Title: Identification and characterisation of *Botrylloides* species from Aotearoa New Zealand coasts

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ABSTRACT

Botryllid ascidians possess diverse biological abilities like whole-body regeneration (WBR), hibernation/aestivation, blastogenesis, metamorphosis, and natural chimerism. However, the absence of distinctive morphological features often makes identification problematic. *Botrylloides diegensis* is a botryllid ascidian that has been misidentified in previous studies and is recorded in GenBank as *Botrylloides leachii* due to the high morphological similarity between the sister species. More available sequences and strategies around identification would help resolve some of the confusion currently surrounding its ambiguous nature. We collected several *Botrylloides* samples from 7 locations around New Zealand and barcoded the species based on Cytochrome Oxidase I, Histone 3, 18S, and 28S ribosomal RNA markers. Network and Bayesian trees confirmed three *Botrylloides* species: *B. diegensis*, *B. jacksonianum,* and *B. aff. anceps.* Additionally, recognition assays were applied to analyse the histocompatibility between distinct morphs qualitatively.

Keywords: ascidian, tunicate, New Zealand, Pacific, barcoding, COI

INTRODUCTION

Ascidiacea includes approximately 3000 identified species that are filter-feeding marine invertebrates, many with global distribution [1]. Botryllid ascidians, including the genera of *Botryllus* and *Botrylloides,* are sessile colonial styelid tunicates found within the intertidal and shallow subtidal zones [2]. The colony is comprised of numerous genetically identical zooids located inside the tunic, an extracellular gelatinous matrix. The tunic contains a cellulose-like substance called tunicine and covers the colonial systems, and protects from the outer environment as a barrier [3].

Compound tunicates belong in the sedentary group, which possesses two life stages. The first is a free-swimming tadpole larva without any extrinsic feeding ability until it attaches to a substratum. These free-living larvae can attach to many substrates such as stones, ship hulls, mussels, and seaweed. The sedentary life stage starts after the attachment is followed by metamorphosis resulting in the loss of chordate features such as the notochord, external pharyngeal gill slits, nerve cord, and post-anal tail [4]. After metamorphosis, the individual adults, termed zooids, can reproduce asexually through a weekly asexual budding cycle called blastogenesis [5]. Individuals form buds from their endothelial epithelium during this cycle, generating new zooids while the original zooid is absorbed.

Tunicates are more closely related to the subphylum Vertebrata than Cephalochordata, and therefore of interest in evolution studies [6]. Botryllid ascidians additionally have several notable abilities. First, they can undergo whole-body regeneration (WBR), the ability to form a new adult from a vascular fragment, following the loss of all zooids [7]. Second, colonies can enter a dormancy period in suboptimal

environmental conditions, where all the zooids are absorbed, and only a compact vasculature is left [8]. Third, natural chimerism, the fusion of two contacting colonies that share similar histocompatibility alleles, also known as allorecognition, results from recognising the self from xeno-recognition [9-11]. WBR, blastogenesis, hibernation/aestivation, and natural chimerism are reported only within the botryllid ascidians. To date, examples of WBR have been described for *Botrylloides violaceus, Botrylloides diegensis* (formerly assigned as *Botrylloides leachii)*, *Botryllus primigenus,* and *Botryllus schlosseri* [4, 12, 13]. *B. diegensis* can regenerate continuously from a little vascular tissue that includes at least ~200 cells in as little as 10 days [7, 14]. Hibernation/aestivation is also only reported for several botryllids as *B. diegensis* (reported as *B. leachii*) and *Botrylloides gascoi* thus far [15]. Investigating these major and minute differences between the tunicate species gives highly profound information about their evolutionary history.

Ascidians have been studied ecologically and regularly monitored to understand if they are indigenous or invasive species, but due to the absence of solid biogeographical or historical evidence, it is challenging to develop a list for their origin or introductions; nevertheless, this is important as spatial competition is a vital phenomenon for the survival of sessile species which can have harmful impacts on native species[16]. Due to the increase in sea transportation and construction of shipping channels, new introductions have increased in the last century, altering habitat structures and biodiversity [17]. For example, *B. schlosseri* and *B. violaceus* are known to be invasive and reported to affect aquaculture negatively [18]. Once these species are introduced to a new habitat, they can overgrow and dominate the available spaces, including the mobile spaces such as the outer layer of crustaceans, which might adversely affect

the animal's mobility. *Botrylloides diegensis* (Ritter & Forsyth, 1917) [19] is commonly seen in the intertidal zone throughout New Zealand's coasts and is thought to have originated from the Western or Southern Pacific, while *B. leachii* is stated to have Mediterranean origins [20-23]. *B. diegensis* is thought to be introduced to Atlantic and Northern Pacific [20, 23], unlike *B. leachii*, which was stated to be non-indigenous for Australia and Tasmania [24].

Ascidians contain crypticity due to morphological plasticity and slight differences in anatomical distinctions between species. The identification of *Botrylloides* species has been particularly challenging. Their separation from its sister species is unclear due to a lack of morphological divergence or a defined distance for inter/intra-species delineation [25, 26]. The sister species which lack this are *Botrylloides leachii, Botrylloides violaceus, Botrylloides niger,* or *Botrylloides diegensis* [25-28]. *Botrylloides perspicuus, Botrylloides giganteus,* and *Botrylloides pizoni* have similar colonial and zooidal features, although they are distinct species [29]. The similarities between these sister botryllid species have resulted in ambiguous identification and sometimes misidentification, which demonstrates the necessity for clear taxonomical identification.

DNA barcoding is a powerful tool and, when combined with morphological data, enables the identification of species, including genus *Botrylloides* [13, 25, 29-31]. The selection of molecular markers to estimate the divergence is essential. These studies often use mitochondrial cytochrome oxidase subunit I (COI), a polymorphic but conserved region, and other nuclear gene markers such as 12S rRNA, 16S rRNA, 18S rRNA, and 28S rRNA genes can also be used to increase the resolution of

identification [25]. Improving the quality and quantity of database sequences will play an important role in future barcoding approaches and improving global biodiversity monitoring. For example, barcoding combined with the morphological investigation has found that *Ciona robusta* was erroneously assigned as *Ciona intestinalis*, a different species [32]. In other cases, mitogenomics, a whole mitochondrial barcoding technique, has been used to separate species. A recent study found that a European clade of *Botryllus schlosseri* was a new species called *Botryllus gaiae* [33]. Significantly, a recent study suggested all the GenBank sequences of *Botrylloides leachii* were incorrectly assigned, and these sequences belong to *Botrylloides diegensis* [26]. Furthermore, it was stated that the spoked wheel morphology in the vicinity of the zooidal buccal siphon is present in *B. leachii* but absent in *B. diegensis*. Considering all these three species are commonly used model invertebrate chordates, due to their prominent phylogenetic position, makes accurate identification essential.

Our group studies regeneration in *Botrylloides*, and previously we had assigned this species as *B. leachii* based on both previous publications and GenBank sequences. Considering the recent studies, we aimed to use DNA barcoding combined with morphology to identify *Botrylloides* species from New Zealand coasts. Additionally, their histocompatibility was analysed via recognition assays to look at similarities between these closely related species.

MATERIALS & METHODS

Sampling Area

In total 40 samples were collected from 7 different intertidal zones around New Zealand (Invercargill, Dunedin, Christchurch, Nelson, Picton, Whangateau, New

Plymouth). Samples were preserved in absolute ethanol, and the samplings were performed from July 2020 to February 2021 (Table S1). Samples were taken from the first few meters of the ocean surface except for a couple of samples acquired from different depths via diving. The collection was based on morphological identification at the sampling sites based on the general knowledge of botryllid ascidian morphology. A colony fragment is taken using a single-edged razor blade. For animal breeding, living tunicate tissue fragments are attached to 5x7.5 cm glass slides [34]. The slides are located in tanks filled with filtered saltwater that is constantly aerated. Animals are fed regularly with a shellfish diet, and their water is replaced every two days.

Morphological Examination

Collected botryllid ascidians were photographed and examined morphologically for the zooid arrangement and colour under the light microscope. The size of the colony and the zooids were measured. Live colonies were monitored regularly.

Molecular Analysis

DNA extraction was performed based on Gemmell and Akiyama [35]. The alcohol was removed from the samples, and 300 µl lysis buffer (100 mM NaCl, 50 mM TrisCl, 1% SDS, 50 mM EDTA pH 8) was added to each tube. Proteinase K (20 mg/ml) was added to a final concentration of 100 µg/ml. The colonies were homogenised and left to incubate for 2 h at 50°C. Following tissue digestion, 300 µl of 5 M LiCl was added to the tubes. The lysate was mixed for 1 min through inversion. Next, 600 µl of chloroform was added, and the samples were left on a rotating wheel for 30 min. The samples were spun for 15 min at max speed. The supernatant was placed in a new tube. Two volumes of absolute ethanol were added to the tubes and were inverted

several times. DNA was precipitated by centrifuging at max speed for 30 min. The supernatant was discarded, and the pellet was washed with 70% ethanol and centrifuged at max speed for 5 min. Excess ethanol was removed, and the pellets were left to air dry for 10 min. Finally, 100-200 µl TE buffer (10 mM TrisCl, 1 mM EDTA – pH 7.5) was used to resuspend the pellet, and the tubes were left overnight at 4°C. DNA samples were stored at -20°C.

DreamTaq Green PCR Master Mix (ThermoFisher) was used with 10 ng/µl of diluted DNA. PCR was performed as following: 1) Denaturation: 95^{o}C – 3min 2) Annealing: 95^{o}C – 30sec, 55^{o}C – 30sec, 72^{o}C – 1min for 35 times 3) Extension: 72^{o}C – 10min. The primers used in this study are given in Table 1. Tun1 primer set is adapted from Folmer, Black [36]. Tun2 primers are taken from Stefaniak, Lambert [37]. 18S, 28S, H3 primer sequences are adapted from Reem, Douek [25].

All PCR products are cleaned with ExoSAP-IT™ PCR Product Cleanup Reagent. 5 µl of post-PCR product is mixed with 2 µl of ExoSAP reagent and incubated at 37^{o}C for 15 minutes and then 80^{o}C for 15 min. Cleaned samples were sequenced by Otago University Sequencing Facility. The tubes were prepared as 3.2 pmol of 1 µl forward primer and 4 µl of cleaned post-PCR template per sample.

Sequences are aligned, edited, and trimmed using BioEdit version 7 per marker [38]. COI haplotypes are demonstrated using NETWORK version 10 with the median-joining algorithm [39]. Bayesian probabilities and branch lengths were acquired for COI locus via MrBayes 3.2 [40]. The Bayesian tree was optimized using FigTree 1.4.4 [41].

For comparative analysis, *Botrylloides* sequences from GenBank were also added to the Network and Phylogenetic analyses. For bayesian phylogeny, we used the present GenBank *Botrylloides* COI sequences (Supplementary File). Besides mitochondrial marker COI, three other nuclear regions Histone 3 (H3), 18S, and 28S, were analysed. Based on H3, 18S, and 28S, bayesian trees were constructed.

Recognition Assay

Recognition assay tests self-nonself discrimination. Two colonies can fuse when they share at least one allele at a highly polymorphic Fu/HC locus (allorecognition) similar to the MHC locus in humans. The absence of common alleles results in non-fusion (xenorecognition). Two botryllid colonies of different morphs were placed beside each other on the slide where their growing ampullae could interact. After placing the colonies, they were left in a moist chamber for 30 min. Then, the colonies were put back into aerated tanks. Within the next day, the interaction between colonies could be seen as their ampullae contacted. Colonies were monitored under Olympus light microscope using CellSens Software.

RESULTS

Morphological observations

Botrylloides zooids are lined side by side as branching double row systems, also called "*leachii"* type, with their dorsal lamina faces the surrounding environment. Different *B. diegensis* morphs from New Zealand coasts were observed, including orange, brown-orange, brown, and brown-white or purple-white (Fig. 1). It is difficult to differentiate the brown-white morph from purple-white in some cases; thus, they might be the same or different, or can be a transition morph. The zooid sizes and the area of the zooids

of *B. diegensis* and *B. affinis anceps* are very similar (~130 um length & ~7500 um^2 area) (Fig. 2A, B, D, E, F). while zooids of *B. jacksonianum* are larger in size and area (~160 um & ~12800 um^2) (Fig. 2C, E, F). The shapes of the zooids are similar within *B. diegensis* and *B. affinis anceps,* elliptical egg-like individuals (Fig. 2A, B, D) while *B. jacksonianum* zooids are more nodal and finger-like towards the atrial tongue (Fig. 2C). All zooids are equal in tentacle numbers (4 large, 4 smaller, and 8 smallest) (Fig. 2A-D). There are white-pigmented cells on the tentacles of *B. jacksonianum* (Fig. 2C). The two largest-lateral tentacles are distinct at the buccal siphon of *B. affinis anceps* (Fig. 2D).

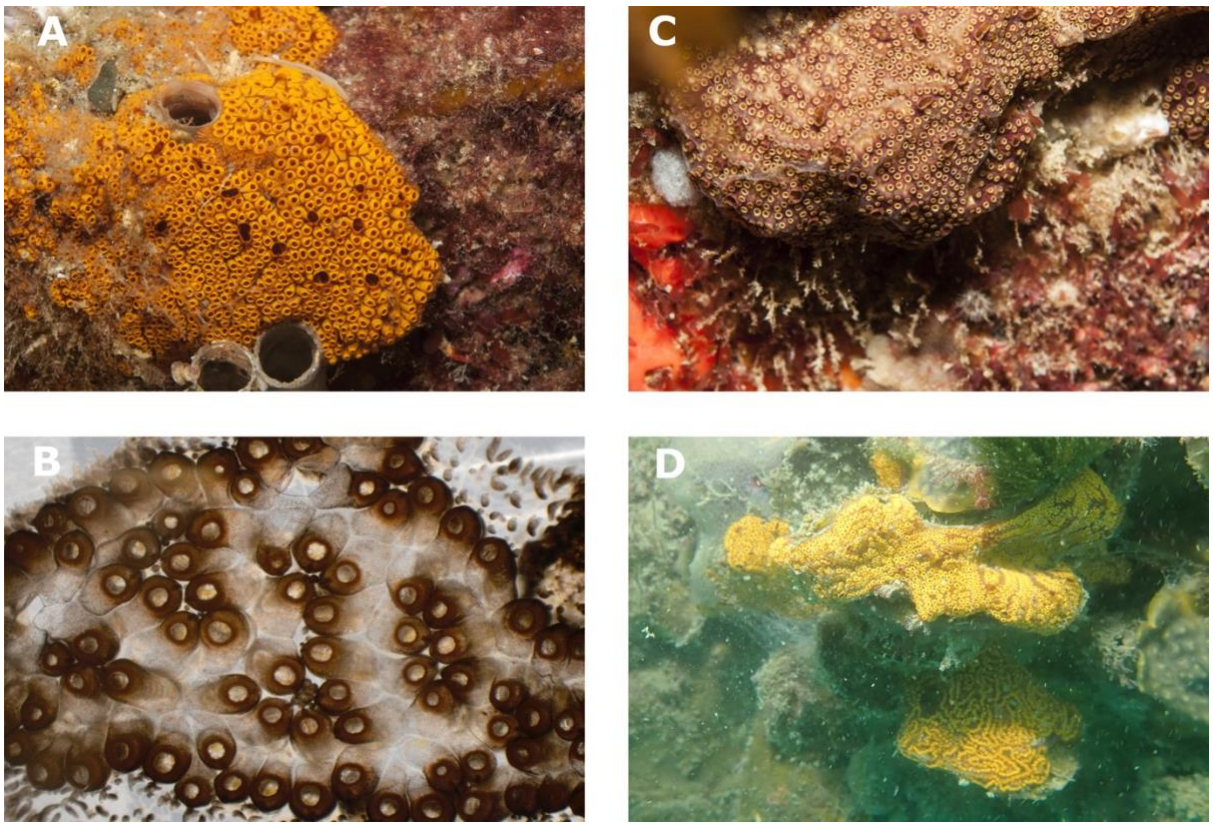

Figure 1. Different colour morphs of *Botrylloides diegensis* from Aotearoa New Zealand. A) Common orange morph. **B)** A brown-orange *B. diegensis* colony. **C)** Brown-white morph. **D)** Brown-orange colony.

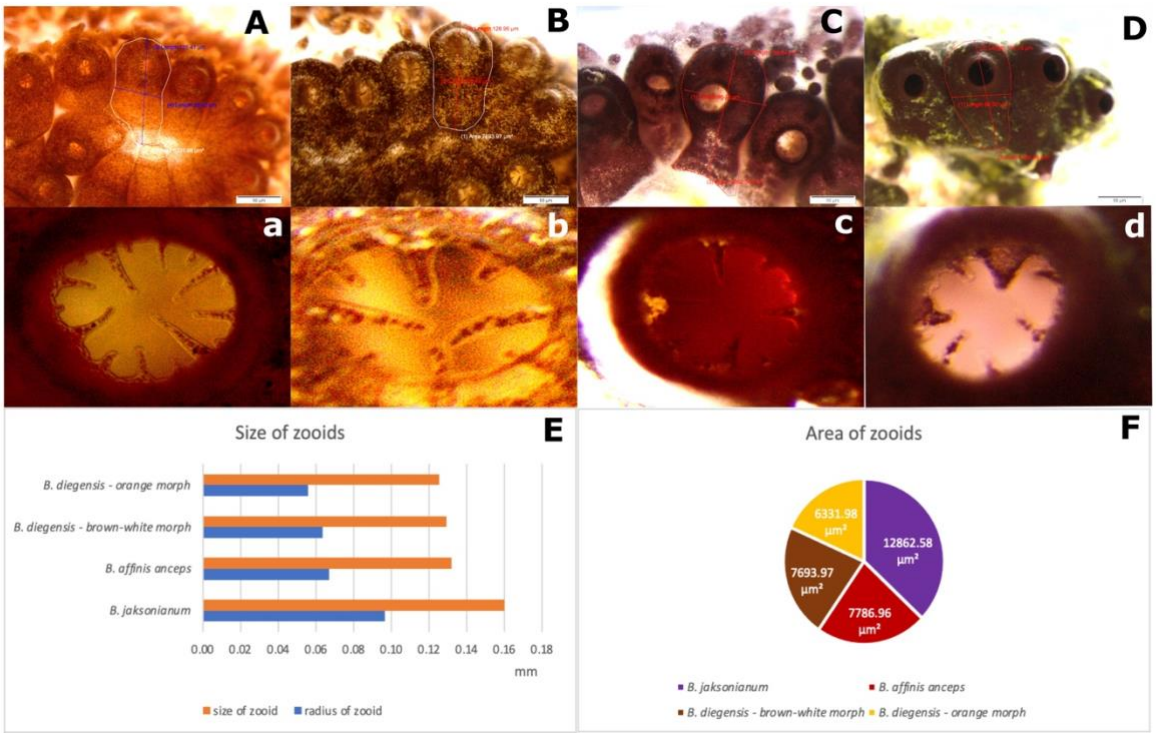

Figure 2. Differences in zooidal size and morphologies of Botryllid species A) Zooid of the orange morph of *B. diegensis.* **a)** Tentacles of the orange morph of *B. diegensis* **B)** Zooid of the brown-white *B. diegensis* colony. **b)** Tentacles of brown-white *B. diegensis.* **C)** Zooid of *B. jacksonianum* **c)** Tentacles of *B. jacksonianum.* **D)** Zooid of *B. affinis anceps* **d)** Tentacles of *B. affinis anceps.* **E)** Bar chart summarising the size and radius of different botryllid zooids. **F)** Pie chart represents the area of the given zooids. Example measurements are shown in the earlier panels A-D.

Genetic analysis of haplotype diversity

Seven haplotypes were found based on the clustering of the COI Network from the 7 sampling locations (Fig. 3). The distance values (0.2-1%) of the sequences support their identification as *B. leachii* or *B. diegensis* based on the COI species delineation threshold (<2%) [42] compared to database sequences based on BLAST mismatch ratio.

Additionally, three samples from Whangateau, Dunedin, and Invercargill were determined to be distinct species based on their evolutionary distances (Fig. 3). A phylogeny based on COI locus was constructed to evaluate their taxonomy (Fig. 4).

Whangateau and Invercargill colonies clustered with *B. cf. anceps* COI barcode sequence from Australia (Accession no: MT873573) and the botryllid sample from Dunedin grouped within *B. jacksonianum* from Australia (Accession no: MT873572). While the distance is high within the *B. anceps* cluster and thus identified as *B. affinis anceps*, the botryllid species from Dunedin is nominated as *B. jacksonianum* due to the low mismatch ratio (<1%). The rest of the sequences clustered with *B. diegensis* sequences from GenBank (Fig. 4).

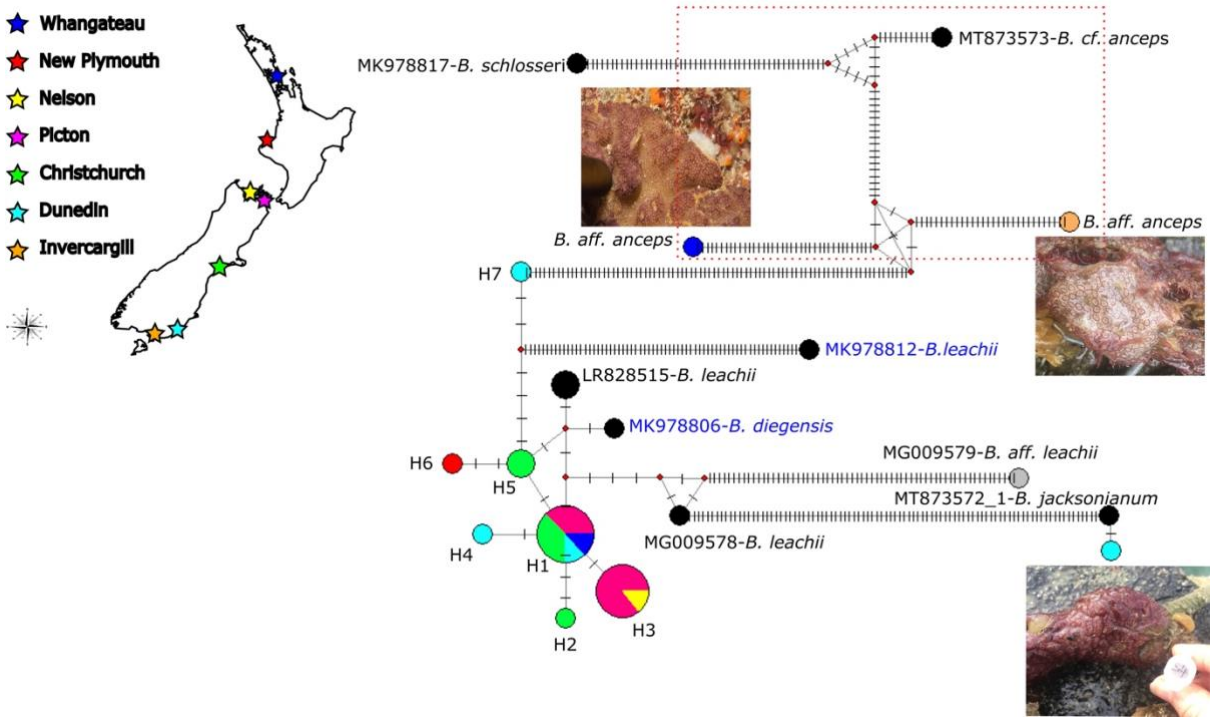

Figure 3. Phylogeography of the New Zealand Botryllid ascidians. Stripes on lines indicate the mutation steps between the haplotypes. Size differences of circles represent frequency. Colors marked the regions where the samples were collected as given in the map (Turquoise: Dunedin, Green: Christchurch, Orange: Invercargill, Yellow: Nelson, Pink: Picton, Blue: Whangateau, Red: New Plymouth). Analyzed partial COI sequences are 647 bp. Grey and black circles indicate the database haplotypes. Black is for B. *diegensis/leachii*, grey is for *B. aff. leachii* from the GenBank. Blue writings demonstrate *B. diegensis* sequences specifically. *B. anceps* cluster is highlighted with a red dotted line.

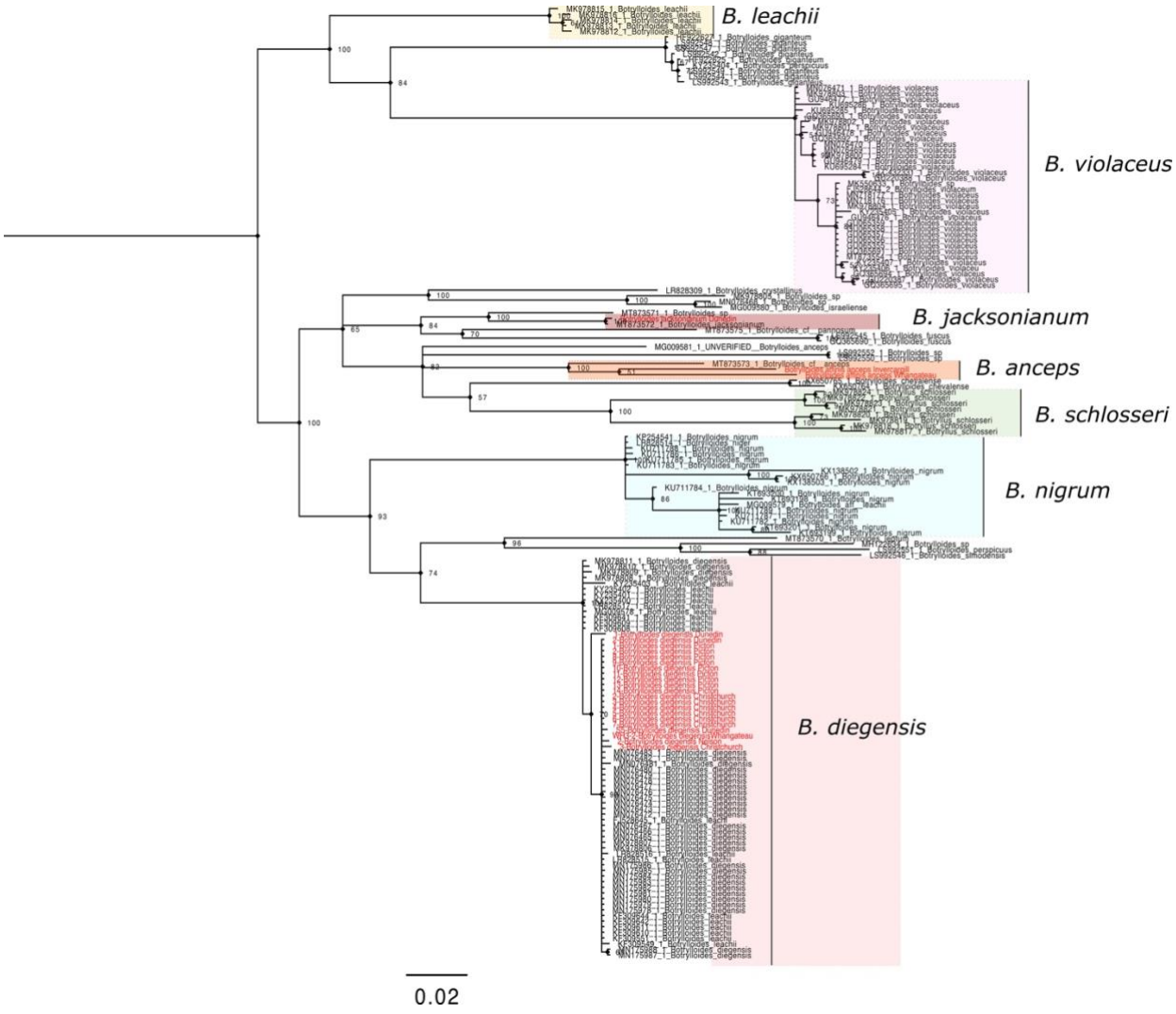

Figure 4. Bayesian tree of *Botrylloides* based on mitochondrial COI locus. Current study sequences and all database *Botrylloides* sequences from GenBank were used to construct the tree. Two independent runs were executed with Monte-Carlo Markov Chains. Ten million generations were measured, and the sampling frequency was 1000 for each generation. Split frequencies were lower than 0.01. Red sequences indicate the current study sequences.

Besides mitochondrial marker COI, three other nuclear regions as Histone 3 (H3), 18S, and 28S were analysed. Based on H3, 18S, and 28S, bayesian trees were constructed (Fig. 5). The H3 tree (Fig. 5A) confirms the clustering of the New Zealand samples with previous samples labeled as *B. leachii* (*B. diegensis)*. Furthermore, the low mismatch ratio (<1%) with database *B. leachii* (*B. diegensis*) sequences based on

18S, and 28S barcodes further confirm the identification of current study sequences as *B. diegensis* (Fig. 5B, C). Moreover, *B. jacksonianum* from Dunedin is more distant to *B. diegensis* from Dunedin based on H3 compared to 18S, while *B. affinis anceps* from Whangateau is farther from the *Botrylloides anceps* from Israel (indicated as unverified on GenBank) based on 28S.

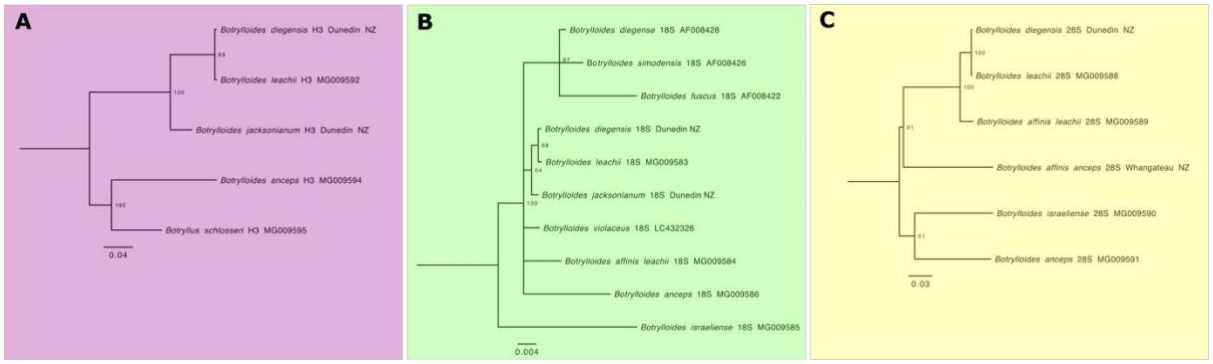

Figure 5. Bayesian trees based on nuclear markers A) Tree is based on Histone 3 **B)** Phylogeny is based on 18S ribosomal subunit gene **C)** 28S ribosomal subunit tree. Phylogenies are constructed with the study sequences and all database *Botrylloides* sequences from GenBank. Two independent runs were executed with Monte-Carlo Markov Chains. Ten million generations were measured, and the sampling frequency was 1000 for each generation. Split frequencies were lower than 0.01. Numbers on the tree indicate the bootstrap frequencies.

Colony Histocompatibility

Two colonies can fuse when they both carry at least one allele at a highly polymorphic Fu/HC locus. This is commonly seen among botryllids called natural chimerism as an evolutionary advantage to recognize genetically similar ones from the distant colonies. While the organism is generally competing for space with the distant or the different, chimerism allows for reconnection, so enables the similar ones to become one again and increase in size instead of competing. To qualitatively analyse the histocompatibility of two colonies based on the ability to fuse, different morphs of *B. diegensis* were located closely so their ampullae could interact. The brown and brown-

orange *B. diegensis* morphs resulted in a non-fusion reaction (Fig. 6). Necrotic spot formation was observed during day 3 of the interaction as an indicator of the non-fusion response. This indicates these two systems are different from each other based on Fu/Hc locus with their blood cells resisting each other instead of fusing and becoming a continuous circulatory system. The second recognition assay was conducted between orange and purple-white morphs (Fig. 7). This assay resulted in the non-interaction of the colonies as the ampullae of two morphs interacted and their ampullae are contracted back from each other instead of continuing to interact.

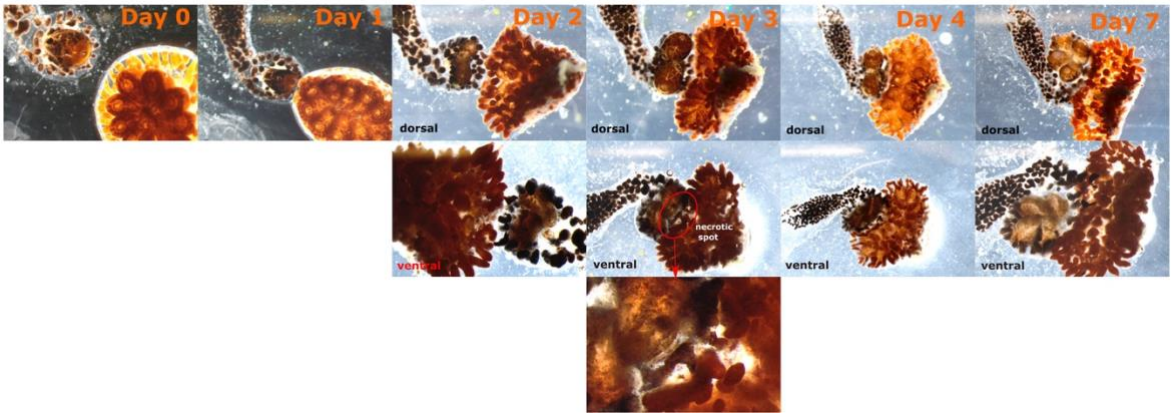

Figure 6. Histocompatibility of different *B. diegensis* morphs. Non-fusion of orange and brown-orange colonies. The interaction was monitored for seven days. The interaction was initiated by the elongation of ampullae. The necrotic spots were detected in between the contact points where the ampullae interacted.

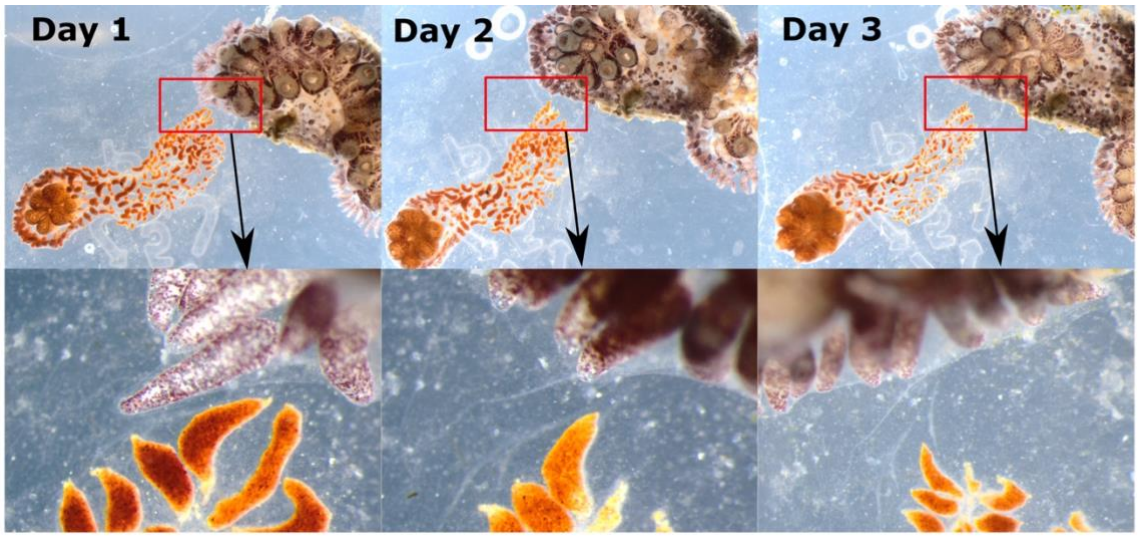

Figure 7. Xeno-recognition in *B. diegensis* morphs. Non-interaction of orange and purple-white morphs. Interactions were monitored for three days. The interaction was initiated by the elongation of ampullae. The ampullae of the two systems moved away in the following days.

DISCUSSION

Botrylloides colonies were collected from seven different regions of the North, and South Island of New Zealand and phylogenetic analysis was based on mitochondrial and nuclear markers. Due to the absence of the spoked wheel morphology and based on the comparative molecular results, most of the collected samples from New Zealand are identified as *Botrylloides diegensis* [26]. For this reason, the previously reported studies from our lab that denoted our model organism as *Botrylloides leachii* are *Botrylloides diegensis* [4, 14, 34, 43, 44].

Two other *Botrylloides* species collected are the first molecular records from New Zealand: *Botrylloides jacksonianum* and *Botrylloides affinis anceps.* The species from Whangateau was assigned as *B. aff. anceps* due to its high molecular affinity to the record from Australia (GenBank Accession No: MT873573) [28]. Although we were

not able to obtain a detailed anatomical comparison for both these records, our result looks coherent, particularly on the determination of *B. anceps* as it is previously stated to be indigenous to the Indo-Pacific region [27]. The presence of two larger lateral tentacles given for *B. anceps* from Israel also confirms the genetic affinity regarding our assignment [27]. Besides a recent study, there is no recently reported record of *B. jacksonianum* [28]. The authors stated that *B. jacksonianum* which was synonymized with *B. leachii* by Kott [45, 46] is not accurate and essentially a distinct species. Our network construction and phylogenetic tree based on COI also validates as it is a separate species. They also indicated the Folmer's fragments are insufficient for amplifying the COI region of botryllid ascidians. A similar problem arose with the barcoding of *B. aff. anceps* and *B. jacksonianum,* which was overcome by cloning these regions into a plasmid vector and then sequencing.

Colonies are not collected in a sterile environment as they come from the ocean, so the isolated DNA often contains the DNA of other organisms such as algae, bacteria, and nearby organisms from the same substratum or the organism itself is attached to them. This means barcoding of these animals is often not easy and can result in contamination. A new primer pair called 860-COI was designed to control for these issues [28, 47]. It provides sufficient resolution for the barcoding of botryllid ascidians even if they are cryptic or a new species. However, as the 860-COI primer was efficient to amplify the COI barcode, we were not able to elongate the sequence and thus only used the Folmer's region.

As a single monophyletic cluster, we observed seven different haplotypes from the New Zealand coasts. Although a small population of the species was barcoded, a

relatively higher number of haplotypic variations were recorded from Dunedin. No phylogeographic divergence within these colonies was recorded. The highest nucleotide variation was observed within COI barcodes and H3, also providing an adequate resolution for the species delineation. 18S and 28S had low resolution compared to COI and H3. No sequence variation was observed among the nuclear markers.

We found the zooid sizes of *B. diegensis* and *B. aff. anceps* is similar while *B. jacksonianum* was bigger. As a general observation, we commonly found *B. diegensis* during the summertime. However, *B. jacksonianum* was observed more often during the winter during our regular samplings in Dunedin.

As compatible with the formerly reported orange colonies, dichromatic morphs of *B. diegensis* can undergo whole-body regeneration which takes approximately twelve days [34, 43]. Dichromatic and monochromatic colonies of *B. diegensis* were not histocompatible due to the non-fusion reaction. The necrotic spot formation in between the contact points supports the non-histocompability observation. The non-interaction of the colonies may be explained more of a behavioral scope as these colonial systems seem to choose alternative actions instead of competing or joining each other.

Overall, our findings suggest that COI is a reliable marker in terms of botryllid ascidian identification. Among nuclear markers, H3 is adequately resolving the species taxonomy compared to 18S and 28S. For our previous publications, we had checked our COI barcodes which matched at the time with the *B. leachii* sequences in the GenBank database. Since then, these sequences were determined as incorrectly

399 annotated and belong to *B. diegensis*. As one of the first barcoding studies on botryllid
400 ascidians from New Zealand, this study represents valuable insights into *Botrylloides*
401 species diversity. In total, we found three *Botrylloides* species which are *Botrylloides*
402 *diegensis, Botrylloides jacksonianum,* and *Botrylloides affinis anceps* which are all
403 indigenous to the Pacific indicating an absence of a new introduction.
404
405 DATA AVAILABILITY
406 The haplotype sequences are uploaded to the GenBank nucleotide database. The
407 accession numbers can be found in the Supplementary File.
408
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416
417 COMPETING INTERESTS
418 The authors declare no competing interests.
419
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TABLES

Table 1. Primer sequences, annealing temperature, expected product length for *COI, H3, 18S*, and *28S* target genes used in this study.

Primer name	Primer sequence	Ann. Temp. (ºC)	Product length (bp)	Analysed length (bp)	Reference
Tun1_F/LCOI1490	GGTCAACAAATCATAAAGATATTGG	55	~650	647	Folmer et al 1994
Tun1_R/HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	55	~650	647	Folmer et al 1994
Tun_F	TCGACTAATCATAAAGATATTA	55	~650	647	Stefaniak et al 2009
Tun_R	AACTTGTATTTAAATTACGATC	55	~650	647	Stefaniak et al 2009
dinF	CGTTGRTTTATRTCTACWAATCATAARGA	52	~700	647	Brunetti et al 2017
Nux1R	GCAGTAAAATAWGCTCGRGARTC	52	~700	647	Brunetti et al 2017
28S_F	ACCCGCTGAATTTAAGCAT	62	~950	774	Reem et al 2017
28S_R	TCCGTGTTTCAAGACGGG	62	~950	774	Reem et al 2017
18S_F	AACCTGGTTGATCCTGCCAGT	60	~1750	1033	Reem et al 2017
18S_R	GATCCTTCTGCAGGTTCACCTAC	60	~1750	1033	Reem et al 2017
H3_F	ATGGCTCGTACCAAGCAGACVGC	55	~350	300	Reem et al 2017
H3_R	ATATCCTTRGGCATRATRGTGAC	55	~350	300	Reem et al 2017

SUPPORTING INFORMATION

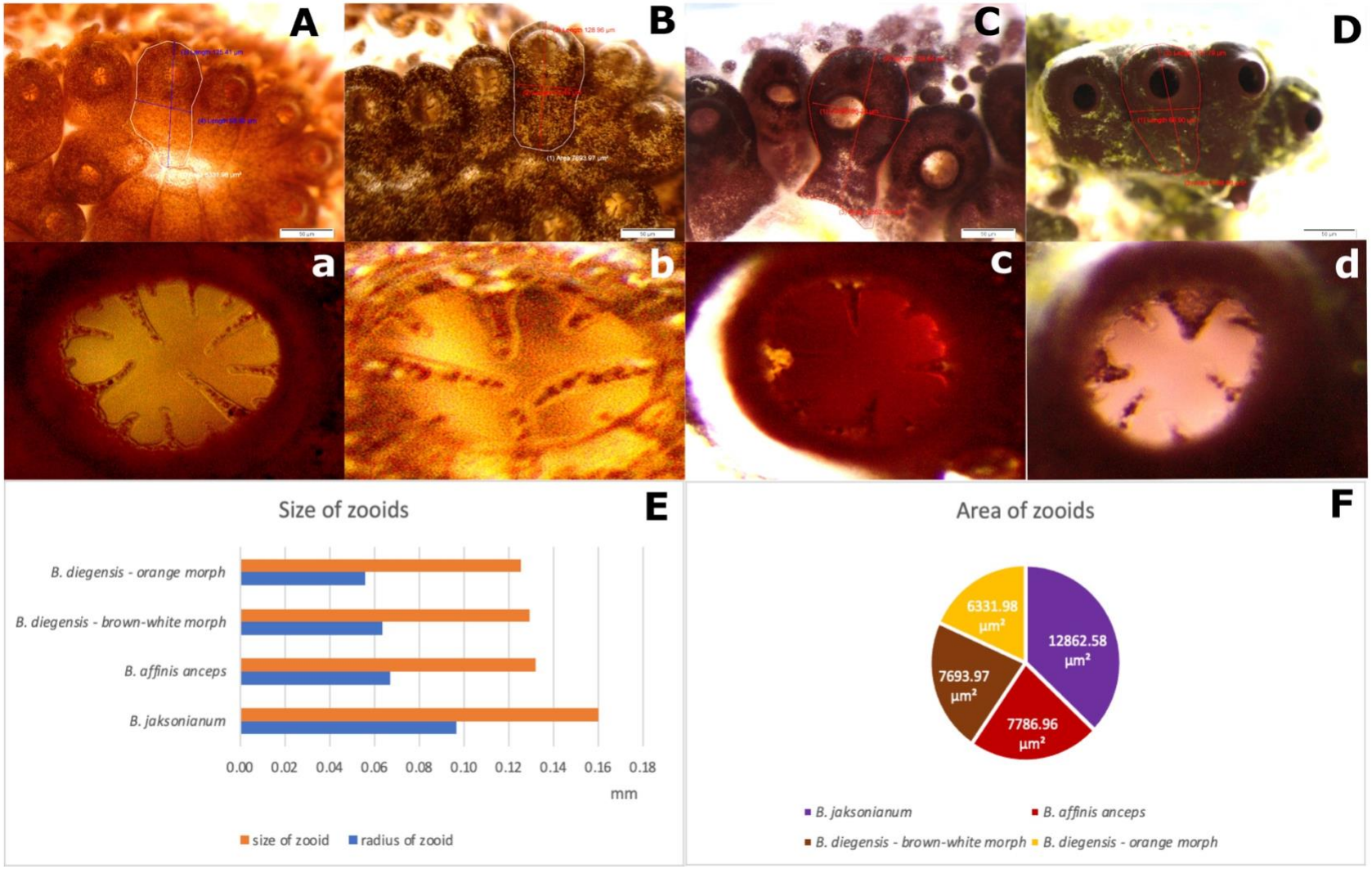
Table S1. Sampling details

Sapling Area	Coordinates	Date
Dunedin	45°52'17"S, 170°31'43"E; 45°49'41"S, 170°38'29"E	11/6/20
Nelson	41°15'54.5"S, 173°16'44.8"E	11/6/20
Picton	41°17'21.5"S, 174°00'32.9"E	20/7/20
Christchurch	43°36'17.89"S, 172°42'44.71"E	14/7/20
Whangateau	36°19'11.03"S, 174°46'55.36"E	23/9/20
New Plymouth	39° 3'17.91"S, 174°3'20.85"E	8/9/2020

Table S2. GenBank Accession Numbers

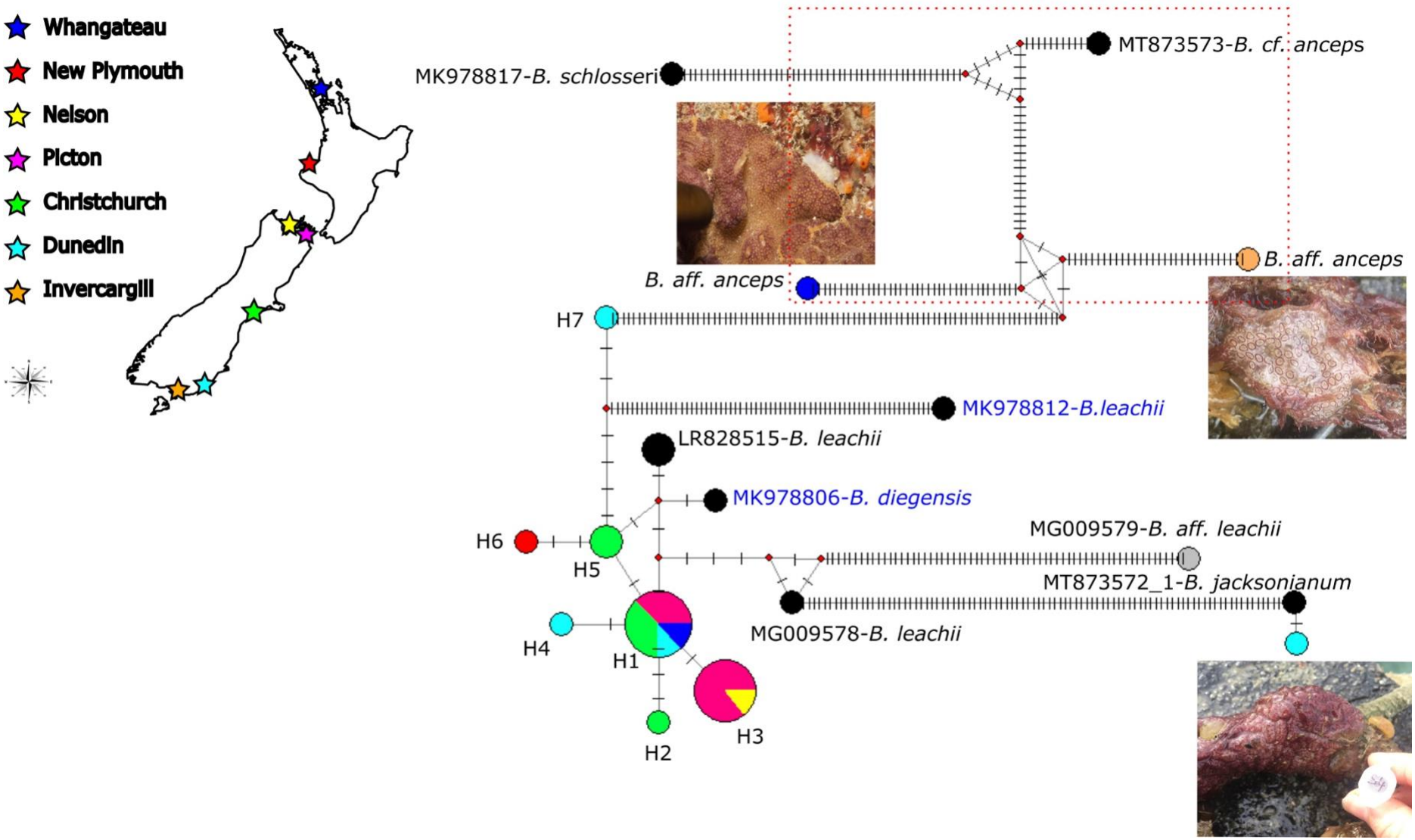
Sequence	Accession Number
1. *Botrylloides diegensis* COI -Dunedin	MZ533117
2. *Botrylloides jacksonianum* COI -Dunedin	MZ533119
3. *Botrylloides affinis anceps* COI -Whangateu	MZ533118
4. *Botrylloides affinis anceps* COI -Invercargill	MZ533120
5. *Botrylloides diegensis* H3 -Dunedin	MZ506871
6. Botrylloides jacksonianum H3 -Dunedin	MZ506872
7. *Botrylloides diegensis* 18S -Dunedin	MZ412536
8. *Botrylloides affinis anceps* 18S -Invercargill	MZ412535
9. *Botrylloides diegensis* 28S -Dunedin	MZ506611
10. *Botrylloides affinis anceps* 28S -Whangateu	MZ506612

570


A
B
C
D
a
b
c
d
Size of zooids
E
B. diegensis - orange morph
B. diegensis - brown-white morph
B. affinis anceps
B. jaksonianum
0.00 0.02 0.04 0.06 0.08 0.10 0.12 0.14 0.16 0.18
mm
size of zooid
radius of zooid
Area of zooids
F
6331.98 µm²
12862.58 µm²
7693.97 µm²
7786.96 µm²
B. jaksonianum
B. affinis anceps
B. diegensis - brown-white morph
B. diegensis - orange morph

571

572


Whangateau
New Plymouth
Nelson
Picton
Christchurch
Dunedin
Invercargill
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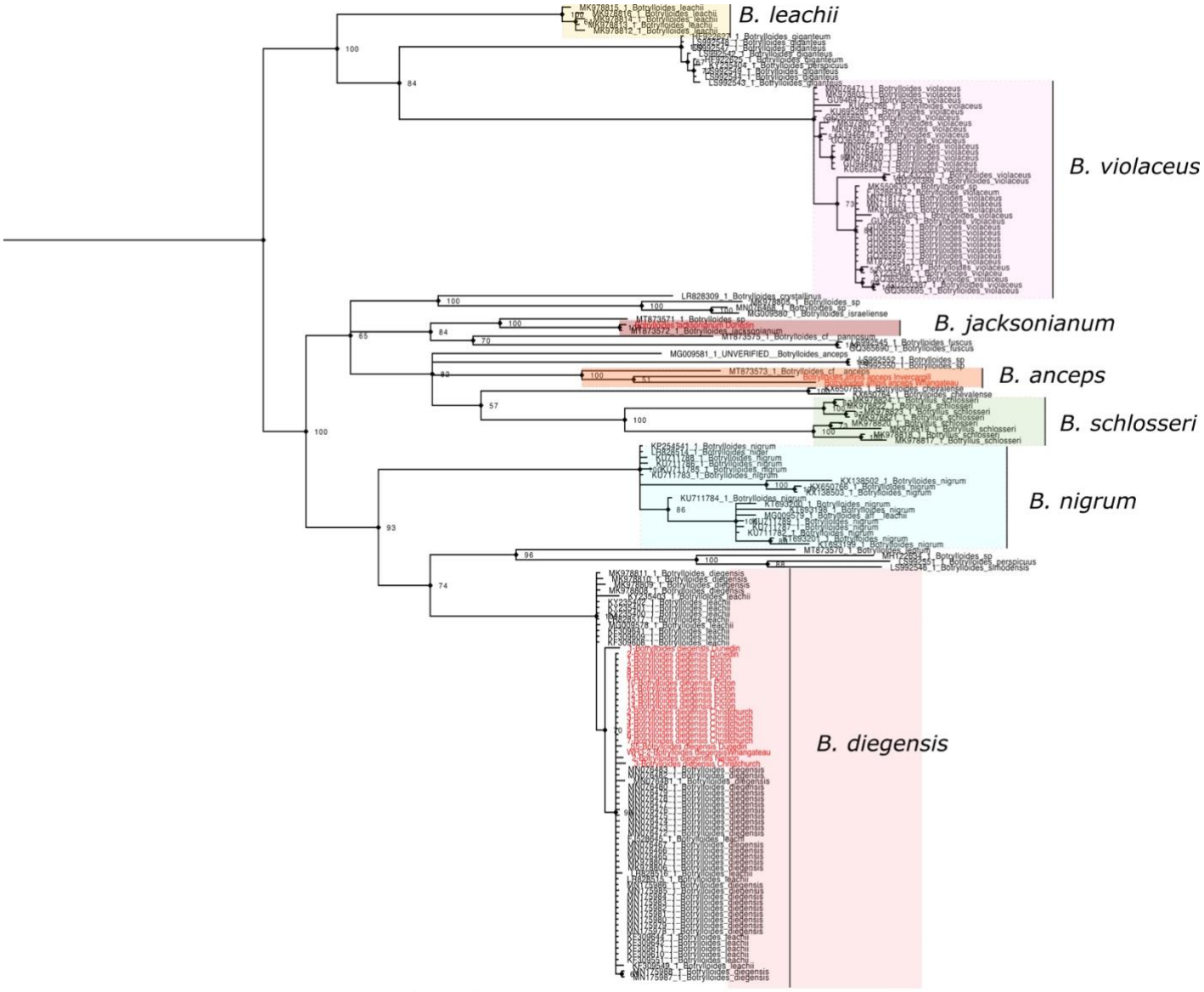
MT873573-B. cf. anceps
B. aff. anceps
B. aff. anceps
H7
MK978812-B.leachii
LR828515-B. leachii
MK978806-B. diegensis
MG009579-B. aff. leachii
MT873572_1-B. jacksonianum
MG009578-B. leachii
H6
H5
H4
H1
H3
H2

573

574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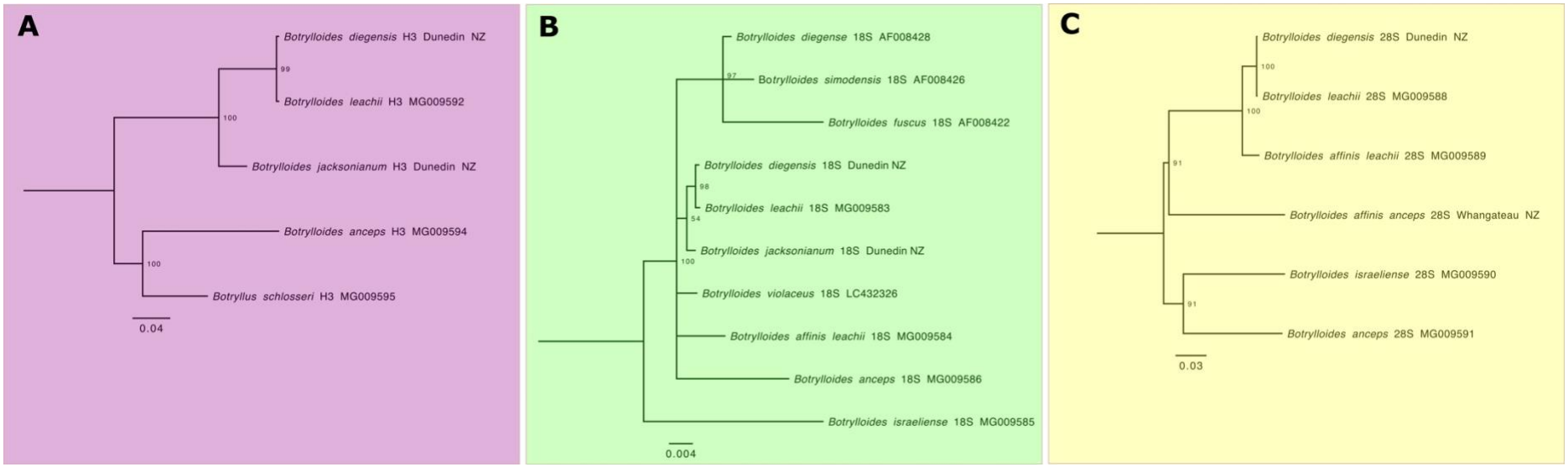
B. violaceus
B. jacksonianum
B. anceps
B. schlosseri
B. nigrum
B. diegensis
0.02

575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Botrylloides leachii H3 MG009592
100
Botrylloides jacksonianum H3 Dunedin NZ
Botrylloides anceps H3 MG009594
100
Botryllus schlosseri H3 MG009595
0.04
B
Botrylloides diegense 18S AF008428
97
Botrylloides simodensis 18S AF008426
Botrylloides fuscus 18S AF008422
Botrylloides diegensis 18S Dunedin NZ
98
Botrylloides leachii 18S MG009583
54
Botrylloides jacksonianum 18S Dunedin NZ
100
Botrylloides violaceus 18S LC432326
Botrylloides affinis leachii 18S MG009584
Botrylloides anceps 18S MG009586
Botrylloides israeliense 18S MG009585
0.004
C
Botrylloides diegensis 28S Dunedin NZ
100
Botrylloides leachii 28S MG009588
100
Botrylloides affinis leachii 28S MG009589
91
Botrylloides affinis anceps 28S Whangateau NZ
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91
Botrylloides anceps 28S MG009591
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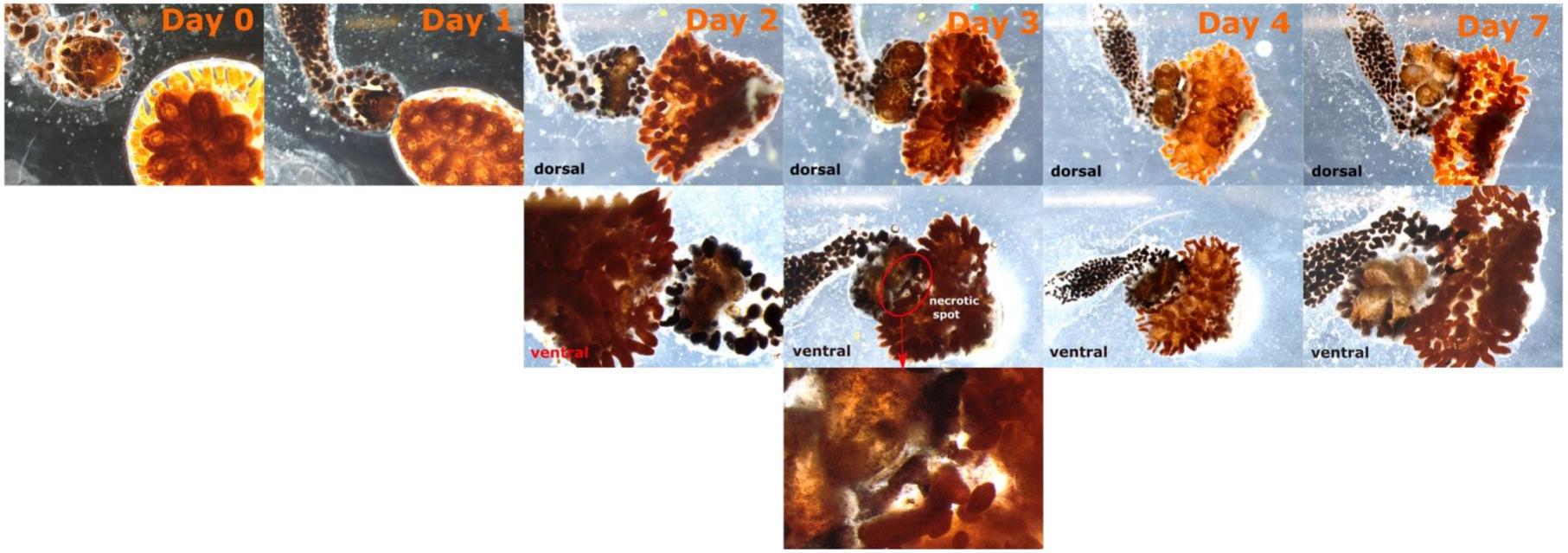
576

577


Day 0
Day 1
Day 2
Day 3
Day 4
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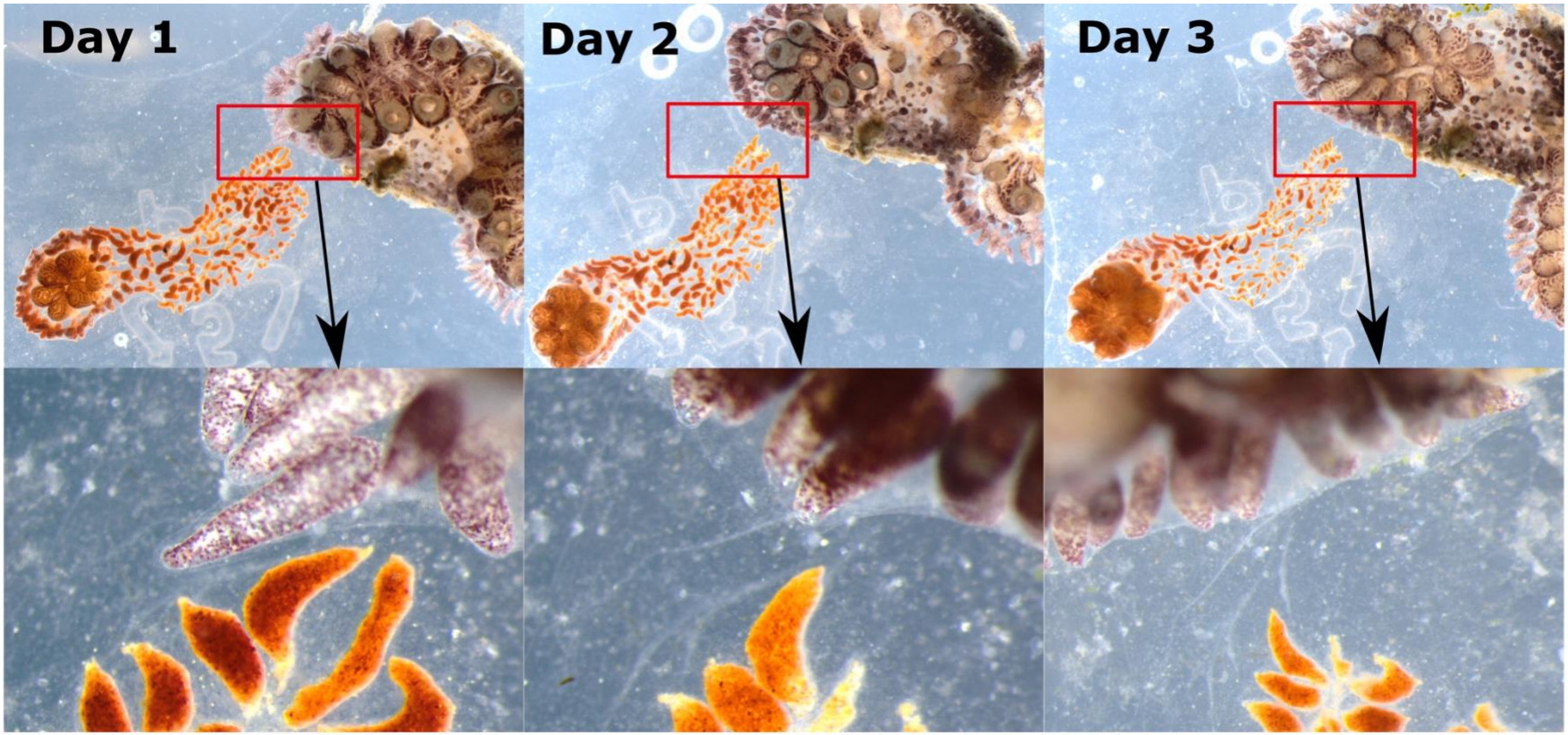
dorsal
dorsal
ventral
ventral
necrotic spot
ventral
ventral

578

579

580



581