondrial COI gene and presented the divergence between the California and Washingtonian populations. Phylogeography of an ascidian, *Microcosmus squamiger*, was studied on Australian coasts and found that the species is native to Australia and expands its range through shipping (Rius et al., 2008). Lejeusne et al. (2010) studied the comparative phylogeography of *Botryllus schlosseri* and *Botryllodies violacceus*, revealing that each species has different colonization histories *Botryllus schlosseri* invade by fouling activity, *Botrylloides violaceus* introduction happened because of the aquaculture activities.

Jaffar et al., (2016) studied the distribution of *Didemnum psammathodes* along the Indian coasts, revealing that distribution of the species is affected by the substrate rather than hydrographical parameters (2016). The population genetic structure of *Styela rustica* was studied in the arctic to detect whether the populations lack genetic diversity as expected due to recent colonization after the glaciation process. They found that the population with the lowest genetic variation was closest to Bloomstrandbreen, the glacier with a fast retreating rate (Demarhci et al., 2008). Phylogenetic analysis differentiated *Molgula manhattensis* and *Molgula socialis* species; moreover, the phylogeographic analysis revealed that *Molgula manhattensis* is native to North-east America (Haydar et al., 2010).

4.4 As an Introduced Species; *Botrylloides anceps* (Herdman, 1891)

Second Mediterranean and first Tukish coasts records are given for *Botrylloides anceps* in this study. Although the species were encountered at the sampling stations in 2018, *Botrylloides anceps* was not present in the area according to the sampling done by Karahan in 2012 and 2014 along the Konacık-Alanya coastlines. Possibly *B. anceps* was introduced to the Turkish coast after 2014 and spread to along the coasts in four years. Massive ship trafficking in the Mediterranean Sea (Muñoz et al., 2009) and the currents (Robinson et al., 2001; Zhan

et al., 2015) are possible reasons for the introduction of *Botrylloides anceps* to the Turkish coasts of Mediterranean Sea.

No polymorphism was detected between *Botrylloides anceps* individuals collected from Konacık, Mezitli, and Alanya for COI, CytB, and 18S gene regions. During the blast analysis, the COI and 18S regions records of the present study *Botrylloides anceps* species gave 100% match with the records from Israel (MG009581.1, MG009586.1). According to the result of the COI and 18S gene fragments, it can be interpreted that *Botrylloides anceps* species in the Mediterranean Sea is not polymorphic and genetically differentiated, it formed one monophyletic group. CytB gene region data for the *Botrylloides anceps* were given for the first time in this study; thus, it could not be compared to any database record. For the H3 nuclear region, three haplotypes were calculated; $H_{H3\text{-}I}$ was seen in all sampling areas (Konacık, Hatay, Alanya), $H_{H3\text{-}II}$, and $H_{H3\text{-}III}$, were seen in only Konacık. HH3-I gave an exact match with the Israel record (MG009594.1). Thus, it can be concluded that $H_{H3\text{-}II}$ and $H_{H3\text{-}III}$ adapted to the area according to the environmental variables.

According to the parameters described to measure the population and subpopulation variation, nucleotide diversity (Pi) and haplotype diversity (h) was found zero for each mitochondrial marker (COI, CytB) and 18S in the all areas and other regions (Konacık, Mezitli, Alanya). The number of polymorphic sites and informative sites were also found zero in all samples for the COI, CytB and 18S gene fragments. Mitochondrial diversity has been preserved in *B. anceps* when the species was colonizing the Turkish coasts. Thus, neutral mitochondrial evolution was observed for *B. anceps* in the study. Although nucleotide diversity (all: 0.002; Konacık: 0.003) and haplotype diversity (All: 0.607; Konacık: 0.733) were calculated higher for the H3 gene region than other gene regions used (COI, CytB, 18S), the values were still low for the populations. The number of polymorphic sites (2) and the number of informative (1) sites were also found very low in both populations for the H3 gene fragment. Tajima's D-statistic was not significant for neither all (D= 0.069) nor for the Konacık samples (D= -0.612) considering H3 gene region, which means that there was no selective pressure acting on the H3 gene

region for the *B. anceps.* The Raggedness index (all: 0, 096; Konacık: -0.222) based on the mismatch distribution, Fu and Li's D (all: -0.149; Konacık: -0.612), Fu and Li's F (all: -0.108; Konacık: -0.479), Fu's FS (all: -0.224; Konacık: 0.172) test and R2 test (all: 0.213; Konacık: 0.239) were done to detect the population growth and all the values were found to be low and not significant supporting neither all population nor Konacık population is expanding concerning H3 region.

According to a study about the phylogeography of *B. schlosseri,* two areas (Gibraltar, Motril-Span) share only one COI haplotype thus have zero nucleotide and haplotype diversity, as we found in our study areas (Konacık, Mezitli, Alanya), on the other hand, Rovinj-Croatia presents the higher variability with 17 haplotypes, 0.792 haplotype diversity and 0.016 nucleotide diversity (Reem et al, 2017). For the overall sampling, the COI gene region of 288 samples resulted in 54 haplotypes and strong genetic divergence between East and West Mediterranean was detected (Reem et al., 2017). For the West Mediterranean, the number of haplotypes, haplotype diversity and nucleotide diversity were calculated as 22; 0.386; 0.018 respectively. For the East Mediterranean, the same parameters were recorded as 34; 0.857; 0.033, respectively, implying stronger genetic structure in the eastern part. For the overall, on the other hand, calculations resulted in 54 haplotypes with 127 polymorphic sites, 0.719 haplotype diversity and 0.026 nucleotide diversity.

The neutrality test parameters (Tajima's D, Fu' Fs, Fu and Li'F, Fu and Li's D) were calculated for the entire Mediterranean as well as the Eastern and Mediterranean. Neither the entire basin nor the east and west parts was rejected for the neutrality, and the constant population size was found for the populations (Reem et al., 2017). In the present study, constant population size for all and Kızkalesi samples was detected for the H3 gene of *B. anceps*. When the results of *B. anceps* of this study were compared to the *B. schlosseri* of Reem et al. (2017), apparently *B. anceps* does not have a genetic variation on the contrary to invasive *B. schlosseri.* Although *B. anceps* was stated as invasive species by Reem and Rinkevich (2014), it can conclude that *B. anceps* has low invasive potential because of low genetic diversity (Rius et al., 2008). This homogeneity and lack of variation among

populations indicate that *B. anceps* species has introduced to the Turkish coasts of Levantine Basin by a single event. It spread along the Turkish coasts through anthropogenic ways, including most possibly shipping and recreational boat activities (Zhan et al., 2015). Also, this lack of genetic variability shows that *B. anceps* populations along the Turkish coast are young (Reem et al., 2017), which agree with the fact that *B. anceps* species present on Levantine coast Turkey outmost for six years.

Marine invertebrates show different degrees of color variation (Tarjuelo, 2004). Different color morphs (reddish-orange, brownish-red, pink, purple, brown, dark brown, blackish-brown) were observed during the sampling of the present study. Yet, Brunetti (2009) stated violet, cream, and grey color morphs of living colonies. Ascidians have many color morph varieties, and color morphs were detected for possible speciation for some species (*Didemnum molle, Pseudodistoma crucigaster, Cystodytes dellechiajei*). On the other hand, the color morphs of some species (*Ecteinascidia turbinata*) supported monophyly, and they didn't show any speciation (Tarjuelo, 2004; Lopez-Legentil and Turon, 2006, 2007; Hirose et al., 2008). Different color morphs of this study supported monophyly in the phylogenetic trees constructed from COI, CytB, and 18S gene fragments. Differences between the color morphs presented by Brunetti and this study samples may be the result of either possible sibling speciation or only reflection of their microenvironments (Hirose et al., 2008). Further analysis, including all color morphs, must be done for precise interpretation.

4.5 As a Possible New Species; *Botrylloides sp.*

Botrylloides sp. collected from Mezitli and Konacık results in three haplotypes for the COI gene region and four haplotypes for the CytB gene region, on the other hand, there was only one haplotype for all samples for 18S gene region. H3 gene region of *Botrylloides sp.* could not be amplified, although different PCR reactions were applied. $H_{COI\text{-}I}$ and $H_{COI\text{-}II}$ of *Botrylloides sp.* were encountered in

Mezitli and Kızkalesi while $H_{COI-III}$ was encountered only in Kızkalesi. For the CytB gene region, the recorded haplotypes for Kızkalesi were H_{CytB-I}, $H_{CytB-II}$ and $H_{CytB-III}$. At the same time, it was H_{CytB-I} and $H_{CytB-IV}$ for the Mezitli region, and those gave the closest (%96,38) match with *Botrylloides sp.* (MN076468.1) from Italy in the GenBank. The COI gene region is widely used for the delimitation of marine organisms (Bucklin et al., 2011), and maximum intra-specific distance is accepted 2% for metazoan species (Leray et al., 2013). Thus, it can be speculated that *Botrylloides sp.,* collected from Mezitli and Kızkalesi coasts whose closest match was located in 3.62% distance, is a possible new botryllid endemic ascidian for the Turkish coasts.

According to the parameters described to measure the population and subpopulation variation, nucleotide diversity (Pi) and haplotype diversity (h) were zero for both all samples and sub-samples (Mezitli, Kızkalesi) for 18S gene. As a result of this, the number of polymorphic sites and the number of informative sites were also zero, indicating no variation at all for *Botrylloides sp*. A study of *Botrylloides schlosseri* from California, 131 COI sequences resulted in 13 haplotypes, and haplotype diversity and nucleotide diversity were reported as 0.467 and 0.005, respectively. (Karahan et al., 2016). Highest haplotype diversity (0.800) was recorded for EYC-01/1996 sampling with 5 haplotypes out of 10 sequences, while lowest haplotype diversity was 0.282 for EYC-01/1996 sampling with 4 haplotypes out of 13 sequences (Karahan et. al., 2016). In this study, *Botrylloides sp.* was found to have 3 haplotypes out of 34 COI sequences. Haplotype diversity and nucleotide diversity were calculated as 0.266 and 0.001, respectively for all samples. For the CytB, 4 haplotypes out of 22 CytB sequences were recorded, the haplotype diversity was recorded as 0.680 and the nucleotide diversity as 0,002 for all samples. The species has been found to have 3 haplotypes for COI and 2 haplotypes for CytB in Mezitli. Haplotype diversity was 0.700 and 0.667; nucleotide diversity was 0.002 and 0.002 for COI and CytB genes, respectively, for the Mezitli area. The species has been found to have 2 haplotypes for COI and 3 haplotypes for CytB in Kızkalesi.

Haplotype diversity was 0.069 and 0.647; nucleotide diversity was zero and 0.002 for COI and CytB genes respectively for the Kızkalesi region.

Mezitli had higher haplotype and nucleotide diversity than the Kızkalesi region for the COI marker. However, haplotype diversity was nearly the same in both areas, and nucleotide diversity in Kızkalesi is slightly higher than Mezitli for the CytB gene region. According to the Bayesian tree, 5 sequences are the source to the other haplotype, and 4 are from Mezitli, while only one sequence is from Kızkalesi. For the CytB gene region in the Bayesian tree, source haplotype consists of sequences from both Mezitli and Kızkalesi. On the other hand, one inherited haplotype consists of only sequences from Mezitli; the other inherited haplotype consists of only sequences from Kızkalesi. Since interpreting the main population from the Bayesian tree was not easy, Nh/N (number of haplotypes over the number of samples) calculation was used for interpretation. Nh/N resulted as; 0.60 (COI-Mezitli); 0.03 (COI-Kızkalesi); 0.16 (CytB-Kızkalesi); 0.50 (CytB-Mezitli). For both markers, Mezitli resulted in a higher Nh/N ratio; thus, Mezitli samples were accepted to be the source population, and the species colonized the Kızkalesi region later on. The number of polymorphic sites (2,2,1) and the number of informative (1,0,0) sites were also found very low in all samples and sub-samples (Mezitli, Kızkalesi) for the COI gene fragment. For the CytB gene fragment, all samples and separate regions' samples (Mezitli, Kızkalesi) were also recorded to have a low number of polymorphic sites (3,1,2) and informative sites (3,1,2), respectively.

Although some variation detected in mitochondrial genes, it is still quite low thus mitochondrial diversity has been preserved in *Botrylloides sp.* leading to neutral mitochondrial evolution for *Botrylloides sp.* According to neutrality parameters (Tajima's D, Fu' Fs, Fu and Li'F, Fu and Li's D), most of the populations provided neutrality and were reported to have constant population size; however, populations (the Elkhorn Yacht Club, 2000 and 2007) which the neutrality was rejected was linked to a possible catastrophic event implying population expansion in Karahan's study (2016). For *Botrylloides sp.* of this study, Tajima's D-statistic was not significant neither for all sampling (-0.694^{COI}; 0.325^{CytB}) nor for Mezitli (-0.973^{COI};

1.633^{CytB}) and Kızkalesi (-1.149^{COI}; 1.016^{CytB}) considering each gene region which means that there was no selective pressure acting on the gene regions studied (COI, CytB) for the *Botrylloides sp.* The raggedness index (all: 0.300^{COI}; 0.118^{CytB}; Mezitli; 0.350^{COI}; 0.556^{CytB}; Kızkalesi: 0.748^{COI}; 0.132^{Cytb}) based on the mismatch distribution, Fu and Li's D (all: -0.778^{COI}; 0.992^{CytB}; Mezitli: -0.973^{COI}; 1.633^{CytB}; Kızkalesi: -1.671^{COI}; 0.885^{CytB}), Fu and Li's F (all: -0.872^{COI}; 0.930^{CytB}; Mezitli; -0.954^{COI}; 1.277^{CytB} Kızkalesi: -1.757^{COI}; 1.053^{CytB}), Fu's FS test (all: -0.725^{COI}; -0.085^{CytB}; Mezitli: -0.829^{COI}; 0.540^{CytB}; Kızkalesi: -1.183^{COI}; 0.691^{CytB}) and R2 test (all: 0.106^{COI}; 0.154^{CytB}; Mezitli: 0.245^{COI}; 0.333^{CytB}; Kızkalesi: 0.183^{COI}; 0.206^{CytB}) were done to detect the population growth and all the values were found to be low and not significant thus constant population size was valid for the all Turkey population and sub-populations (Mezitli, Kızkalesi) for both COI and CytB regions.

The overall haplotype diversity is higher than that of nucleotide diversity. Some haplotypes are specific to one area rather than shared among locations, which show that populations (Mezitli, Kızkalesi) of *Botrylloides sp.* evolving independently. Genetic differentiation (F_{ST}) between the Mezitli and Konacık populations of *Botrylloides sp*. species was found to be 0.56 for COI and 0.26 for CytB markers. Thus, genetic isolation between the two populations of *Botrylloides sp.* was found to be high, and gene flow (Nm: 0.400^{COI}, 0.880^{CytB}) between these two populations is quite low. The genetic differentiation (F_{ST}) for the West and East Mediterranean populations of *B. schlosseri* was 0.171 and 0.416 for COI, respectively, indicating a strong population structure for the eastern basin (Reem et al., 2017). Thus, Kızkalesi and Mezitli samples of *Botrylloides sp.* have distinct genetic profiles when the high F_{ST}= 0.56 value is considered. The strong divergence between the eastern and western populations of *B. schlosseri* is coherent because two basins differ in terms of salinity and temperature. For *Botrylloides sp*., salinity (~39-40 ppt) does not vary much for Kızkalesi and Mezitli stations; thus, other mechanisms must be present for the site-specific evolution. Mezitli stream resides nearby Mezitli station, and it is located between Mezitli and Kızkalesi sampling areas, when it is summer, the flow rate of the stream reduces; thus, sea blocks the

stream mouth creating a pond in the mouth (Yalvaç and Deveci, 2007). Ascidians are susceptible to salinity changes. Considering that the reproduction of ascidians mostly happens in summer and they are also intertidal species, this pond formation right on the mouth of the stream may block the migration between these two sampling stations. North Atlantic surface water reaching the Alboran Sea act as a barrier between some Atlantic Sea and Mediterranean marine invertebrates (Duran et al., 2004). Besides, the probable short life cycle of free-swimming larvae of *Botrylloides sp.* species could be another factor that prevents the connection of two populations (Ayre et al., 1997; Pérez-Portela ve Turon, 2008).

4.6 *Botrylloides israeliense* Brunetti, 2009

Botrylloides israeliense was encountered on the Turkish coasts of Mediterranean Sea for the first time in this study. *B. israeliense* sample from Tisan that was characterized by cream and yellow-pigmented strips over the brown, black colored ground on its natural substrate. According to the underwater photographs by Reem et al. (2017), it can be seen that it has reddish-brown, and it does not have pigmented strips as in the Tisan sample. Furthermore, the similarity between Israel (MG009580.1) and Turkey (Tisan-G3) samples were found to be 97.59%, which corresponds to a greater value than expected maximum distance (2%) or metazoans (Leray et al., 2013). On the other hand, according to Hebert et al., there is not only one threshold for species identification, and the COI gene variation is diverse at different taxa (2003). For example, *Botryllus schlosseri* were found to have a 5.45% divergence among its haplotypes and all belonging to the same species (López-Legentil et al., 2006). However, for *Pseudodistoma crucigaster* (colonial ascidian), there was a 2.12% variation for the COI sequence between the color morphs identified as different species (Tarjuelo et al. 2004). The reason for that higher distance (2.41%) could indicate that *Botrylloides israeliense* (Tisan) is a sub-species of *Botrylloides israeliense* of Israel and the species has high intraspecific variability.

Botrylloides israeliense collected from Tisan resulted in 4.6% distance to the Kızkalesi populations of *Botrylloides sp.* samples which corresponds to only 24 mutation steps and the distance between the two records for CytB was calculated 6.1% which correspond 16 mutation steps. On the other hand, the distance between *B. israeliense* (Tisan) and *Botrylloides sp.* of Kızkalesi and Mezitli samples was zero, which means the 18S sequences of two species is the same with no variation. It can be explained by the self-nonself recognition (allorecognition) ability of ascidians which two colonies of the same or different species fuse to form one individual colony. Probably, *Botrylloides sp.* and *B. israeliense* formed a chimera because they share at least one FuHC allele which formed entity consists of combined genotypes of two partners (Pancer et al., 1995; Stoner and Weissman, 1996; Stoner et al., 1999; Voskoboynik, 2009).

Although chimeric formation is restricted to kin in *B. schlosseri*, fusion experiments performed on the *Diplosoma listerianum* have shown that chimera formations happened for siblings and unrelated individuals (Bishop and Sommerfeldt, 1999). Since *Botrylllus* genus is the closest to *Botrylloides*, chimera formation between *Botrylloides israeliense* and *Botrylloides sp.* can be said limited to the closest kin. Moreover, colony specificity was stated for some *Botrylloides* species such as *Botrylloides simodensis* and *Botrylloides violaceus* (Hirose et al., 1988; 1990). Thus, considering that the distance between two species is 4.6% *Botrylloides sp.* is either sub-species with high intraspecific variability or sibling species of *B. israeliense.*

Natural chimerism was detected in some ascidians such as; *Botryllus schlosseri, Botryllodies nigrum, Diplosoma listerianum, Didemnum vexillum Perophora japonica* (Casso et al., 2019). According to fusion experiment done on the invasive colonial ascidian *Didemnum vexillum*, 44% of 9 colonies (45 pairs) studied were chimeric with 2-3 different genotypes; thus Casso et al. (2019) stated the high occurrence of chimerism in *D. vexillum* and its possible role in the species' invasive success. Smith et al. (2012) studied genetic diversity and the chimerism of New Zealand (introduced) and Japan (native) populations of *D. vexillum*. They found

that a higher degree of fusion rate was detected in less genetically diverse New Zealand colonies (80% chimera formation) than highly genetically distinct Japanese colonies (27% chimerism).

Microsatellites can be used to detect the genetic diversity and chimerism in the populations when the individuals share at least two alleles, for example, highest chimera frequency on the worldwide population of *B. schlosseri* was found as 30% from 35 individuals followed by Portugal with 25.7% from 56 individuals (Ben Shlomo, 2017). When the chimeras share the same allele, they cannot be detected; thus, Ben Shlomo (2017) states that actual chimeric formations are much more than estimated. The mitochondrial COI markers could be useful with additional markers for detecting of the chimeras as happened in this study.

CONCLUSION

The distribution record and presence of *Botrylloides anceps, Botrylloides sp.,* and *Botrylloides israeliense* species were given for the first time with this study along the Levantine coasts of Turkey. Whereas *Botrylloides anceps* is an Indo-Pacific originated species, *Botrylloides israeliense* was identified for the first time from the Israel coasts as possible endemic species and *Botrylloides sp.* were encountered in the Turkish coastlines for the first time as a possible new species. There was no difference between the Konacık, Mezitli, and Alanya populations of *B. anceps,* and the population expansion could not be detected for any of these locations. However, population differentiation was observed between Mezitli and Kızkalesi populations of *Botrylloides sp.,* although both populations were under the constant population size. Besides, this study indicates that species identification is based on the COI gene region and creates a base for related studies such as phylogeographic, phylogenetic, and conservation. And it was concluded that both

mitochondrial and nuclear gene regions must be used for the species identification. Detection of botryllid ascidians diversity along the Turkish coasts is vital, considering the recording biodiversity of the area. Moreover, when the wide range of ascidians uses as model organisms for the regeneration, development, aging, allorecognition, cancer studies are considered, *B. anceps* with the experience gained during this study will be a significant source for the future studies on these areas.

REFERENCES

Aligner, C. (2019). CodonCode Corporation, version 9.0.1. https://www.codoncode.com/aligner/

Allio, R., Donega, S., Galtier, N., & Nabholz, B. (2017). Large variation in the ratio of mitochondrial to nuclear mutation rate across animals: implications for genetic diversity and the use of mitochondrial DNA as a molecular marker. *Molecular Biology and Evolution*, *34*(11), 2762-2772.

Ananthan, G., & Murugan, R. (2018). Molecular phylogeny of four ascidian species inferred from mitochondrial Cytochrome Oxidase subunit I (COI) sequence. *Mitochondrial DNA Part A*, *29*(1), 121-125.

Aslan, H. (2006). Ascidians and their distribution in the Mediterranean Sea. *Ege Journal of Fisheries and Aquatic Sciences*, *23*(1), 29-31.

Atsumi, M. O., & Saito, Y. (2011). Studies on Japanese botryllid ascidians. V. A new species of the genus *Botrylloides* very similar to *Botrylloides simodensis* in morphology. *Zoological Science*, *28*(7), 532-542.

Avise, J. C. (2000). *Phylogeography: the history and formation of species*. Harvard university press.

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., ... & Saunders, N. C. (1987). Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual review of ecology and systematics*, *18*(1), 489-522.

Avise, J. C., Bowen, B. W., Lamb, T., Meylan, A. B., & Bermingham, E. (1992). Mitochondrial DNA evolution at a turtle's pace: evidence for low genetic variability and reduced microevolutionary rate in the Testudines. *Molecular Biology and Evolution*, *9*(3), 457-473.

Avise, J. C., Ball, R. M., & Arnold, J. (1988). Current versus historical population sizes in vertebrate species with high gene flow: a comparison based on mitochondrial DNA lineages and inbreeding theory for neutral mutations. *Molecular Biology and Evolution*, *5*(4), 331-344.

Aydin-Onen, S. (2018). Distribution of ascidians with a new record of the non-indigenous species *Polyclinum constellatum* Savigny, 1816 from the Aegean coast of Turkey. *Turkish Journal of Fisheries and Aquatic Sciences*, *18*(9), 1077-1089.

Ayre, D. J., Davis, A. R., Billingham, M., Llorens, T., & Styan, C. (1997). Genetic evidence for contrasting patterns of dispersal in solitary and colonial ascidians. *Marine Biology*, *130*(1), 51-61.

Băcescu, M. (1961). Le role des iles dans la dispersion récente des espèces indo-pacifiques en Méditerranée occidentale et quelques observations sur la faune marine de L'ile des serpents, en comparaison avec celle peuplants les parages prébosphoriques de la Mer Noire. In: V. Le peuplement des iles Méditerraneennes et la problème de l'insularité, Banyulssur-Mer. Paris, France: Centre National de la Recherche Scientifique, pp. 241–253 (in French).

Bak, R. P., Joenje, M., De Jong, I., Lambrechts, D. Y. M., & Nieuwland, G. (1998). Bacterial suspension feeding by coral reef benthic organisms. *Marine Ecology Progress Series*, *175*, 285-288.

Ballard, J. W. O., & Whitlock, M. C. (2004). The incomplete natural history of mitochondria. *Molecular Ecology*, *13*(4), 729-744.

Bandelt, H. J., Forster, P., & Rohl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, *16*(1), 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036

Ben-Shlomo, R. (2008). The molecular basis of allorecognition in ascidians. *Bioessays*, *30*(11-12), 1048-1051.

Ben-Shlomo, R. (2017). Invasiveness, chimerism and genetic diversity. *Molecular Ecology*, *26*(23), 6502-6509.

Berrill, N. J. (1950). The Tunicata. 354pp The Ray Society.

Berrill, N.J. (1975). Chordata: tunicata. In Giese, A. C., & Pearse, J. S. (Eds.). *Reproduction of marine invertebrates* (v.2, pp. 241-282). New York, Academic Press, Inc.

Bishop, J. D., & Sommerfeldt, A. D. (1999). Not like *Botryllus*: indiscriminate post–metamorphic fusion in a compound ascidian. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *266*(1416), 241-248.

Blanchoud, S., Rinkevich, B., & Wilson, M. J. (2018). Whole-Body regeneration in the colonial tunicate *Botrylloides leachii*. In *Marine Organisms as Model Systems in Biology and Medicine* (pp. 337-355). Springer, Cham.

Blanchoud, S., Rutherford, K., Zondag, L., Gemmell, N. J., & Wilson, M. J. (2018). De novo draft assembly of the *Botrylloides leachii* genome provides further insight into tunicate evolution. *Scientific Reports*, *8*(1), 1-18.

Blum, J. C., Chang, A. L., Liljesthröm, M., Schenk, M. E., Steinberg, M. K., & Ruiz, G. M. (2007). The non-native solitary ascidian *Ciona intestinalis* (L.) depresses species richness. *Journal of Experimental Marine Biology and Ecology*, *342*(1), 5-14.

Braun, K., Leubner, F., & Stach, T. (2019). Phylogenetic analysis of phenotypic characters of Tunicata supports basal Appendicularia and monophyletic Ascidiacea. *Cladistics*, *36*(3), 259-300.

Brunetti, R. (2009). Botryllid species (Tunicata, Ascidiacea) from the Mediterranean coast of Israel, with some considerations on the systematics of Botryllinae. *Zootaxa*, *2289*(1), 18-32.

Brunetti, R., & Mastrototaro, F. (2012). *Botrylloides pizoni*, a new species of Botryllinae (Ascidiacea) from the Mediterranean Sea R. *Zootaxa*, *3258*(1), 28-36.

Bucklin, A., Steinke, D., & Blanco-Bercial, L. (2011). DNA barcoding of marine metazoa. *Annual Review of Marine Science*, *3*, 471-508.

Cariton, J. T., & Geller, J. B. (1993). Ecological roulette: the global transport of nonindigenous marine organisms. *Science*, *261*(5117), 78-82.

Carman, M. R., Bullard, S. G., & Donnelly, J. P. (2007). Water quality, nitrogen pollution, and ascidian diversity in coastal waters of southern Massachusetts, USA. *Journal of Experimental Marine Biology and Ecology*, *342*(1), 175-178.

Carver, C. E., Mallet, A. L., & Vercaemer, B. (2006). *Biological synopsis of the colonial tunicates (Botryllus schlosseri and Botrylloides violaceus)*. Bedford Institute of Oceanography. Darthmouth, Nova Scotia.

Casso, M., Tagliapietra, D., Turon, X., & Pascual, M. (2019). High fusibility and chimera prevalence in an invasive colonial ascidian. *Scientific Reports*, *9*(1), 1-10.

Cihangir, H. A., Izquierdo Munoz, A., Papadopoulou, M. A. P., Ramos Espla, A. A. and Yilmaz, E. C. 2011. *Microcosmus polymorphus* Heller, 1877 (Tunicata: Ascidiacea: Pyuridae) - a new addition to the fauna of the Turkish coasts. Turkish Journal of Fisheries and Aquatic Sciences, *11*, 157-159.

Çinar, M. E. (2014). Checklist of the phyla Platyhelminthes, Xenacoelomorpha, Nematoda, Acanthocephala, Myxozoa, Tardigrada, Cephalorhyncha,

Nemertea, Echiura, Brachiopoda, Phoronida, Chaetognatha, and Chordata (Tunicata, Cephalochordata, and Hemichordata) from the coasts of Turkey. *Turkish Journal of Zoology*, *38*(6), 698-722.

Çinar, M. E. (2016). The alien ascidian *Styela clava* now invading the Sea of Marmara (Tunicata: Ascidiacea). *ZooKeys*, (563), 1.

Çinar, M. E., Bilecenoglu, M., Öztürk, B., & Can, A. (2006). New records of alien species on the Levantine coast of Turkey. *Aquatic Invasions*, *1*(2), 84-90.

Cloney, R. A. (1982). Ascidian larvae and the events of metamorphosis. *American Zoologist*, *22*(4), 817-826.

Cohen, C. S., Saito, Y., & Weissman, I. L. (1998). Evolution of allorecognition in botryllid ascidians inferred from a molecular phylogeny. *Evolution*, *52*(3), 746-756.

Coles, S. L., DeFelice, R. C., Eldredge, L. G., & Carlton, J. T. (1999). Historical and recent introductions of non-indigenous marine species into Pearl Harbor, Oahu, Hawaiian Islands. *Marine Biology*, *135*(1), 147-158.

Coll, M., Piroddi, C., Steenbeek, J., Kaschner, K., Lasram, F. B. R., Aguzzi, J., ... & Danovaro, R. (2010). The biodiversity of the Mediterranean Sea: estimates, patterns, and threats. *PloS One*, *5*(8), e11842.

Connell, S. D. (2000). Floating pontoons create novel habitats for subtidal epibiota. *Journal of Experimental Marine Biology and Ecology*, *247*(2), 183-194.

Crisp, D. J., & Ghobashy, A. F. A. A. (1971). Responses of the larvae of *Diplosoma listerianum* to light and gravity. In *Fourth European marine biology symposium* (pp. 443-465). Cambridge University Press, Cambridge.

Davidson, B., Jacobs, M. W., Swalla, B. J., Schlosser, G., & Wagner, G. (2004). The individual as a module: solitary-to-colonial transition in metazoan evolution and development. In *Modularity in development and evolution* (pp. 443-465). University of Chicago Press.

Davidson, B., & Swalla, B. J. (2002). A molecular analysis of ascidian metamorphosis reveals activation of an innate immune response. *Development*, *129*(20), 4739-4751.

Degnan, B. M., Souter, D., Degnan, S. M., & Long, S. C. (1997). Induction of metamorphosis with potassium ions requires development of competence and an anterior signalling centre in the ascidian *Herdmania momus*. *Development Genes and Evolution*, *206*(6), 370-376.

Dehal, P., Satou, Y., Campbell, R. K., Chapman, J., Degnan, B., De Tomaso, A., ... & Harafuji, N. (2002). The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science*, *298*(5601), 2157-2167.

Demarchi, M., Chiappero, M., Laudien, J., & Sahade, R. (2008). Population genetic structure of the ascidian *Styela rustica* at Kongsfjorden, Svalbard, Arctic. *Journal of Experimental Marine Biology and Ecology*, *364*(1), 29-34.

Demir, M. (1952). Boğazlar ve adalar sahillerinin omurgasız dip hayvanları. İstanbul Üniversitesi Fen Fakültesi Hidrobiyoloji Araştırma Enstitüsü Yayınları, 3, 1-615 (in Turkish).

Dinçaslan, Y. E., & Öber, A. (2004). İzmir Körfezi'nde Yaşayan Bazı Ascidiacea (Tunicata) Üyelerinin Dorsal Tüberkül Yapıları. *Su Ürünleri Dergisi*, *21*(1).

DNA Sequence Assembler v 5.15.0 (2017). Heracle BioSoft, www.DnaBaser.com

D-maps (2020). *Eastern Mediterranean Sea.* Retrieved from https://d-maps.com/index.php?lang=en

Duran, S., Palacin, C., Becerro, M. A., Turon, X., & Giribet, G. (2004). Genetic diversity and population structure of the commercially harvested sea urchin *Paracentrotus lividus* (Echinodermata, Echinoidea). *Molecular Ecology*, *13*(11), 3317-3328.

Eri, R., Arnold, J. M., Hinman, V. F., Green, K. M., Jones, M. K., Degnan, B. M., & Lavin, M. F. (1999). Hemps, a novel EGF-like protein, plays a central role in ascidian metamorphosis. *Development*, *126*(24), 5809-5818.

Franchi, N., & Ballarin, L. (2017). Immunity in protochordates: the tunicate perspective. *Frontiers in Immunology*, *8*, 674.

Féral, J. P. (2002). How useful are the genetic markers in attempts to understand and manage marine biodiversity? *Journal of Experimental Marine Biology and Ecology*, *268*(2), 121-145.

Fu, Y. X., & Li, W. H. (1993). Statistical tests of neutrality of mutations. *Genetics*, *133*(3), 693-709.

Fu, Y. X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, *147*(2), 915-925.

Galil, B. S. (2009). Taking stock: inventory of alien species in the Mediterranean Sea. *Biological Invasions*, *11*(2), 359-372.

Geller, J. B., Carlton, J. T., & Powers, D. A. (1993). Interspecific and intrapopulation variation in mitochondrial ribosomal DNA sequences of *Mytilus spp.* (Bivalvia: Mollusca). *Molecular Marine Biology and Biotechnology*, *2*(1), 44.

Geneious Prime 2020.1.2.(https://www.geneious.com)

Giese, A. (Ed.). (2012). *Reproduction of Marine Invertebrates V2: Entoprocts and Lesser Coelomates*. Elsevier.

Grave, C., & Nicoll, P. A. (1935). Accelerators of metamorphosis of larvae of *Phallusia nigra* and species of Polyandrocarpa. *Annual Reports of Tortugas Laboratory, Carnegie Institute of Washington*, *1936*, 84-85.

Grave, C., & Woodbridge, H. (1924). *Botryllus schlosseri* (Pallas): The behavior and morphology of the free? swimming larva. *Journal of Morphology*, *39*(1), 207-247.

Google Earth (2020). *The North-Eastern Mediterranean Sea.* Google. Retrieved from https://earth.google.com/web

Griggio, F., Voskoboynik, A., Iannelli, F., Justy, F., Tilak, M. K., Xavier, T., ... & Gissi, C. (2014). Ascidian mitogenomics: comparison of evolutionary rates in closely related taxa provides evidence of ongoing speciation events. *Genome Biology and Evolution*, *6*(3), 591-605.

Halim, Y., & Messeih, M. A. (2016). Aliens in Egyptian waters. A checklist of ascidians of the Suez Canal and the adjacent Mediterranean waters. *The Egyptian Journal of Aquatic Research*, *42*(4), 449-457.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Nucleic acids symposium series* (Vol. 41, No. 41, pp. 95-98). [London]: Information Retrieval Ltd., c1979-c2000.

Hastings, A.B. (1931) Tunicata. *Scientific Reports of the Great Barrier Reef Expedition*, 4(3), 69-109.

Harpending, H. C. (1994). Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human Biology*, *66*(4), 591-600.

Haven, N. D. (1971). Temporal patterns of sexual and asexual reproduction in the colonial ascidian *Metandrocarpa taylori* Huntsman. *The Biological Bulletin*, *140*(3), 400-415.

Haydar, D., Hoarau, G., Olsen, J. L., Stam, W. T., & Wolff, W. J. (2011). Introduced or glacial relict? Phylogeography of the cryptogenic tunicate Molgula manhattensis (Ascidiacea, Pleurogona). *Diversity and Distributions*, *17*(1), 68-80.

Hebert, P. D., Ratnasingham, S., & De Waard, J. R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(suppl_1), S96-S99.

Hellberg, M. E. (1994). Relationships between inferred levels of gene flow and geographic distance in a philopatric coral, *Balanophyllia elegans*. *Evolution*, *48*(6), 1829-1854.

Herdman, W. A. (1891). A revised classification of the Tunicata, with definitions of the orders, suborders, families, subfamilies, and genera, and analytical keys to the species. *Zoological Journal of the Linnean Society*, *23*(148), 558-652.

Hirose, E., Saito, Y., & Watanabe, H. (1988). A new type of the manifestation of colony specificity in the compound ascidian, *Botrylloides violaceus* Oka. *The Biological Bulletin*, *175*(2), 240-245.

Hirose, E., Saito, Y., & Watanabe, H. (1990). Allogeneic rejection induced by cut surface contact in the compound ascidian, *Botrylloides simodensis*. *Invertebrate Reproduction & Development*, *17*(2), 159-164.

Hirose, M., Yokobori, S., & Hirose, E. (2008). Potential speciation of morphotypes in the photosymbiotic ascidian *Didemnum molle* in the Ryukyu Archipelago, Japan. *Coral Reefs*, *28*(1), 119-126.

Holland, P. W., Garcia-Fernàndez, J., Williams, N. A., & Sidow, A. (1994). Gene duplications and the origins of vertebrate development. *Development*, *1994*(Supplement), 125-133.

Holland, L. Z. (2016). Tunicates. *Current Biology*, *26*(4), R146-R152.

Huang, D., Meier, R., Todd, P. A., & Chou, L. M. (2007). Slow mitochondrial COI sequence evolution at the base of the metazoan tree and its implications for DNA barcoding. *Journal of Molecular Evolution*, *66*(2), 167-174.

Huelsenbeck, J. P., & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, *17*(8), 754-755.

Izquierdo Muñoz, A., Díaz Valdés, M., & Ramos-Esplá, A. A. (2009). Recent non-indigenous ascidians in the Mediterranean Sea. *Aquatic Invasions, 4*(1), 59-64.

Jeffery, W. R., Swalla, B. J., & Gilbert, S. (1997). Embryology of the Tunicates. In *Embryology: Constructing the organisms*. (pp. 331-364). Oxford University Press.

Jaffar, H. A., Akram, A. S., Arshan, M. K., Sivakumar, V., & Tamilselvi, M. (2016). Distribution and invasiveness of a colonial ascidian, *Didemnum psammathodes*, along the southern Indian coastal water. *Oceanologia*, *58*(3), 212-220.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., & Madden, T.L. (2008). "NCBI BLAST: a better web interface". *Nucleic Acids Research*, 36, W5-W9.

Karahan, A. (2020). Kuzeydoğu botryllid tulumlularının (ascidians) moleküler teknikler kullanılarak tanımlanması ve karşılaştırılması. (Unpublish report in Turkish-Proje No: YÖP-701-2018-2666). Institute of Marine Sciences, Middle East Technical University.

Karahan, A., Douek, J., Paz, G., & Rinkevich, B. (2016). Population genetics features for persistent, but transient, *Botryllus schlosseri* (Urochordata) congregations in a central Californian marina. *Molecular Phylogenetics and Evolution*, *101*, 19-31.

Katow, H., & Watanabe, H. (1980). Fine structure of fusion reaction in compound ascidian *Botryllus primigenus* Oka. *Developmental Biology*, *76*(1), 1-14.

Kayiş, Ş. (2011). Ascidian Tunicate, *Botryllus schlosseri* (Pallas, 1766) infestation on seahorse. *Bulletin of European Association of Fish Pathologist*, *31*(2), 81.

Kim, W. J., Lee, C. I., Kim, H. S., Han, H. S., Jee, Y. J., Kong, H. J., ... & Lee, S. J. (2012). Population genetic structure and phylogeography of the ascidian, *Halocynthia roretzi*, along the coasts of Korea and Japan, inferred from mitochondrial DNA sequence analysis. *Biochemical Systematics and Ecology*, *44*, 128-135.

Kocak, F., & Kucuksezgin, F. (2000). Sessile fouling organisms and environmental parameters in the marinas of the Turkish Aegean coast. *Indian Journal of Marine Sciences, 29*, 149-157.

Konuklugil, B.., Heydari, H., Genc, Y.., & Ozgen, U. (2018). Bioactivity screening of some marine species from Turkey's coasts. *Farmacia*, *66*(2), 342-346.

Koukouras, A., Vouldtiadou-Koukoura, E., Kevrekidis, T., & Vafidis, D. (1995). Ascidian fauna of the Aegean Sea with a check list of the eastern Mediterranean and Black Sea species. In *Annales de l'Institut Océanographique (Monaco)* (Vol. 71, No. 1, pp. 19-34).

Korf, I., Yandell, M., & Bedell, J. (2003). *Blast*. " O'Reilly Media, Inc."

Knowlton, N. (2000). Molecular genetic analyses of species boundaries in the sea. In *Marine genetics* (pp. 73-90). Springer, Dordrecht.

Kott, P. (1952). Observations on compound ascidians of the Plymouth area, with descriptions of two new species. *Journal of the Marine Biological Association of the United Kingdom*, *31*(1), 65-83.

Kott, P., & Mather, P. (1969). Antarctic Ascidiacea: Monographic Account of the Known Species Based on Specimens Collected Under US Government Auspices, *American Geophysical Union, 13*, 1947-1965.

Kumar, S., Stecher, G., & Tarra, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw0547

Lambert, G. (2001). A global overview of ascidian introductions and their possible impact on the endemic fauna. In *The biology of ascidians* (pp. 249-257). Springer, Tokyo.

Lambert, C. C., & Lambert, G. (1998). Non-indigenous ascidians in southern California harbors and marinas. *Marine Biology*, *130*(4), 675-688.

Lambert, G. (2007). Invasive sea squirts: a growing global problem. *Journal of Experimental Marine Biology and Ecology*, *342*(1), 3-4.

Leray, M., Yang, J. Y., Meyer, C. P., Mills, S. C., Agudelo, N., Ranwez, V., ... & Machida, R. J. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Frontiers in Zoology*, *10*(1), 34.

Lejeusne, C., Bock, D. G., Therriault, T. W., MacIsaac, H. J., & Cristescu, M. E. (2010). Comparative phylogeography of two colonial ascidians reveals contrasting invasion histories in North America. *Biological Invasions*, *13*(3), 635-650.

Librado, P., & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, *25*(11), 1451–1452. https://doi.org/10.1093/bioinformatics/btp187

Lopez-Legentil, S., & Turon, X. (2006). Population genetics, phylogeography and speciation of Cystodytes (Ascidiacea) in the western Mediterranean Sea. *Biological Journal of the Linnean Society*, *88*(2), 203-214.

Lopez-Legentil, S., Turon, X., & Planes, S. (2006). Genetic structure of the star sea squirt, *Botryllus schlosseri*, introduced in southern European harbours. *Molecular Ecology*, *15*(13), 3957-3967.

Lopez-Legentil, S., & Turon, X. (2007). Lack of genetic variation in mtDNA sequences over the amphiatlantic distribution range of the ascidian *Ecteinascidia turbinata*. *Molecular Phylogenetics and Evolution*, *45*(1), 405-408.

Lynch, W. F. (1961). Extrinsic factors influencing metamorphosis in bryozoan and ascidian larvae. *American Zoologist*, 59-66.

Lynch, M., & Crease, T. J. (1990). The analysis of population survey data on DNA sequence variation. *Molecular Biology and Evolution*, *7*(4), 377-394.

Maddison, W. P. (1997). Gene trees in species trees. *Systematic biology*, *46*(3), 523-536.

Matthysse, A. G., Deschet, K., Williams, M., Marry, M., White, A. R., & Smith, W. C. (2004). A functional cellulose synthase from ascidian epidermis. *Proceedings of the National Academy of Sciences*, *101*(4), 986-991.

Manni, L., Anselmi, C., Cima, F., Gasparini, F., Voskoboynik, A., Martini, M., ... & Ballarin, L. (2018). Sixty years of experimental studies on the blastogenesis of

the colonial tunicate *Botryllus schlosseri*. *Developmental Biology*, *448*(2), 293-308.

Manni, L., & Burighel, P. (2006). Common and divergent pathways in alternative developmental processes of ascidians. *BioEssays*, *28*(9), 902-912.

Manni, L., Zaniolo, G., Cima, F., Burighel, P., & Ballarin, L. (2006). *Botryllus schlosseri*: a model ascidian for the study of asexual reproduction. *Developmental dynamics: an official publication of the American Association of Anatomists*, *236*(2), 335-352.

Makabe, K. W., Kawashima, T., Kawashima, S., Minokawa, T., Adachi, A., Kawamura, H., ... & Arioka, S. (2001). Large-scale cDNA analysis of the maternal genetic information in the egg of *Halocynthia roretzi* for a gene expression catalog of ascidian development. *Development*, *128*(13), 2555-2567.

Maldonado, M., & Uriz, M. J. (1995). Biotic affinities in a transitional zone between the Atlantic and the Mediterranean: a biogeographical approach based on sponges. *Journal of Biogeography*, 89-110.

Michaelsen, V. W. (1927). Einige neue westaustralische ptychobranchiate Ascidien. *Zoologischer Anzeiger*, *71*, 193-203.

Millar, R. H. (1966). Port Phillip survey 1957–1963. Ascidiacea. *Memoirs of the Natural Museum Victoria*, *27*, 357-384.

Miroğlu, A., & Yalçin, M. (2017). Ascidians (Tunicata, Urochordata) Fauna of Turkey Coasts. The 3rd International Symposium on EuroAsian Biodiversity, Minsk-BELARUS, 05-08 July.

Monniot, F. (1988). Ascidies de Nouvelle-Calédonie. V: Polycitoridae du lagon. *Bulletin du Muséum national d'histoire naturelle. Section A, Zoologie, biologie et écologie animales*, *10*(2), 197-235.

Mukai, H. (1977). Comparative studies on the structure of reproductive organs of four botryllid ascidians. *Journal of Morphology*, *152*(3), 363-379.

Nabholz, B., Glémin, S., & Galtier, N. (2009). The erratic mitochondrial clock: variations of mutation rate, not population size, affect mtDNA diversity across birds and mammals. *BMC Evolutionary Biology*, *9*(1), 54.

Nakauchi, M. (1981). Development and budding in the oozooids of *Polyclinum aurantium* (a colonial ascidian) (Protochordata). *Journal of Zoology*, *194*(1), 1-9.

Nakauchi, M. (1982). Asexual development of ascidians: its biological significance, diversity, and morphogenesis. *American Zoologist*, *22*(4), 753-763.

Naranjo, S. A., Carballo, J. L., & Garcia-Gomez, J. C. (1996). Effects of environmental stress on ascidian populations in Algeciras Bay (southern Spain). Possible marine bioindicators? *Marine Ecology Progress Series*, *144*, 119-131.

Nei, M. (1982). Evolution of human races at the gene level. *Human genetics, part A: the unfolding genome. Alan R. Liss, New York*, *167.*

Nichols, R. (2001). Gene trees and species trees are not the same. *Trends in Ecology & Evolution*, *16*(7), 358-364.

Nomaguchi, T. A., Nishijima, C., Minowa, S., Hashimoto, M., Haraguchi, C., Amemiya, S., & Fujisawa, H. (1997). Embryonic thermosensitivity of the ascidian, *Ciona savignyi*. *Zoological science*, *14*(3), 511-515.

Nydam, M. L., & Harrison, R. G. (2010). Polymorphism and divergence within the ascidian genus *Ciona. Molecular Phylogenetics and Evolution*, *56*(2), 718-726.

Oka, H., & Watanabe, H. (1957). Vascular budding, a new type of budding in *Botryllus*. *The Biological Bulletin*, *112*(2), 225-240.

Oka, H., & Watanabe, H. (1959). Vascular budding in *Botrylloides*. *The Biological Bulletin*, *117*(2), 340-346.

Okuş, E., Sur, H. I., Yüksek, A., Yılmaz, I. N., Aslan-Yılmaz, A., Karhan, S. Ü., ... & Müftüoğlu, A. E. (2004). Datça-Bozburun özel çevre koruma bölgesinin denizsel ve kıyısal alanlarının biyolojik çeşitliliğinin tespiti projesi. *Ankara: TC Çevre ve Orman Bakanlığı Özel Çevre Koruma Kurumu Başkanlığı (in Turkish)*.

Okuş, E., Altiok, H., Yüksek, A., Yılmaz, N., Yılmaz, A. A., Karhan, S. Ü., ... & Kalkan, E. (2007). Biodiversity in western part of the Fethiye Bay. *Journal of the Black Sea/Mediterranean Environment*, *13*(1).

Palumbi, S. R. (1994). Genetic divergence, reproductive isolation, and marine speciation. *Annual review of Ecology and Systematics*, *25*(1), 547-572.

Palumbi, S. R., Grabowsky, G., Duda, T., Geyer, L., & Tachino, N. (1997). Speciation and population genetic structure in tropical Pacific sea urchins. *Evolution*, *51*(5), 1506-1517.

Pancer, Z., Gershon, H., & Rinkevich, B. (1995). Coexistence and possible parasitism of somatic and germ cell lines in chimeras of the colonial urochordate *Botryllus schlosseri*. *The Biological Bulletin*, *189*(2), 106-112.

Paz, G., Douek, J., Mo, C., Goren, M., & Rinkevich, B. (2003). Genetic structure of *Botryllus schlosseri* (Tunicata) populations from the Mediterranean coast of Israel. *Marine Ecology Progress Series*, *250*, 153-162.

Pérez-Portela, R., & Turon, X. (2008). Cryptic divergence and strong population structure in the colonial invertebrate *Pycnoclavella communis* (Ascidiacea) inferred from molecular data. *Zoology*, *111*(2), 163-178.

Pérez-Portela, R., Bishop, J. D. D., Davis, A. R., & Turon, X. (2009). Phylogeny of the families Pyuridae and Styelidae (Stolidobranchiata, Ascidiacea) inferred from mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution*, *50*(3), 560-570.

Pineda, M. C., López-Legentil, S., & Turon, X. (2011). The whereabouts of an ancient wanderer: global phylogeography of the solitary ascidian *Styela plicata*. *PLoS One*, *6*(9), e25495.

Pınar, E. (1974). Türkiye'nin bazı limanlarında fouling-boring organizmalar ve antifouling-antiboring boyaların bunlar üzerine etkisi. Ege Üniversitesi Fen Fak İlmi Raporlar Serisi 170, 1-67 (in Turkish).

Pusphabai Rajesh, R., Santhana Ramasamy, M., & Murugan, A. (2010). Anticancer activity of the ascidian *Polyclinum indicum* against cervical cancer cells (HeLa) mediated through apoptosis induction. *Medicinal Chemistry*, *6*(6), 396-405.

Rambaut, A. (2016). FigTree. Tree figure drawing tool. 1.4. 3. *Institute of Evolutionary Biology, University of Edinburgh. URL< http://tree. bio. ed. ac. uk/software/figtree*.

Ramos-Onsins, S. E., & Rozas, J. (2002). Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution*, *19*(12), 2092-2100.

Ranasinghe, J. A., Mikel, T. K., Velarde, R. G., Weisberg, S. B., Montagne, D. E., Cadien, D. B., & Dalkey, A. (2005). The prevalence of non-indigenous species in southern California embayments and their effects on benthic macroinvertebrate communities. *Biological Invasions*, *7*(4), 679-686.

Reem, E., & Rinkevich, B. (2014). *Botryllus schlosseri* and *Botrylloides leachii* (Chordata, Ascidiacea) have not been recorded in the Red Sea. *Marine Biodiversity*, *44*(4), 585-587.

Reem, E., Douek, J., Paz, G., Katzir, G., & Rinkevich, B. (2017). Phylogenetics, biogeography and population genetics of the ascidian *Botryllus schlosseri* in the Mediterranean Sea and beyond. *Molecular Phylogenetics and Evolution*, *107*, 221-231.

Reem, E., Douek, J., & Rinkevich, B. (2017). Ambiguities in the taxonomic assignment and species delineation of botryllid ascidians from the Israeli Mediterranean and other coastlines. *Mitochondrial DNA Part A*, *29*(7), 1073-1080.

Reynolds, J., Weir, B. S., & Cockerham, C. C. (1983). Estimation of the coancestry coefficient: basis for a short-term genetic distance. *Genetics*, *105*(3), 767-779.

Rinkevich, B., (2005). Natural chimerism in colonial urochordates. *Journal of Experimental Marine Biology and Ecology*, 322, 93-109.

Rinkevich, B. (2011). Quo vadis chimerism? *Chimerism*, *2*(1), 1-5.

Rinkevich, B. (2017). 11 Senescence in modular animals. *The Evolution of Senescence in the Tree of Life*, 220.

Rinkevich, B., & Weissman, I. L. (1992). Allogeneic resorption in colonial protochordates: consequences of nonself recognition. *Developmental & Comparative Immunology*, *16*(4), 275-286.

Rius, M., & Shenkar, N. (2012). Ascidian introductions through the Suez Canal: The case study of an Indo-Pacific species. *Marine Pollution Bulletin*, *64*(10), 2060-2068.

Rius, M., Pascual, M., & Turon, X. (2008). Phylogeography of the widespread marine invader *Microcosmus squamiger* (Ascidiacea) reveals high genetic diversity of introduced populations and non-independent colonizations. *Diversity and Distributions*, *14*(5), 818-828.

Robinson, A. R., Leslie, W. G., Theocharis, A., & Lascaratos, A. (2001). Mediterranean sea circulation. *Ocean Currents*, *1*, 19

Rocha, R. M., Salonna, M., Griggio, F., Ekins, M., Lambert, G., Mastrototaro, F., ... & Gissi, C. (2019). The power of combined molecular and morphological analyses for the genus *Botrylloides*: identification of a potentially global invasive ascidian and description of a new species. *Systematics and Biodiversity*, *17*(5), 509-526.

Rodriguez, D., Sanders, E. N., Farell, K., Langenbacher, A. D., Taketa, D. A., Hopper, M. R., ... & De Tomaso, A. W. (2014). Analysis of the basal chordate *Botryllus schlosseri* reveals a set of genes associated with fertility. *BMC Genomics*, *15*(1), 1183.

Sabbadin, A. (1971). Self-and cross-fertilization in the compound ascidian *Botryllus schlosseri*. *Developmental Biology*, *24*(3), 379-391.

Sabbadin, A., & Zaniolo, G. (1979). Sexual differentiation and germ cell transfer in the colonial ascidian *Botryllus schlosseri*. *Journal of Experimental Zoology*, *207*(2), 289-304.

Saito, Y., Shirae, M., Okuyama, M., & Cohen, S. (2001). Phylogeny of botryllid ascidians. In *The Biology of Ascidians* (pp. 315-320). Springer, Tokyo.

Saito, Y., Hirose, E., & Watanabe, H. (1994. Allorecognition in compound ascidians. *International Journal of Developmental Biology*, *38*(2), 237-247.

Saretzki, G. (2010). Cellular senescence in the development and treatment of cancer. *Current Pharmaceutical Design*, *16*(1), 79-100.

Satoh, N., Deno, T., Nishida, H., Nishikata, T., & Makabe, K. W. (1990). Cellular and molecular mechanisms of muscle cell differentiation in ascidian embryos. In *International Review of Cytology* (Vol. 122, pp. 221-258). Academic Press.

Scofield, V. L., & Nagashima, L. S. (1982). Morphology and genetics of rejection reactions between oozooids from the tunicate *Botryllus schlosseri*. *The Biological Bulletin*, *165*(3), 733-744.

Schoener, A. (1982). Artificial substrates in marine environments. *Artificial Substrates. Ann Arbor Scientific Publications, Ann Arbor, MI, USA*, 1-22.

Seo, H. C., Kube, M., Edvardsen, R. B., Jensen, M. F., Beck, A., Spriet, E., ... & Chourrout, D. (2001). Miniature genome in the marine chordate *Oikopleura dioica*. *Science*, *294*(5551), 2506-2506.

Shenkar, N., & Swalla, B. J. (2011). Global diversity of Ascidiacea. *PLoS One*, *6*(6), e20657.

Shenkar N., Gittenberger A., Lambert G., Rius M., Moreira Da Rocha R., Swalla B.J. & Turon X. (2019). WoRMS Ascidiacea: Ascidiacea World Database (version 2019-03-05). In: Species 2000 & ITIS Catalogue of Life, 2019 Annual Checklist (Roskov Y., Ower G., Orrell T., Nicolson D., Bailly N., Kirk P.M., Bourgoin T., DeWalt R.E., Decock W., Nieukerken E. van, Zarucchi J., Penev L., eds.). Digital resource at www.catalogueoflife.org/annual-checklist/2019. Species 2000: Naturalis, Leiden, the Netherlands. ISSN 2405-884X.

Sherwood, N. M., Tello, J. A., & Roch, G. J. (2006). Neuroendocrinology of protochordates: insights from *Ciona* genomics. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *144*(3), 254-271.

Simkanin, C., Fofonoff, P. W., Larson, K., Lambert, G., Dijkstra, J. A., & Ruiz, G. M. (2016). Spatial and temporal dynamics of ascidian invasions in the continental United States and Alaska. *Marine Biology*, *163*(7), 163.

Simmen, M. W., Leitgeb, S., Clark, V. H., Jones, S. J., & Bird, A. (1998). Gene number in an invertebrate chordate, *Ciona intestinalis*. *Proceedings of the National Academy of Sciences*, *95*(8), 4437-4440.

Sims, L. L. (1984). Osmoregulatory capabilities of three macrosympatric stolidobranch ascidians, *Styela clava* Herdman, *S. plicata* (Lesueur), and *S. montereyensis* (Dall). *Journal of Experimental Marine Biology and Ecology*, *82*(2-3), 117-129.

Sloan, D. B., Alverson, A. J., Chuckalovcak, J. P., Wu, M., McCauley, D. E., Palmer, J. D., & Taylor, D. R. (2012). Rapid evolution of enormous, multichromosomal genomes in flowering plant mitochondria with exceptionally high mutation rates. *PLoS Biology*, *10*(1), e1001241.

Smith, K. F., Stefaniak, L., Saito, Y., Gemmill, C. E., Cary, S. C., & Fidler, A. E. (2012). Increased inter-colony fusion rates are associated with reduced COI haplotype diversity in an invasive colonial ascidian Didemnum vexillum. *PloS One*, *7*(1), e30473.

Swalla, B. J., Cameron, C. B., Corley, L. S., & Garey, J. R. (2000). Urochordates are monophyletic within the deuterostomes. *Systematic Biology*, *49*(1), 52-64.

Swalla, B. J. (2006). Building divergent body plans with similar genetic pathways. *Heredity*, *97*(3), 235-243.

Stoner, D. S., Rinkevich, B., & Weissman, I. L. (1999). Heritable germ and somatic cell lineage competitions in chimeric colonial protochordates. *Proceedings of the National Academy of Sciences*, *96*(16), 9148-9153.

Stoner, D. S., & Weissman, I. L. (1996). Somatic and germ cell parasitism in a colonial ascidian: possible role for a highly polymorphic allorecognition system. *Proceedings of the National Academy of Sciences*, *93*(26), 15254-15259.

Svane, I., & Young, C. M. (1989). The ecology and behaviour of ascidian larvae. *Oceanography and Marine Biology*, *27*, 45-90.

Ulman, A., Ferrario, J., Occhpinti-Ambrogi, A., Arvanitidis, C., Bandi, A., Bertolino, M., ... & Ramos-Esplá, A. (2017). A massive update of non-indigenous species records in Mediterranean marinas. *PeerJ*, *5*, e3954.

Uysal, A. 1976. Ascidians in Turkish Waters (in Turkish). İ.Ü. Fen Fakültesi Hidrobioloji, Araştırma Enstitüsü Yayınları. Sayı:15, 29.

Wada, H., Makabe, K. W., Nakauchi, M., & Satoh, N. (1992). Phylogenetic relationships between solitary and colonial ascidians, as inferred from the sequence of the central region of their respective 18S rDNAs. *The Biological Bulletin*, *183*(3), 448-455.

Watanabe, H. (1953). Studies on the regulation in fused colonies in *Botryllus primigenus* (Ascidiae Compositae). *Science Reports of Tokyo Bunrika Daigaku section B*, *7*, 183-198.

Watanabe, H., Taneda, Y., 1982. Self or non-self recognition in compound ascidians. American Zoologist, 22, 775–782.

Watterson, R. L. (1945). Asexual reproduction in the colonial tunicate, *Botryllus schlosseri* (Pallas) Savigny, with special reference to the developmental history of intersiphonal bands of pigment cells. *The Biological Bulletin*, *88*(1), 71-103.

Wilson-Sanders, S. E. (2011). Invertebrate models for biomedical research, testing, and education. *ILAR Journal*, *52*(2), 126-152.

Vandepas, L. E., Oliveira, L. M., Lee, S. S., Hirose, E., Rocha, R. M., & Swalla, B. J. (2015). Biogeography of *Phallusia nigra*: is it really black and white?. *The Biological Bulletin*, *228*(1), 52-64.

VanName, W. G. (1945). North and South American ascidians. *Bulletin of the American Museum of Natural History*, *84*, 1-462.

Virata, M. J., & Zeller, R. W. (2010). Ascidians: an invertebrate chordate model to study Alzheimer's disease pathogenesis. *Disease Models & Mechanisms*, *3*(5-6), 377-385.

Voskoboynik, A. (2009). Chimerism a natural ability to tolerate kin, evolutionary traits connecting mammalian and protochordates. *Invertebrate Survival Journal*, *6*(1 (Suppl)), S9-S20.

Voskoboynik, A., Neff, N. F., Sahoo, D., Newman, A. M., Pushkarev, D., Koh, W., ... & Ishizuka, K. J. (2013). The genome sequence of the colonial chordate, *Botryllus schlosseri*. *elife*, *2*, e00569.

Voskoboynik, A., & Weissman, I. L. (2015). *Botryllus schlosseri*, an emerging model for the study of aging, stem cells, and mechanisms of regeneration. *Invertebrate Reproduction & Development*, *59*(sup1), 33-38.

Voskoboynik, A., Newman, A. M., Kowarsky, M., & Weissman, I. L. (2018). Natural Chimerism and Tolerance Induction in a Colonial Chordate. In *Advances in Comparative Immunology.* (pp. 503-519). *Springer, Cham*.

Tajima, F. (1993). Simple methods for testing the molecular evolutionary clock hypothesis. *Genetics*, *135*(2), 599-607.

Tanaka, K., & Watanabe, H. (1973). Allogeneic inhibition in a compound ascidian, *Botryllus primigenus* Oka. I. Processes and features of "nonfusion" reaction. *Cellular Immunology*, *7*(3), 410-426.

Tarjuelo, I., Posada, D., Crandall, K. A., Pascual, M., & Turon, X. (2004). Phylogeography and speciation of colour morphs in the colonial ascidian *Pseudodistoma crucigaster*. *Molecular Ecology*, *13*(10), 3125-3136.

Tiozzo, S., Brown, F. D., & De Tomaso, A. W. (2008). Regeneration and stem cells in ascidians. In *Stem Cells* (pp. 95-112). Springer, Dordrecht.

Tokioka, T. (1961). Ascidians collected during the Melanesia expedition of the Osaka Museum of Natural History-I. Ascidians presented by Dr. Rla Catala ot the Aquarium of Noumea.

Tracy, B. M., & Reyns, N. B. (2014). Spatial and temporal patterns of native and invasive ascidian assemblages in a Southern California embayment. *Aquatic Invasions*, *9*(4).

Yalçın, M. (2018). *Ordu İli (Türkiye) Sahil Şeridindeki Ascidiacea (Tunicata, Urochordata) Faunası Üzerine Bir Araştırma* (Master's thesis, Ordu Üniversitesi, Ordu, Turkey). Retrieved from http://earsiv.odu.edu.tr:8080/jspui/handle/11489/788.

Yalvaç M., Deveci E. Ü. (2007). Mezitli Deresi'nin Yağışlı-Yağışsız Dönemlerde Su Kalitesindeki Değişiminin Araştırılması. *Turkish Journal of Aquatic Life,* 5(8), 584-595.

Zeng, L., & Swalla, B. J. (2005). Molecular phylogeny of the protochordates: chordate evolution. *Canadian Journal of Zoology*, *83*(1), 24-33.

Zeng, L., Jacobs, M. W., & Swalla, B. J. (2006). Coloniality has evolved once in stolidobranch ascidians. *Integrative and Comparative Biology*, *46*(3), 255-268.

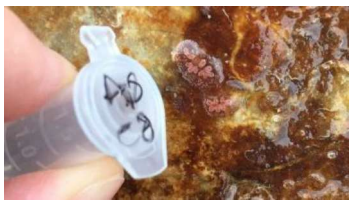
Zhan, A., Briski, E., Bock, D. G., Ghabooli, S., & MacIsaac, H. J. (2015). Ascidians as models for studying invasion success. *Marine Biology*, *162*(12), 2449-2470.

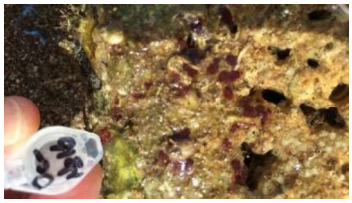
APPENDICES

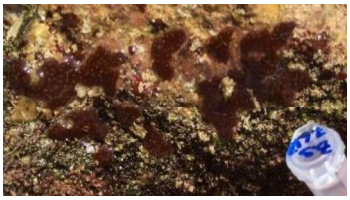
A. ID's and Field Photographs of Samples

Sample IDs	Field Photos	Species	Color	Station	Date
L1		*B. anceps*	Pink	Konacık	26.09.2018
L3		*B. anceps*	Dark brown	Konacık	26.09.2018
L4		*B. anceps*	Pink	Konacık	26.09.2018
L5		*B. anceps*	Purple	Konacık	26.09.2018
L6		*B. anceps*	Pink	Konacık	26.09.2018

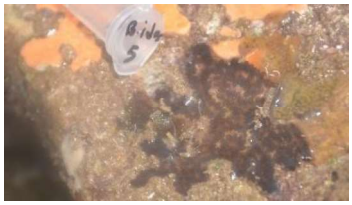
L7		*B. anceps*	Reddish orange	Konacık	26.09.2018
L8		*B. anceps*	Reddish orange	Konacık	26.09.2018
L10		*B. anceps*	Reddish orange	Konacık	26.09.2018
C4		*B. anceps*	Pink	Alanya	24.10.2018
C5		*B. anceps*	Blackish brown	Alanya	24.10.2018
C6		*B. anceps*	Purple	Alanya	24.10.2018

C7		*B. anceps*	Claret red	Alanya	24.10.2018
C8		*B. anceps*	Pink	Alanya	24.10.2018
C9		*B. anceps*	Dark brown	Alanya	24.10.2018
C13		*B. anceps*	Purple	Alanya	24.10.2018
C14		*B. anceps*	Pink	Alanya	24.10.2018
C15		*B. anceps*	Blackish brown	Alanya	24.10.2018
M2-1		*B. anceps*	Purple	Mezitli	06.09.2018

M2-3		*B. anceps*	Brown	Mezitli	06.09.2018
M2-4		*B. anceps*	Dark brown	Mezitli	06.09.2018
M2-5		*B. anceps*	Purple	Mezitli	06.09.2018
M2-11		*B. anceps*	Pink	Mezitli	06.09.2018
M2-16		*B. anceps*	Purple	Mezitli	06.09.2018
M2-18		*B. anceps*	Purple	Mezitli	06.09.2018
M2-19		*B. anceps*	Pink	Mezitli	06.09.2018

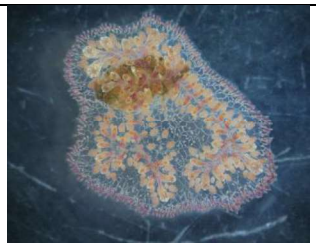
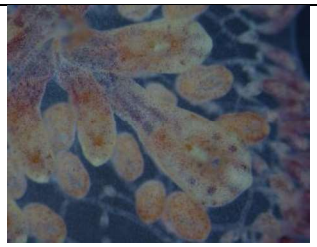
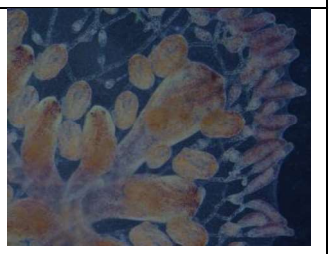
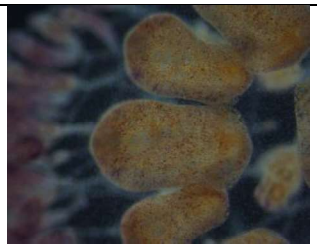
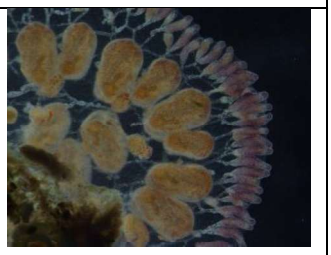
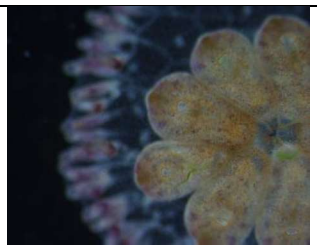
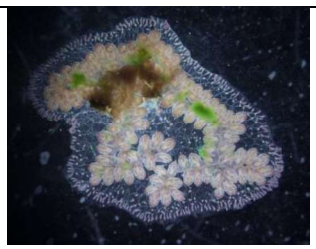
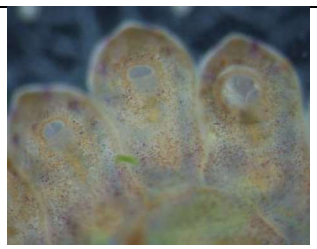
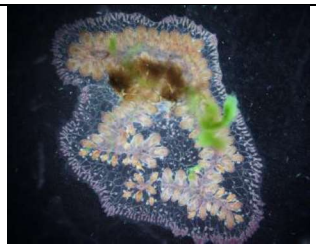
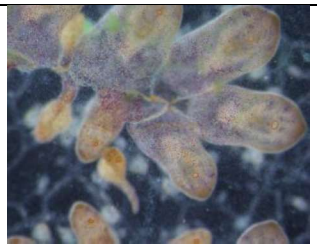
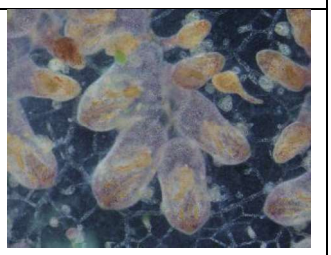
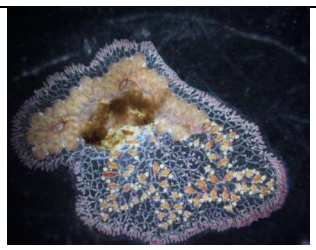
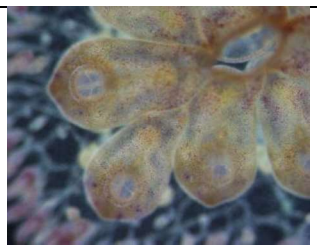
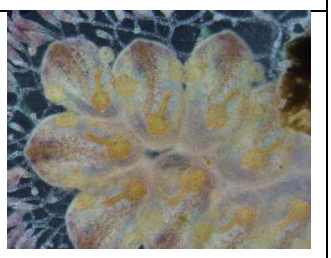
P6		*B. anceps*	Purple	Mezitli	14.10.2019
BSS1		*Botrylloides sp.*	Dark reddish brown with creamy lines	Kızkalesi	05.07.2018
BSS2		*Botrylloides sp.*	Black with creamy lines	Kızkalesi	05.07.2018
BSS3		*Botrylloides sp.*	Reddish brown	Kızkalesi	05.07.2018
BSS4		*Botrylloides sp.*	Brown with yellow pigmentation around oral siphon	Kızkalesi	05.07.2018
Z7		*Botrylloides sp.*	Dark brown with creamy lines	Kızkalesi	03.08.2018
Z17		*Botrylloides sp.*	Brown	Kızkalesi	03.08.2018

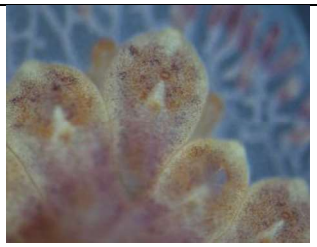
Z18		*Botrylloides sp.*	Brown with yellow pigmentation around oral siphon	Kızkalesi	03.08.2018
J18-BS5		*Botrylloides sp.*	Brown with yellow pigmentation around oral siphon	Kızkalesi	05.07.2018
J18-BS6		*Botrylloides sp.*	Black with creamy lines	Kızkalesi	05.07.2018
J18-BS10		*Botrylloides sp.*	Brown with creamy lines	Kızkalesi	05.07.2018
J18-BS11		*Botrylloides sp.*	Reddish brown	Kızkalesi	05.07.2018
J18-BS12		*Botrylloides sp.*	Black with creamy lines	Kızkalesi	05.07.2018
BS7		*Botrylloides sp.*	Black with creamy lines	Kızkalesi	01.11.2017

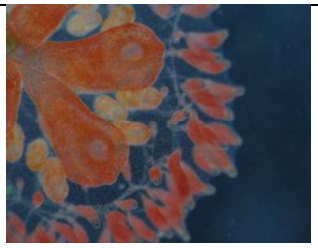
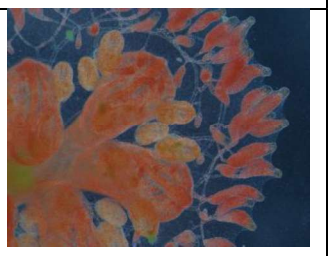
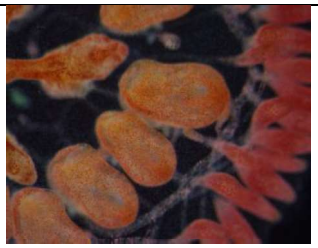
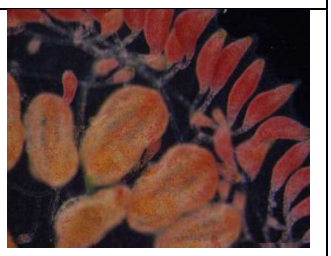
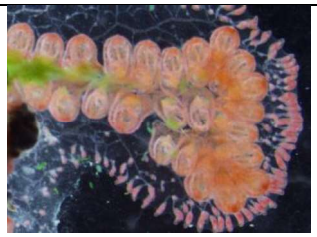
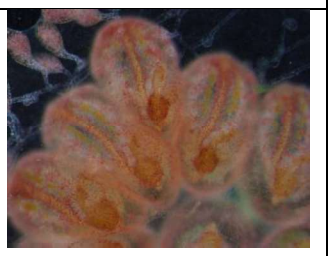
BS9		*Botrylloides sp.*	Brown with creamy lines	Kızkalesi	01.11.2017
Bides3		*Botrylloides sp.*	Light brown with creamy lines	Kızkalesi	10.07.2014
Bides4		*Botrylloides sp.*	Brown	Kızkalesi	10.07.2014
Bides5		*Botrylloides sp.*	Dark brown	Kızkalesi	10.07.2014
Botryllus 1		*Botrylloides sp.*	Brown	Kızkalesi	10.07.2014
Botryllus 2		*Botrylloides sp.*	Brown with creamy lines	Kızkalesi	10.07.2014
GK28		*Botrylloides sp.*	Brown with yellow strips	Kızkalesi	03.10.2018

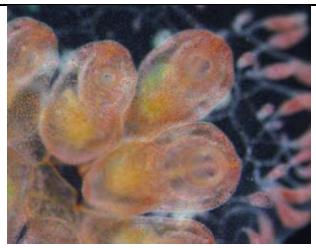
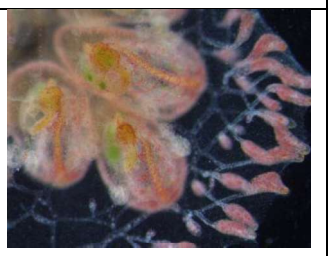
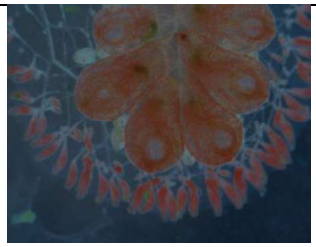
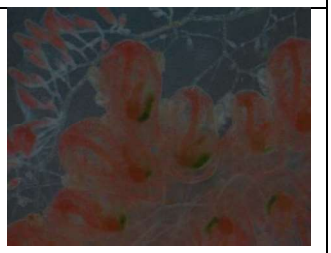
GK42		*Botrylloides sp.*	Brown with yellow pigmentation around oral siphon	Kızkalesi	03.10.2018
G3		*Botrylloides israeliense*	Dark brown with yellow bands	Tisan	03.10.2018
GK23	N.A	*Botrylloides sp.*	-	Kızkalesi	03.10.2018
3M	N.A	*Botrylloides sp.*	-	Kızkalesi	2012
M4	N.A	*Botrylloides sp.*	-	Kızkalesi	2012
M5	N.A	*Botrylloides sp.*	-	Kızkalesi	2012
M6	N.A	*Botrylloides sp.*	-	Kızkalesi	2012
M7	N.A	*Botrylloides sp.*	-	Kızkalesi	2012
8M	N.A	*Botrylloides sp.*	-	Kızkalesi	2012
1MBDES	N.A	*Botrylloides sp.*	-	Kızkalesi	2012
F2	N.A	*Botrylloides sp.*	-	Mezitli	14.10.2019
F3	N.A	*Botrylloides sp.*	-	Mezitli	14.10.2019
F5	N.A	*Botrylloides sp.*	-	Mezitli	14.10.2019
F6	N.A	*Botrylloides sp.*	-	Mezitli	14.10.2019
F7	N.A	*Botrylloides sp.*	-	Mezitli	14.10.2019
P3	N.A	*Botrylloides anceps*	-	Mezitli	14.10.2019

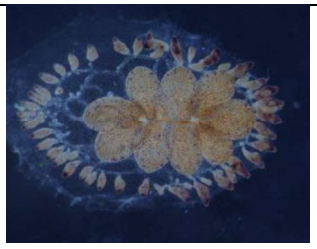
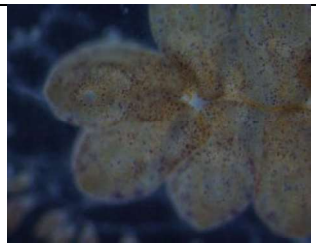
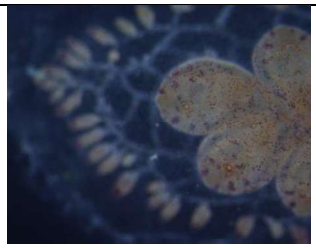
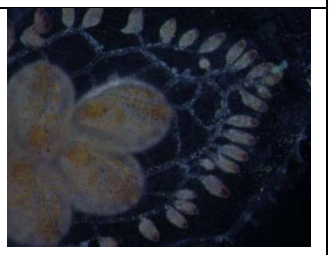
B. Blastogenic Cycle Monitoring of *B. anceps* and *Botrylloides sp.*

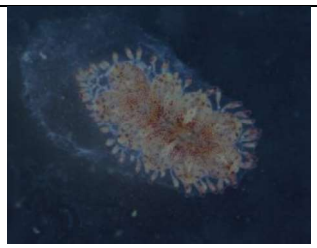
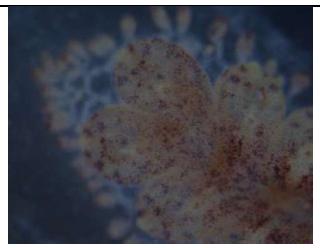
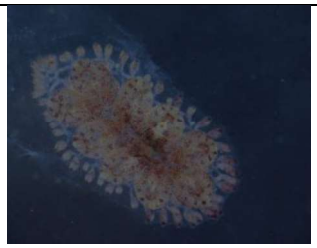
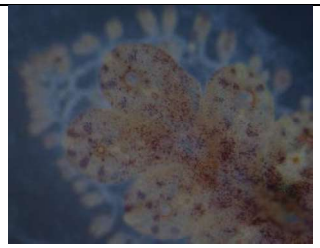
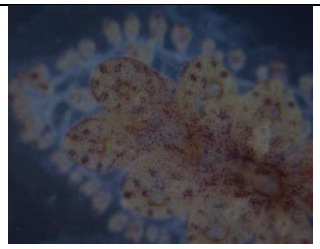
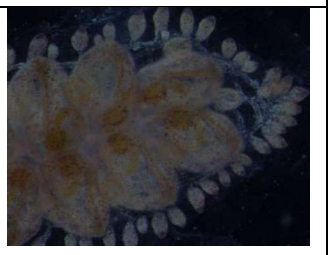
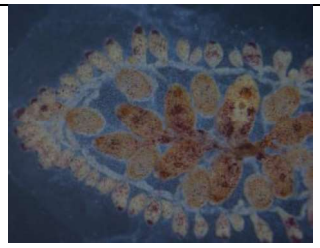
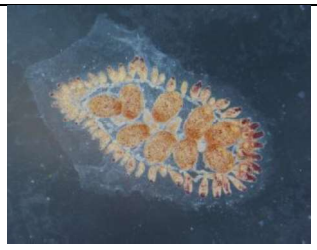
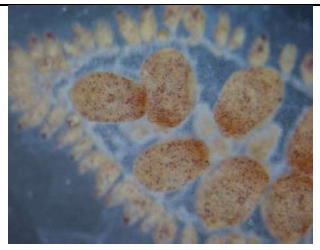
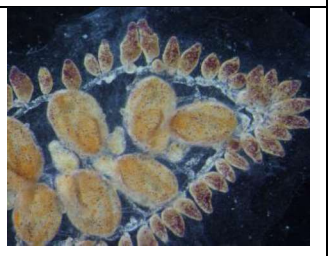
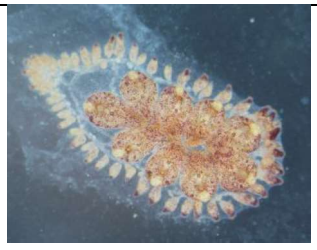
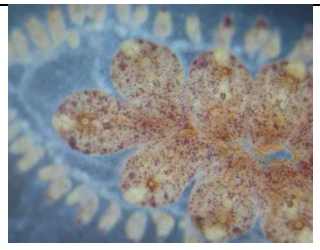
L1	Colony/System	Close up: Front	Close up: Back
Day 1			
Day 2			
Day 3			
Day 4			
Day 5			
Day 6			

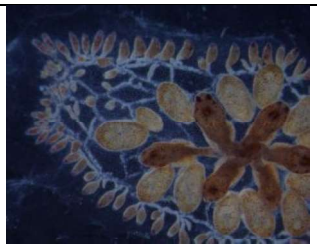
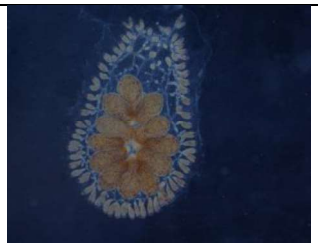
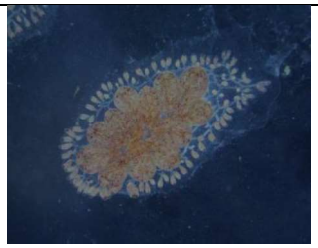
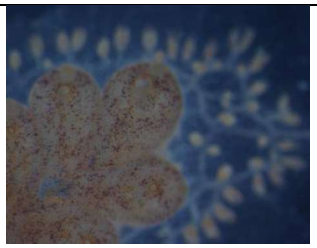
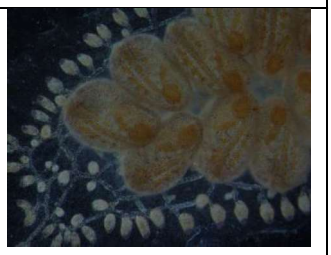
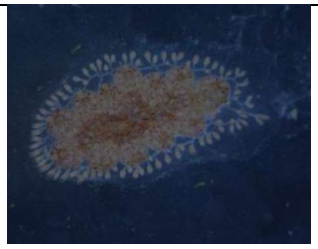
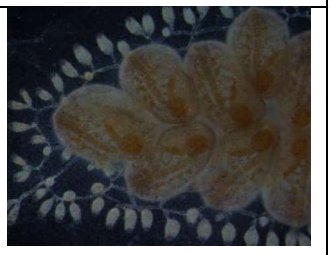
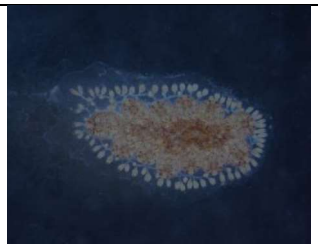
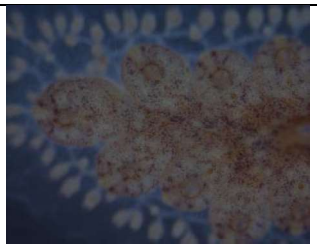
Day 7			

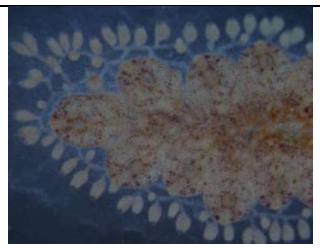
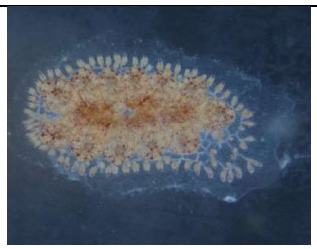
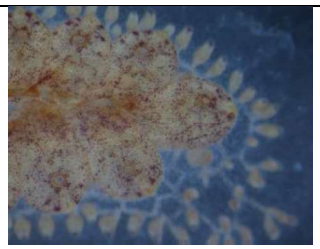
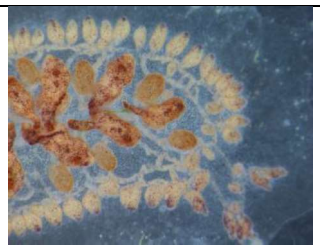
L10	Colony/System	Close up: Front	Close up: Back
Day 1			
Day 2			
Day 3			
Day 4			

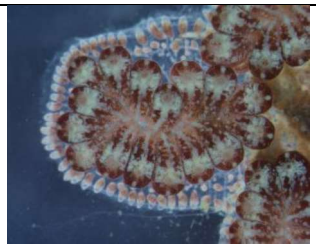
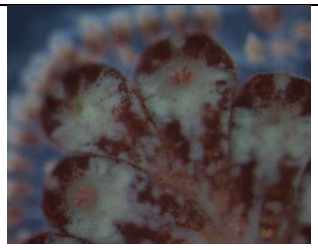
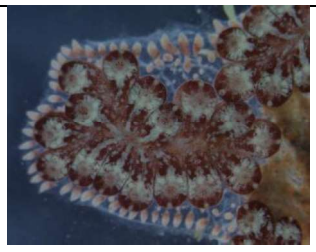
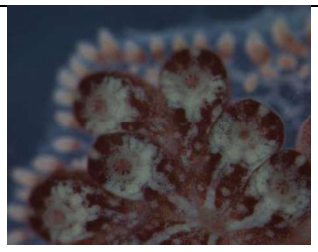
Day 5			
Day 6			
Day 7			

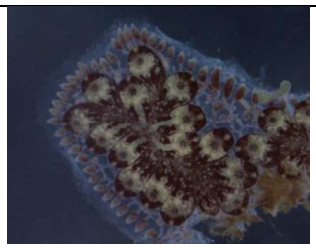
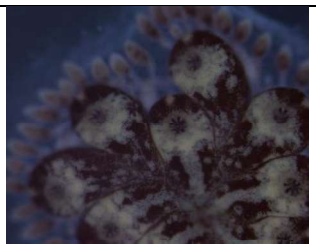
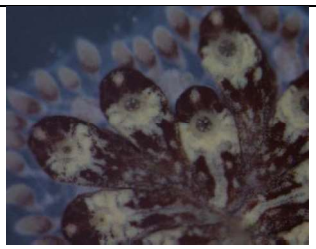
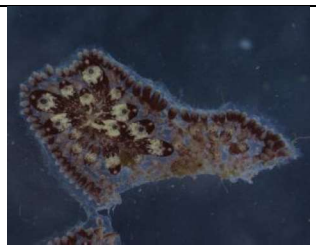
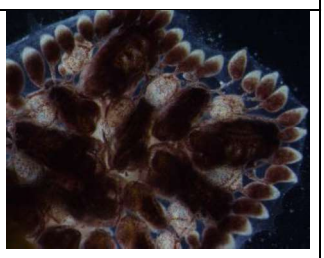
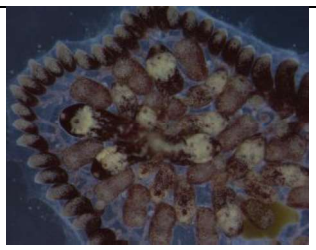
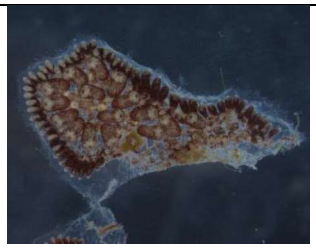
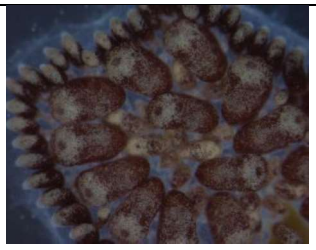
L4	Colony/System	Close up: Front	Close up: Back
Day 1			
Day 2			
Day 3			

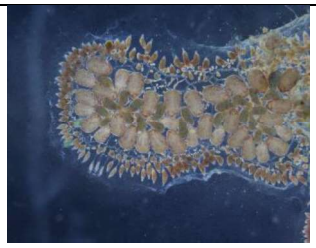
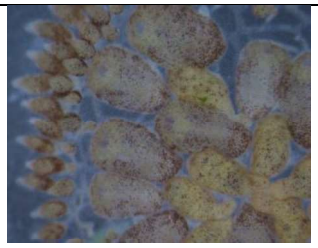
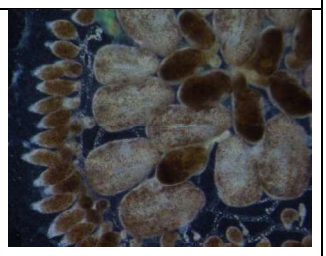
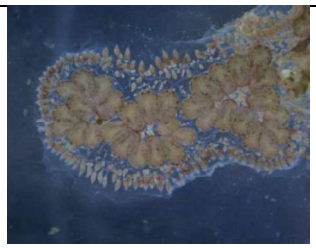
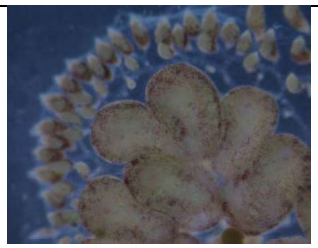
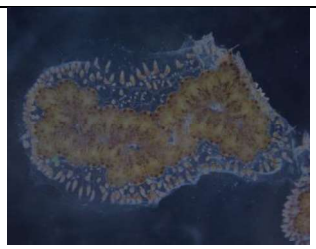
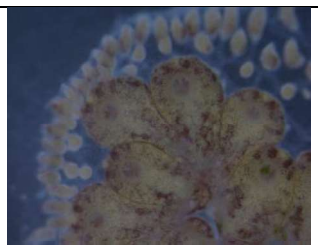
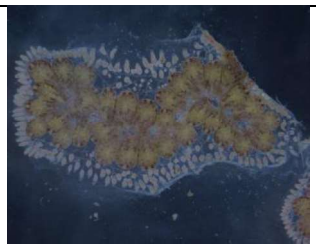
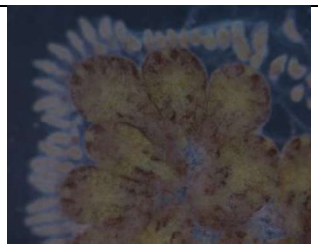
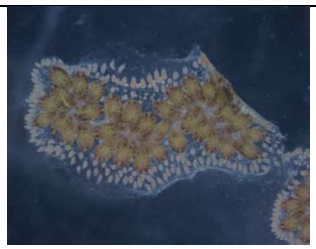
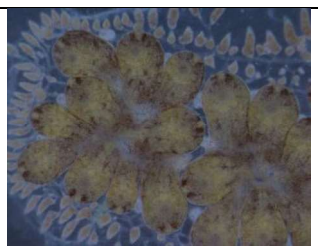
Day 4			
Day 5			
Day 6			
Day 7			
Day 8			
Day 9			

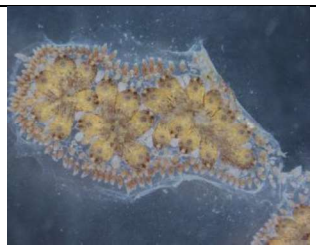
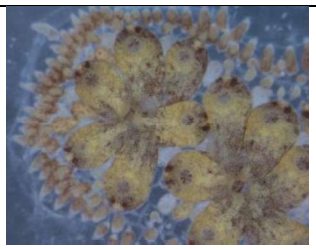
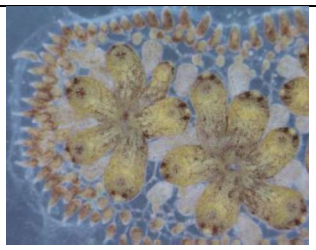
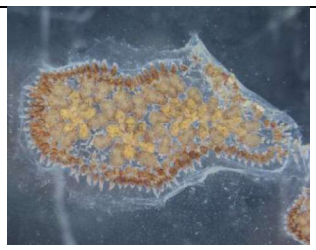
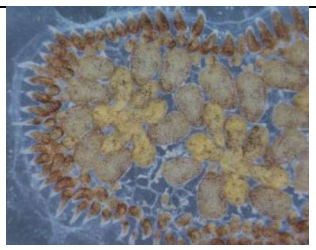
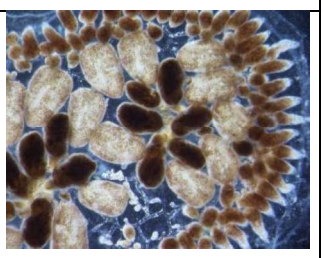
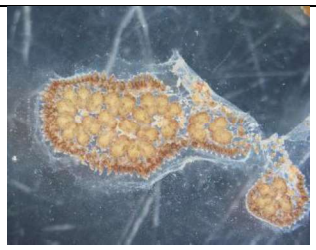
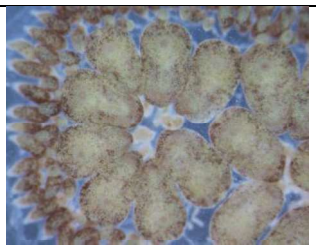
L6	Colony/System	Close up: Front	Close up: Back
Day 1			
Day 2			
Day 3			
Day 4			
Day 5			
Day 6			

Day 7			
Day 8			
Day 9			

GK2 8	Colony/System	Close up: Front	Close up: Back
Day 1			
Day 2			

Day 3			
Day 4			
Day 5			
Day 6			
Day 7			
Day 8			

GK42	Colony/System	Close up: Front	Close up: Back
Day 1			
Day 2			
Day 3			
Day 4			
Day 5			
Day 6			

Day 7			
Day 8			
Day 9			
Day 10			
Day 11			
Day 12