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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

50 INTRODUCTION

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

58

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

69

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

75 environmental conditions, where all the zooids are absorbed, and only a compact
76 vasculature is left [8]. Third, natural chimerism, the fusion of two contacting colonies
77 that share similar histocompatibility alleles, also known as allorecognition, results from
78 recognising the self from xeno-recognition [9-11]. WBR, blastogenesis,
79 hibernation/aestivation, and natural chimerism are reported only within the botryllid
80 ascidians. To date, examples of WBR have been described for *Botrylloides violaceus*,
81 *Botrylloides diegensis* (formerly assigned as *Botrylloides leachii*), *Botryllus*
82 *primigenus*, and *Botryllus schlosseri* [4, 12, 13]. *B. diegensis* can regenerate
83 continuously from a little vascular tissue that includes at least ~200 cells in as little as
84 10 days [7, 14]. Hibernation/aestivation is also only reported for several botryllids as
85 *B. diegensis* (reported as *B. leachii*) and *Botrylloides gascoi* thus far [15]. Investigating
86 these major and minute differences between the tunicate species gives highly
87 profound information about their evolutionary history.

88

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

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

106

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

118

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

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

138

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

145

146 **MATERIALS & METHODS**

147 **Sampling Area**

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

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

159

160 **Morphological Examination**

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

164

165 **Molecular Analysis**

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

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

181

182 DreamTaq Green PCR Master Mix (ThermoFisher) was used with 10 ng/ μ l of diluted
183 DNA. PCR was performed as following: 1) Denaturation: 95°C – 3min 2) Annealing:
184 95°C – 30sec, 55°C – 30sec, 72°C – 1min for 35 times 3) Extension: 72°C – 10min.
185 The primers used in this study are given in Table 1. Tun1 primer set is adapted from
186 Folmer, Black [36]. Tun2 primers are taken from Stefaniak, Lambert [37]. 18S, 28S,
187 H3 primer sequences are adapted from Reem, Douek [25].

188

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

194

195 Sequences are aligned, edited, and trimmed using BioEdit version 7 per marker [38].
196 COI haplotypes are demonstrated using NETWORK version 10 with the median-
197 joining algorithm [39]. Bayesian probabilities and branch lengths were acquired for
198 COI locus via MrBayes 3.2 [40]. The Bayesian tree was optimized using FigTree 1.4.4
199 [41].

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

205

206 **Recognition Assay**

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

216

217 **RESULTS**

218 **Morphological observations**

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

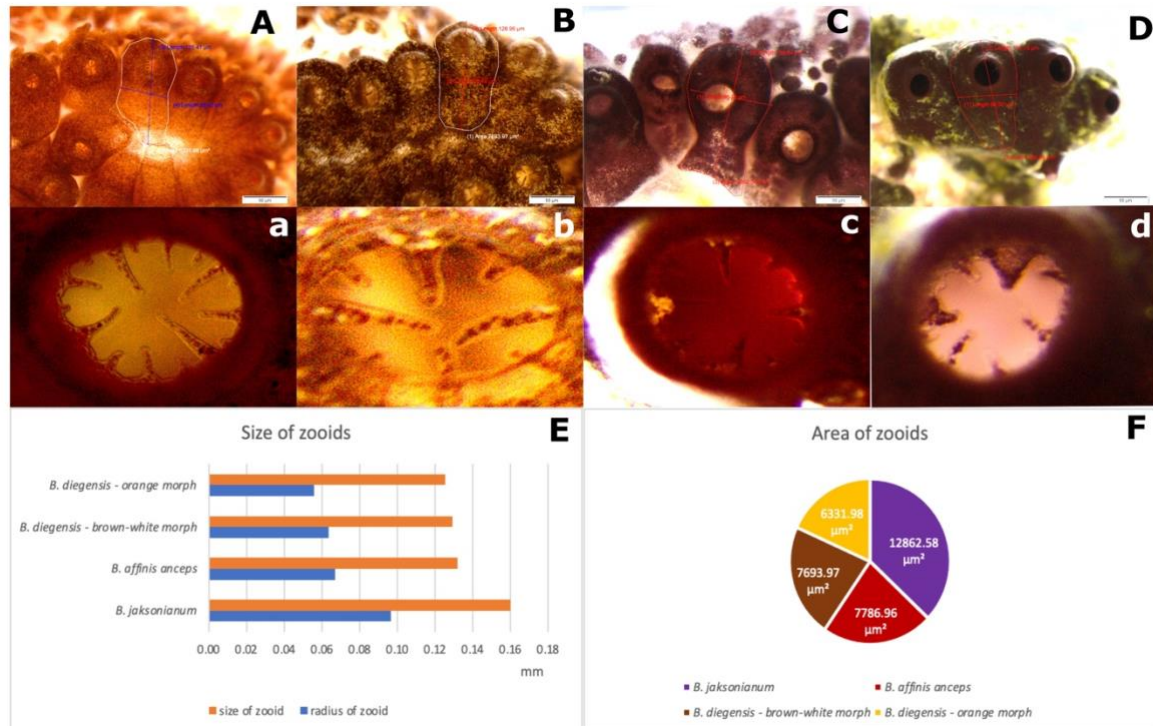
225 of *B. diegensis* and *B. affinis anceps* are very similar (~130 μm length & ~7500 μm^2
226 area) (Fig. 2A, B, D, E, F). while zooids of *B. jacksonianum* are larger in size and area
227 (~160 μm & ~12800 μm^2) (Fig. 2C, E, F). The shapes of the zooids are similar within
228 *B. diegensis* and *B. affinis anceps*, elliptical egg-like individuals (Fig. 2A, B, D) while
229 *B. jacksonianum* zooids are more nodal and finger-like towards the atrial tongue (Fig.
230 2C). All zooids are equal in tentacle numbers (4 large, 4 smaller, and 8 smallest) (Fig.
231 2A-D). There are white-pigmented cells on the tentacles of *B. jacksonianum* (Fig. 2C).
232 The two largest-lateral tentacles are distinct at the buccal siphon of *B. affinis anceps*
233 (Fig. 2D).



234

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

237



238

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

245

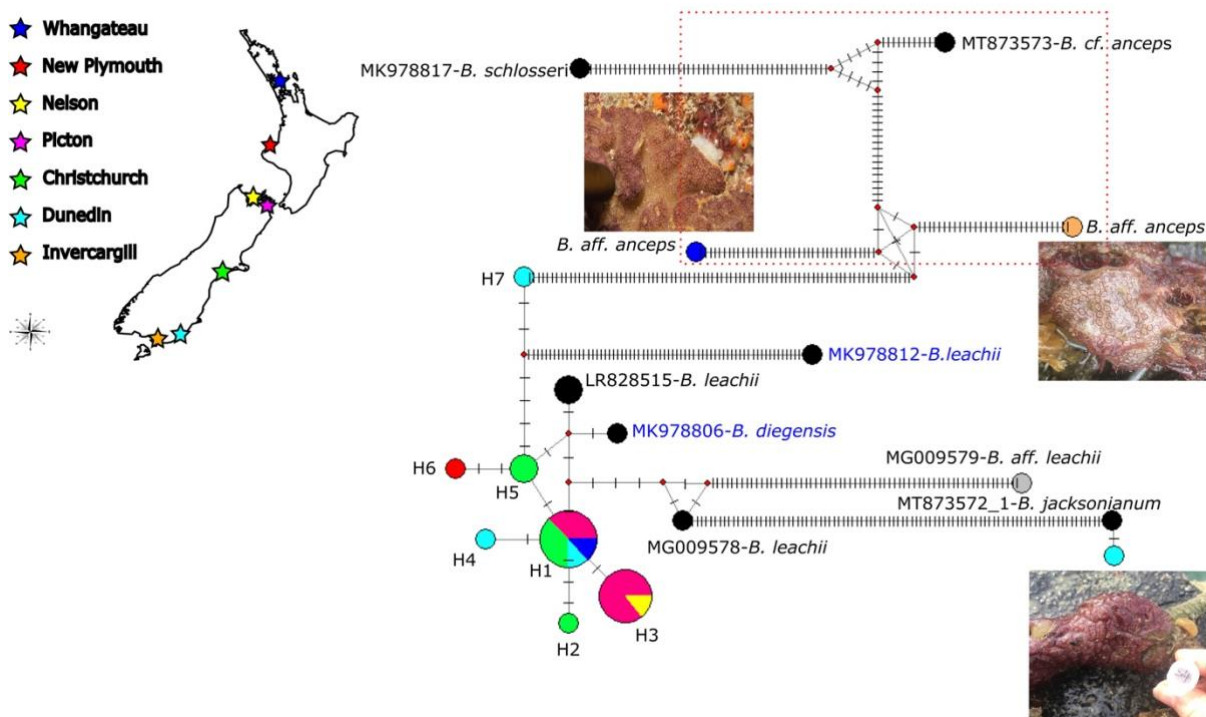
246 Genetic analysis of haplotype diversity

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

252

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

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



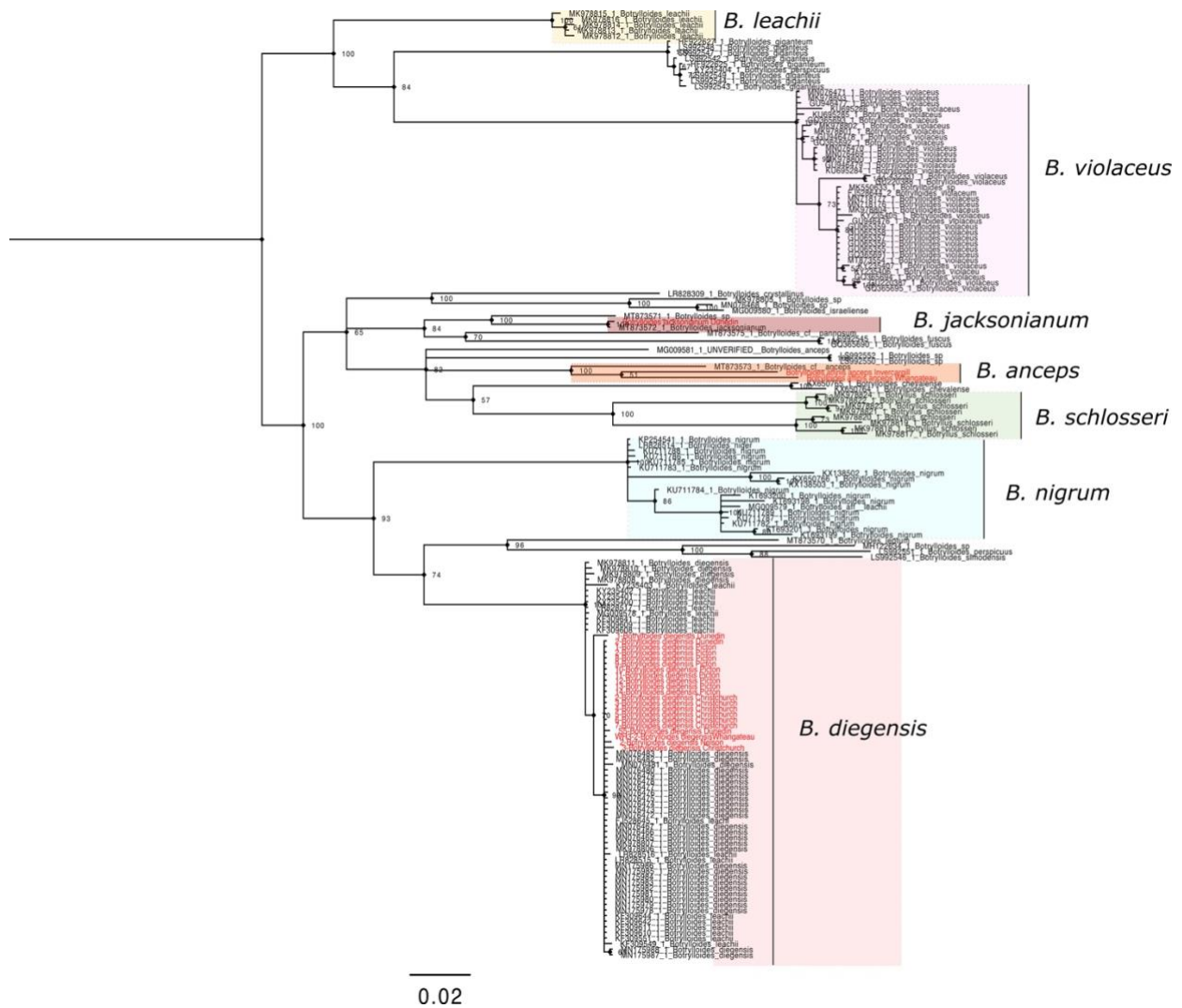
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265 **Figure 3. Phylogeography of the New Zealand Botryllid ascidians.** Stripes on lines indicate the mutation
266 steps between the haplotypes. Size differences of circles represent frequency. Colors marked the regions
267 where the samples were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

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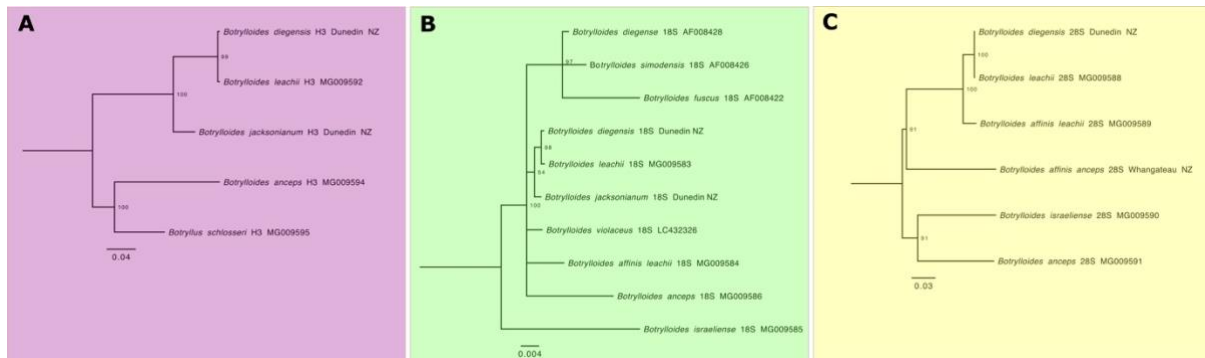
0.02

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

280

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

286 18S, and 28S barcodes further confirm the identification of current study sequences
287 as *B. diegensis* (Fig. 5B, C). Moreover, *B. jacksonianum* from Dunedin is more distant
288 to *B. diegensis* from Dunedin based on H3 compared to 18S, while *B. affinis anceps*
289 from Whangateau is farther from the *Botrylloides anceps* from Israel (indicated as
290 unverified on GenBank) based on 28S.



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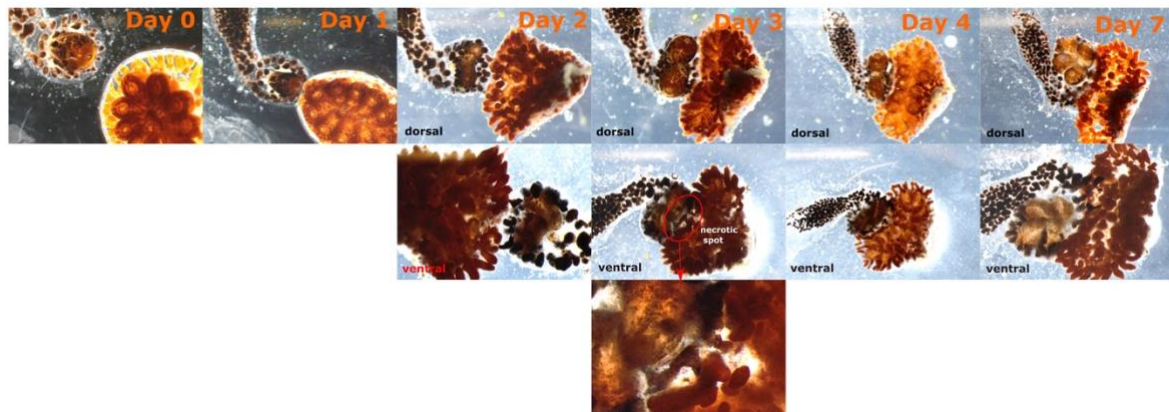
292 **Figure 5. Bayesian trees based on nuclear markers** A) Tree is based on Histone 3 B) Phylogeny is based
293 on 18S ribosomal subunit gene C) 28S ribosomal subunit tree. Phylogenies are constructed with the study
294 sequences and all database *Botrylloides* sequences from GenBank. Two independent runs were executed
295 with Monte-Carlo Markov Chains. Ten million generations were measured, and the sampling frequency was
296 1000 for each generation. Split frequencies were lower than 0.01. Numbers on the tree indicate the
297 bootstrap frequencies.

298

299 Colony Histocompatibility

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

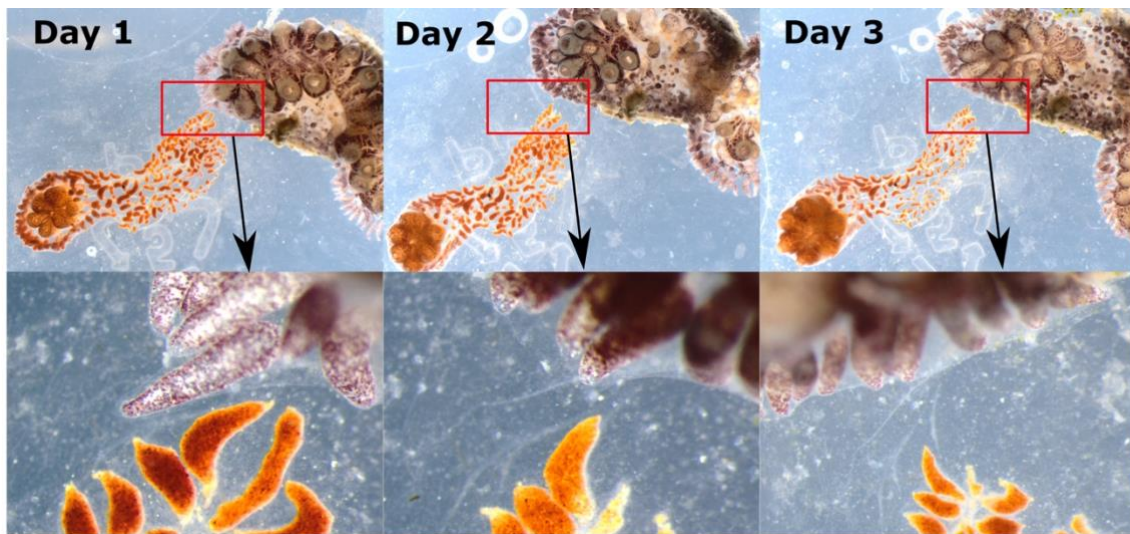
308 orange *B. diegensis* morphs resulted in a non-fusion reaction (Fig. 6). Necrotic spot
309 formation was observed during day 3 of the interaction as an indicator of the non-
310 fusion response. This indicates these two systems are different from each other based
311 on Fu/Hc locus with their blood cells resisting each other instead of fusing and
312 becoming a continuous circulatory system. The second recognition assay was
313 conducted between orange and purple-white morphs (Fig. 7). This assay resulted in
314 the non-interaction of the colonies as the ampullae of two morphs interacted and their
315 ampullae are contracted back from each other instead of continuing to interact.
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319 **Figure 6. Histocompatibility of different *B. diegensis* morphs.** Non-fusion of orange and brown-orange
320 colonies. The interaction was monitored for seven days. The interaction was initiated by the elongation of
321 ampullae. The necrotic spots were detected in between the contact points where the ampullae interacted.

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332 **Figure 7. Xeno-recognition in *B. diegensis* morphs.** Non-interaction of orange and purple-white morphs.

333 Interactions were monitored for three days. The interaction was initiated by the elongation of ampullae.

334 The ampullae of the two systems moved away in the following days.

335

336 DISCUSSION

337 *Botrylloides* colonies were collected from seven different regions of the North, and

338 South Island of New Zealand and phylogenetic analysis was based on mitochondrial

339 and nuclear markers. Due to the absence of the spoked wheel morphology and based

340 on the comparative molecular results, most of the collected samples from New

341 Zealand are identified as *Botrylloides diegensis* [26]. For this reason, the previously

342 reported studies from our lab that denoted our model organism as *Botrylloides leachii*

343 are *Botrylloides diegensis* [4, 14, 34, 43, 44].

344

345 Two other *Botrylloides* species collected are the first molecular records from New

346 Zealand: *Botrylloides jacksonianum* and *Botrylloides affinis anceps*. The species from

347 Whangateau was assigned as *B. aff. anceps* due to its high molecular affinity to the

348 record from Australia (GenBank Accession No: MT873573) [28]. Although we were

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

361

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

371

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

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

380

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

385

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

393

394 Overall, our findings suggest that COI is a reliable marker in terms of botryllid ascidian
395 identification. Among nuclear markers, H3 is adequately resolving the species
396 taxonomy compared to 18S and 28S. For our previous publications, we had checked
397 our COI barcodes which matched at the time with the *B. leachii* sequences in the
398 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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557 TABLES

558 **Table 1.** Primer sequences, annealing temperature, expected product length for

559 *COI, H3, 18S, and 28S* target genes used in this study.

Primer name	Primer sequence	Ann. Temp. (°C)	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	AACTTGATTTAAATTACGATC	55	~650	647	Stefaniak et al 2009
dinF	CGTTGRTTATRTCTACWAATCATAARGA	52	~700	647	Brunetti et al 2017
Nux1R	GCAGTAAATAWGCTCGRGARTC	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	GATCCTTCTGCAGGTTACCTAC	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

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563 SUPPORTING INFORMATION

564 **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

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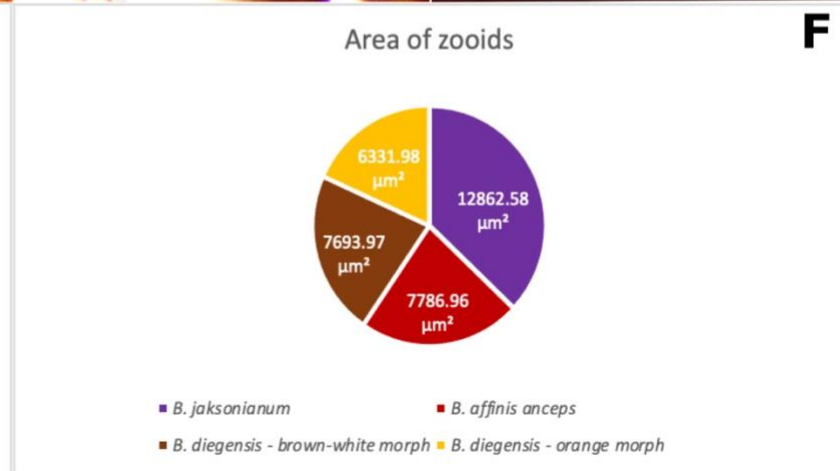
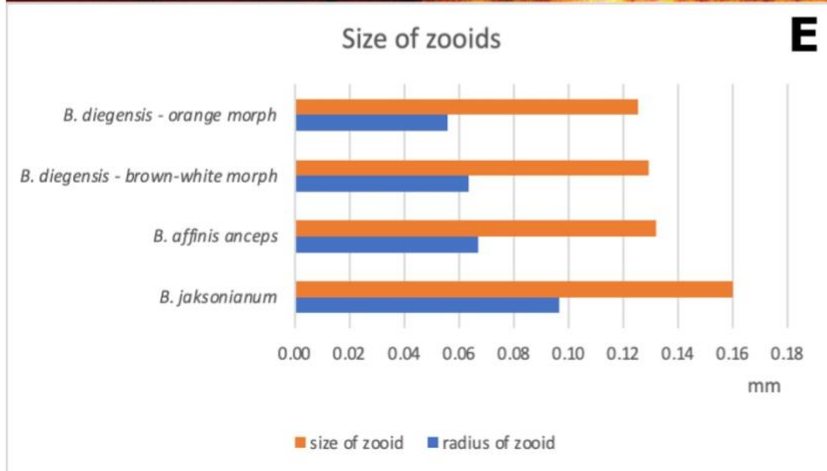
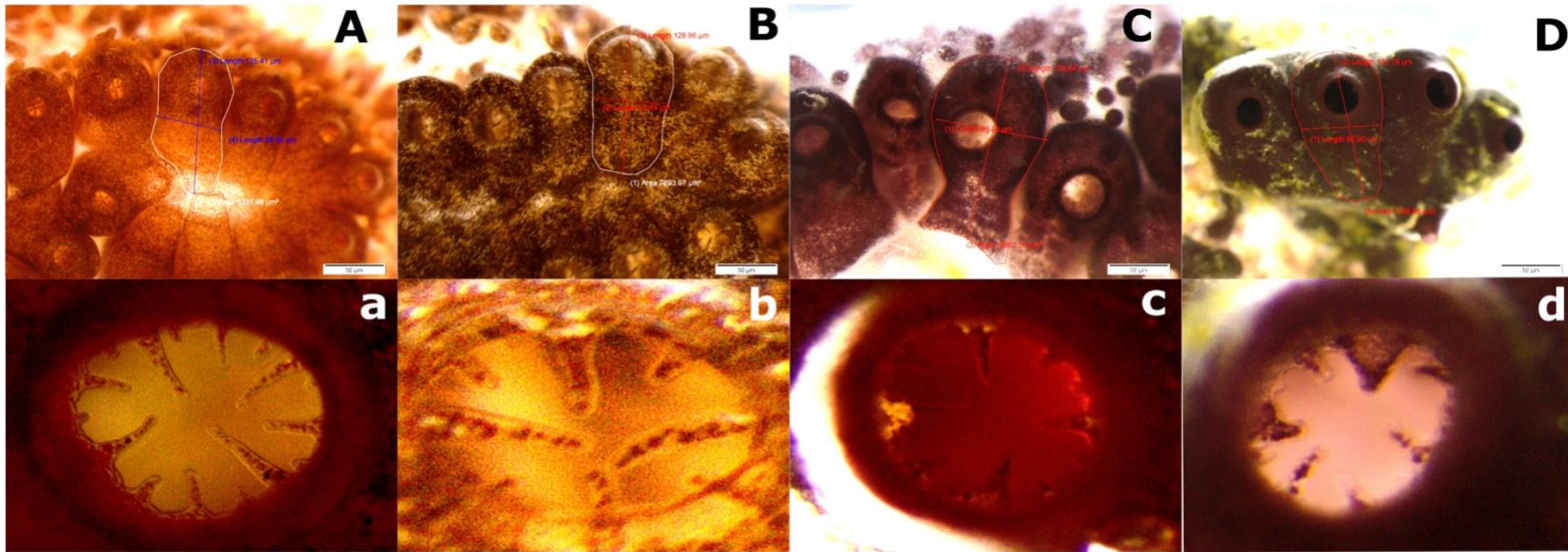
567 **Table S2.** GenBank Accession Numbers

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

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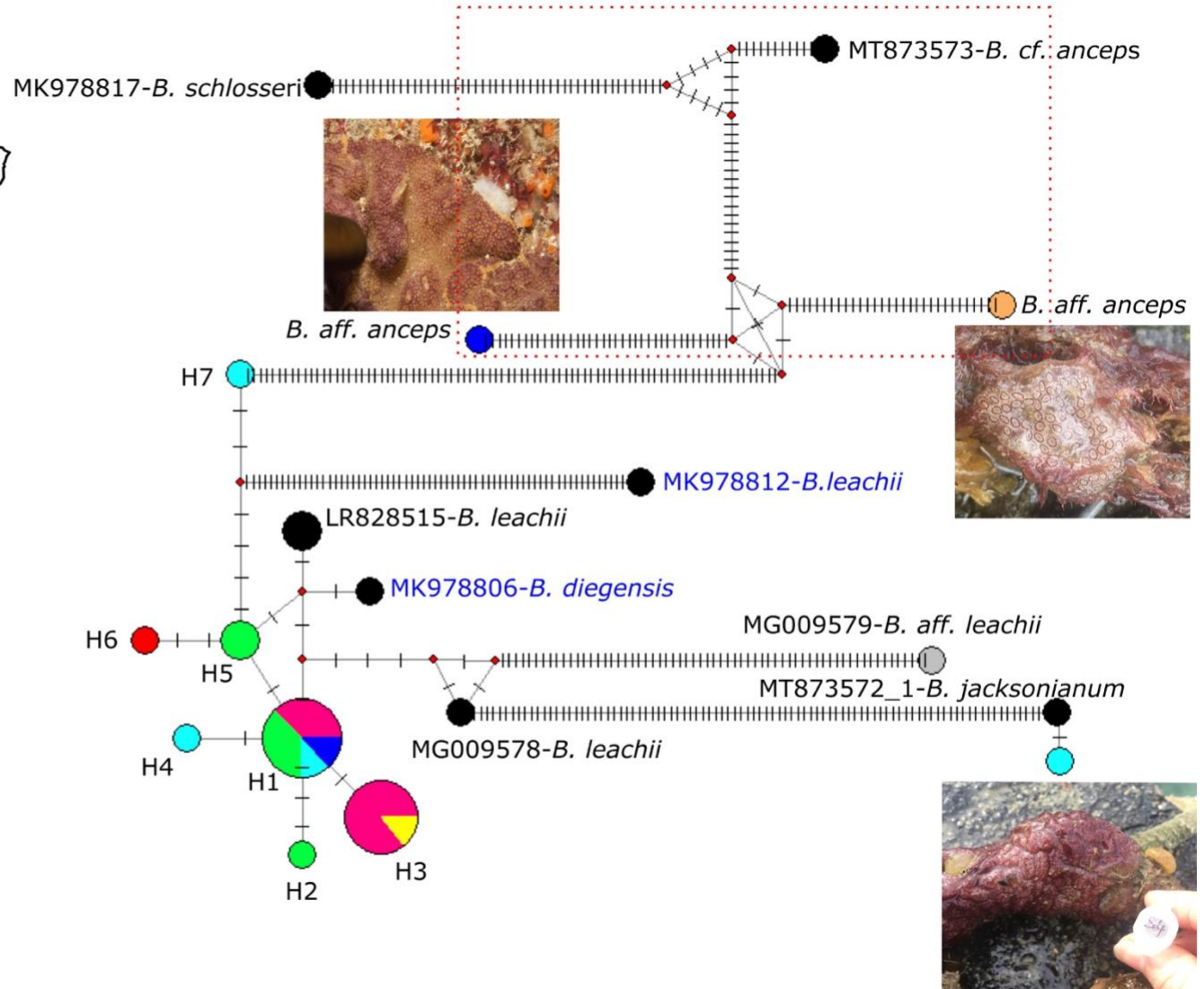
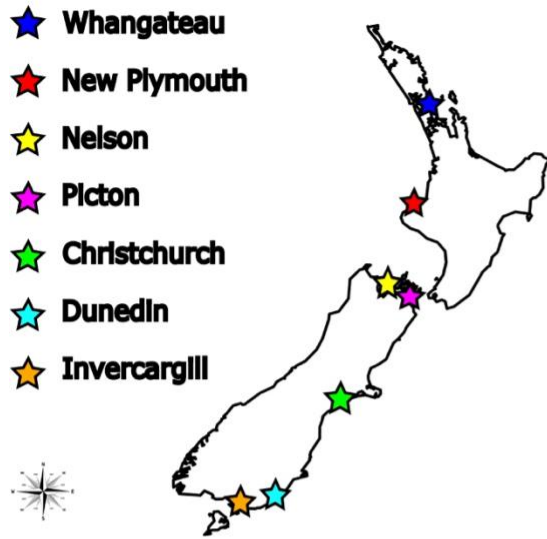
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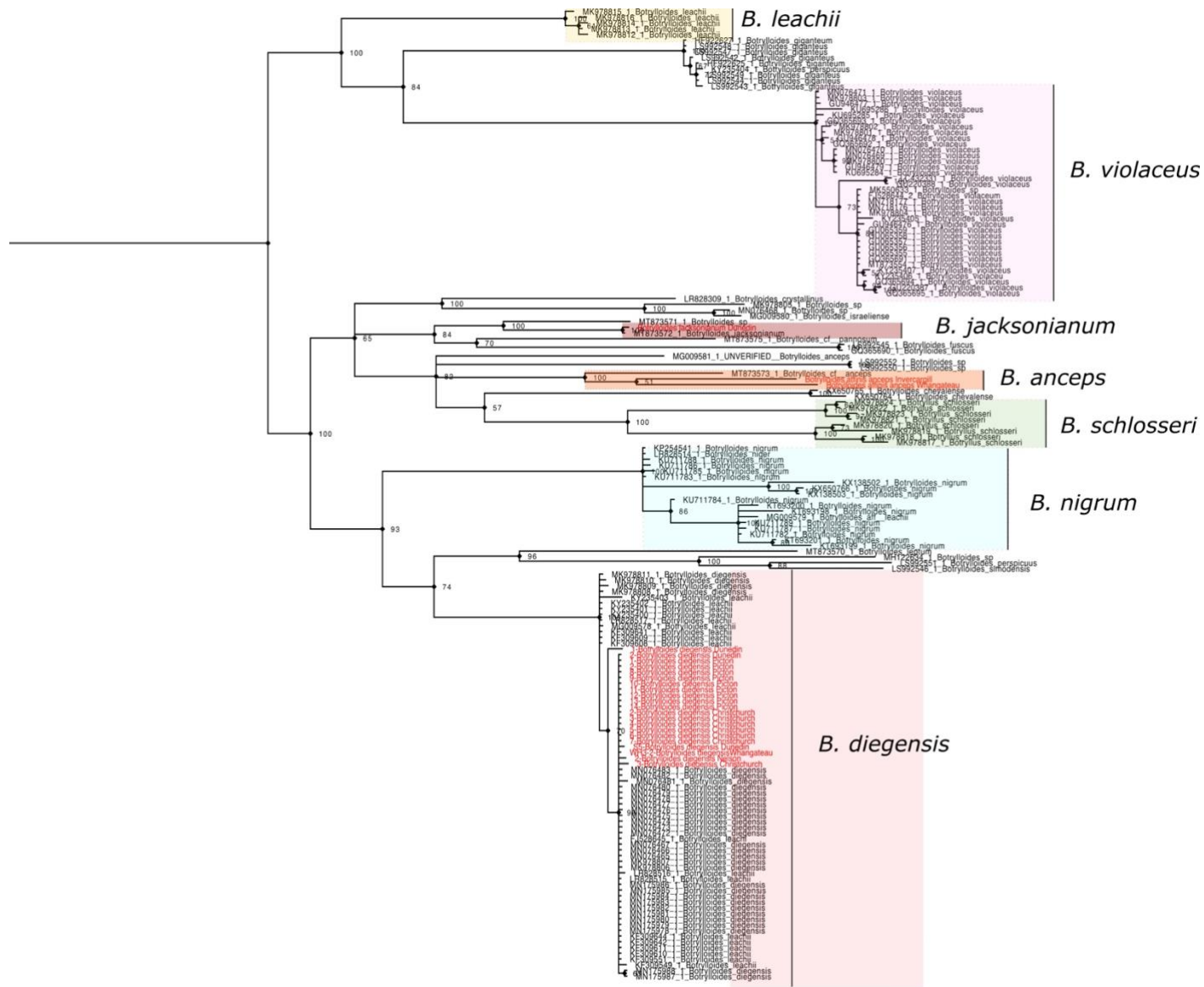
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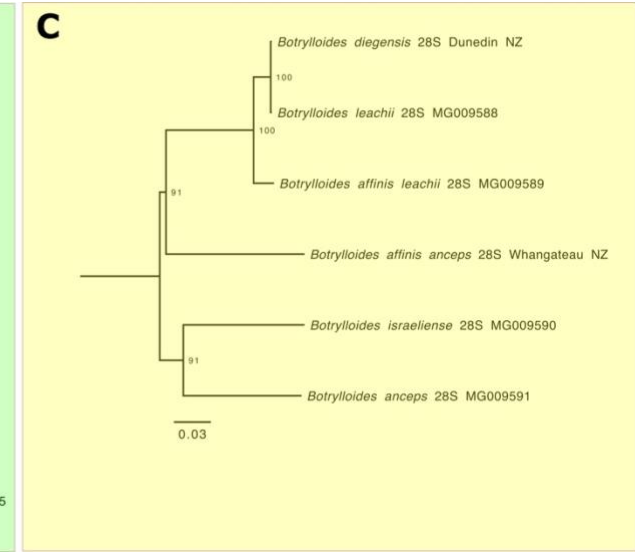
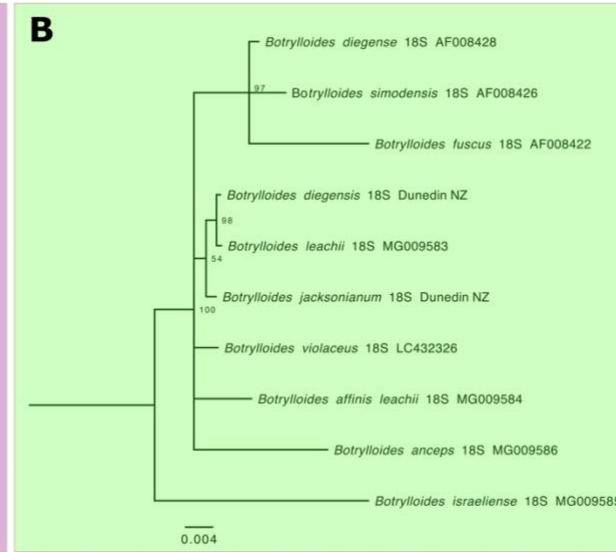
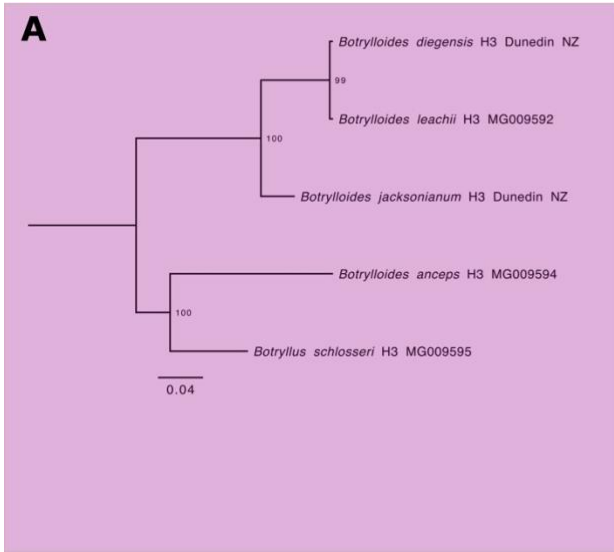
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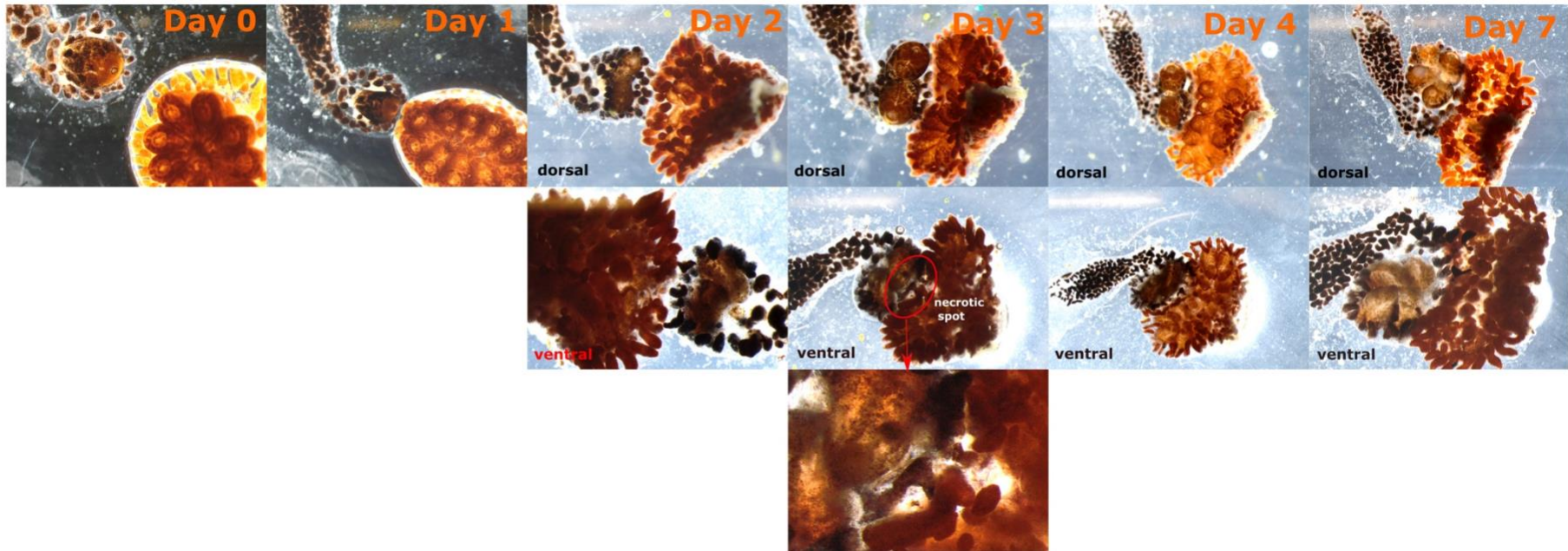
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