NEW ZEALAND JOURNAL OF MARINE AND FRESHWATER RESEARCH
2024, VOL. 58, NO. 2, 255–273
https://doi.org/10.1080/00288330.2023.2215534

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RESEARCH ARTICLE

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Identification and characterisation of *Botrylloides* (Styelidae) species from Aotearoa New Zealand coasts

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ABSTRACT

Ascidians are marine filter-feeder chordates. *Botrylloides* ascidians possess diverse biological properties such as whole-body regeneration (WBR), hibernation/aestivation, blastogenesis, metamorphosis, and natural chimerism. However, the absence of distinctive morphological features often makes identification difficult. *Botrylloides diegensis* is an ascidian that has been misidentified in previous studies and is recorded in GenBank as *Botrylloides leachii* owing to the high morphological similarity between the sister species. More available sequences and strategies for identification would help resolve some of the confusion surrounding its ambiguous nature. We collected several *Botrylloides* samples from seven locations in Aotearoa New Zealand (Dunedin, Christchurch, Picton, Nelson, Whangateau, New Plymouth, and Invercargill) and barcoded the species based on Cytochrome Oxidase I, Histone 3, 18S, and 28S ribosomal RNA markers. Network and Bayesian trees confirmed the presence of three *Botrylloides* species: *B. diegensis*, *Botrylloides jacksonianum,* and *Botrylloides affinis anceps.*

ARTICLE HISTORY
Received 15 March 2023
Accepted 15 May 2023

HANDLING EDITOR
Andrea Alfaro

KEYWORDS
Ascidian; tunicate; Aotearoa New Zealand; Pacific; barcoding; COI

Introduction

Ascidians, a group of marine invertebrates have been extensively studied for ecological purposes such as determining biodiversity, origins, and introductions (Rocha et al. 2005; Shenkar and Swalla 2011; Dias et al. 2013; Page and Kelly 2013; Page et al. 2014; Bae et al. 2023). However, identifying invasive and indigenous species can be challenging due to the absence of solid biogeographical or historical evidence, which can have a significant impact on the survival of native species. The survival of sessile species is dependent on spatial competition, which may have detrimental effects on the native species (Simkanin et al. 2012; Zhan et al. 2015; Zhang et al. 2020). With the increase in sea transportation and the construction of shipping channels, new introductions have altered

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/00288330.2023.2215534.

habitat structures and local biodiversity (Shenkar and Loya 2009; Carman et al. 2010; Fletcher et al. 2013; Halim and Messeih 2016). The morphological plasticity and slight differences in anatomical distinctions between species have also made accurate identification of ascidians challenging, particularly for colonial forms which are in a constant budding cycle. Nevertheless, ascidians exhibit crypticity owing to morphological plasticity and slight differences in anatomical distinctions between species (Iannelli et al. 2007; Viard et al. 2019; Dias et al. 2021; Salonna et al. 2021). This has resulted in challenges in which anatomical and morphometric analyses can be limited, particularly for colonial forms (Berrill 1947; Teske et al. 2011; Reem et al. 2018; Nydam et al. 2021). Despite these challenges, the species of *Botrylloides* (Milne-Edwards 1841) and *Botryllus* (Gaertner 1774) are becoming increasingly important in biomedical research owing to their high regeneration capacities, novel biomolecular content, and lab adaptability (Voskoboynik and Weissman 2015; Blanchoud et al. 2018; Franchi et al. 2023). Thus, accurate identification of ascidians is crucial for biodiversity estimation, control, taxonomic positioning for comparative analyses, and the development of ascidian-derived biomedical applications integrated with stem cell research. (Ben-Hamo and Rinkevich 2021; Temiz and Wilson 2022; Vanni et al. 2022).

Identification of *Botrylloides* species is particularly challenging (Blanchoud et al. 2017; Reem et al. 2018; Viard et al. 2019; Nydam et al. 2021; Salonna et al. 2021; Karahan et al. 2023; Temiz et al. 2023). Their separation from sister species is unclear because of the lack of morphological divergence or a defined distance (barcoding gap) for inter-and intraspecies delineation (Reem et al. 2018; Viard et al. 2019). Some of the sister species that lack this are *Botrylloides leachii* (Savigny 1816), *Botrylloides violaceus* (Oka 1927), *Botrylloides niger* (Herdman 1886), and *Botrylloides diegensis* (Ritter and Forsyth 1917) (Brunetti 2009; Reem et al. 2018; Viard et al. 2019; Salonna et al. 2021; Temiz et al. 2023). Moreover, *Botrylloides perspicuus* (Herdman 1886), *Botrylloides giganteus* (Pérès 1949), and *Botrylloides pizoni* (Brunetti and Mastrototaro 2012) have similar colonial and zooidal features, although they are distinct species (Rocha et al. 2019). The similarities between these sister *Botrylloides* species have resulted in ambiguous identification and sometimes misidentification, demonstrating the need for clear taxonomic identification (Reem et al. 2018; Viard et al. 2019). The synonymisation of three indo-pacific species, *Botrylloides leptum* (Herdman 1899), *Botrylloides pannosum* (Herdman 1899), and *Botrylloides jacksonianum* (Herdman 1899), with *B. leachii,* is another example of this incorrect classification (Salonna et al. 2021). The development of molecular tools for species delimitation is decreasing discrepancies by prevailing solely in anatomical base taxonomy, as they are less prone to error and are more reliable (Carstens et al. 2013; Viard et al. 2019; Puillandre et al. 2021; Salonna et al. 2021). This can also enable the proper detection of species diversity, characterisation of the distribution, and human-mediated species introductions which are crucial for ecological studies. Some ascidian species like *Botryllus schlosseri* (Pallas 1766) and *B. violaceus* are invasive and can negatively affect aquaculture (Westerman 2007; Ben-Shlomo et al. 2010; Reem et al. 2013; Simkanin et al. 2013; Karahan et al. 2016). Once these species are introduced to a new habitat, they can overgrow and dominate the available spaces, including mobile areas, such as the outer layer of crustaceans, which might adversely affect animal mobility. DNA barcoding is a powerful tool and, when combined with morphological data, enables the identification of species, including the genus *Botrylloides* (Miralles et al.

2016; Reem et al. 2018; Rocha et al. 2019). The selection of molecular markers to estimate divergence is essential. These studies often use mitochondrial cytochrome oxidase subunit I (COI), a polymorphic but conserved region, and other gene markers such as 12S rRNA (ribosomal RNA), 16S rRNA, 18S rRNA, and 28S rRNA, which can also be used to increase identification resolution (Puillandre et al. 2009; Reem et al. 2018; Paz and Rinkevich 2021). Improving the quality and quantity of database sequences will play an essential role in future barcoding approaches and in improving global biodiversity monitoring. For example, molecular studies combined with morphological investigation allowed a taxonomic revision of *Ciona intestinalis* and confirmed the existence of both *Ciona robusta* (Hoshino and Tokioka 1967) and *Ciona intestinalis* (Linnaeus 1767), previously synonymised, as distinct species (Brunetti et al. 2015; Gissi et al. 2017). In other cases, mitogenomics, a whole-mitochondrial barcoding technique, has been used to separate species. This supports the findings of a recent study that reported that a European clade of *B. schlosseri* was a new species, identified as *Botryllus gaiae,* using a deeper molecular taxonomy (Brunetti et al. 2020).

The first records of *B. leachii* from Otago Harbour, Aotearoa New Zealand were given by Brewin (1946) in the name of *Botryllus leachii*, an old synonym for *B. leachii*. Records from other localities from Aotearoa New Zealand were presented for *B. leachii* demonstrated its widespread presence (Brewin 1960; Lindsay et al. 1999; Page and Kelly 2013; Page et al. 2014; Cahill et al. 2016; Zondag et al. 2016; Blanchoud et al. 2017; Blanchoud et al. 2018). In recent research, all GenBank sequences of *B. leachii* were stated to have been incorrectly assigned and predicted to belong to *B. diegensis* (Viard et al. 2019). It was also stated that the spoked-wheel morphology in the vicinity of the zooidal buccal siphon is present in *B. leachii* but absent in *B. diegensis*. Thus, this places all the previously identified samples, especially those with no molecular information, in an uncertain position. *B. diegensis* is commonly seen in the intertidal zone throughout Aotearoa New Zealand's coasts and is thought to have originated from the Western or Southern Pacific and was potentially introduced to the Atlantic and Northern Pacific, whereas *B. leachii* is stated to have Mediterranean origins and is recorded to be non-indigenous to the Indo-Pacific. (Carlton 2009; Shenkar and Swalla 2011; Page and Kelly 2013; Page et al. 2014; Viard et al. 2019). *B. diegensis* is globally invasive; therefore, its detection is important for the protection of marine diversity (Viard et al. 2019). Furthermore, considering that these species are emerging models, particularly for regenerative and developmental science because of their prominent phylogenetic position, accurate identification is essential. Our group studies regeneration in *Botrylloides*; we had assigned this species as *B. leachii* based on previous publications and comparisons with GenBank sequences. Thus, in this study, DNA barcoding combined with morphology was to identify *Botrylloides* species in the coastal waters of Aotearoa New Zealand.

Materials & methods

Sampling area

Forty colonies were collected from seven zones around Aotearoa New Zealand (Invercargill, Dunedin, Christchurch, Nelson, Picton, Whangateau, and New Plymouth). Colonies were preserved in absolute ethanol and collected between July 2020 and February 2021.

Sampling details, including the collection date, haplotype information, sampling stations, coordinates, and sampling dates, are listed for all collected samples (Table 1). Colonies were collected from the first few metres of the ocean surface. The collection was based on morphological identification at the sampling sites based on general knowledge of the morphology of *Botrylloides* ascidian: ladder-type zooid distribution and the presence of a gelatinous matrix (Brunetti 2009). Colony fragments were obtained using a single-edged razor blade. For animal breeding, living tunicate tissue fragments were attached to 5 × 7.5 cm glass slides (Zondag et al. 2016). The slides were placed in tanks filled with filtered salt water that was constantly aerated. The animals were fed a shellfish diet (a blend of marine microalgae; LPB Frozen Shellfish Diet^{TM,} Reed Mariculture) regularly, and their water was replaced every two days.

Morphological insights

All morphological examinations were performed qualitatively. This is because the focus was primarily on molecular analysis. However, some general morphotypes, zooid and tentacle pictures were also obtained.

Collected *Botrylloides* ascidians were photographed and morphologically examined for zooid arrangement and colour. Some samples were only taken for molecular analysis; therefore, not all colony images were taken. Orange and brown-white morphotypes of *B. diegensis*, purple morphotypes of *B. jacksonianum,* and purple *Botrylloides affinis* were investigated for their tentacle numbers, sizes, and areas of the zooids. The size and area of the zooids were measured using the cellSens Standard 1.5 imaging suite and a Nikon SMZ745 dissection microscope. Only some of the living morphotypes of the three species during stage A of blastogenesis were examined for zooid size and area.

Molecular analysis

DNA was extracted as described by Gemmell and Akiyama (1996). Alcohol was removed from the samples, and 300 µl lysis buffer (100 mM NaCl, 50 mM Tris-HCl, 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. Colonies were homogenised and incubated 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 by 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 the maximum speed. The supernatant was then transferred to a new tube. Two volumes of absolute ethanol were added to the tubes, which were inverted several times. DNA was precipitated by centrifugation at maximum speed for 30 min. The supernatant was discarded, and the pellet was washed with 70% ethanol and centrifuged at the maximum speed for 5 min. Excess ethanol was removed, and the pellets were left to air dry for 10 min. Finally, 100–200 µl of TE buffer (10 mM Tris-HCl, 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. The DNA concentrations were measured using a Nanodrop (Thermo Fisher).

DreamTaq Green PCR Master Mix (Thermo Fisher) was used with 10 ng/µl diluted genomic DNA. PCR was performed as follows:1) 95 °C for 3 min; 1 cycle; 2) 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min for 35 cycles; and 3) 72 °C for 10 min. Primers

Table 1. Taxonomic assignment of colonies, sampling details including the collection date, haplotype information, sampling stations, their coordinates and the sampling dates. The success of the barcodes of the samples included based on COI, H3, 18S, and 28S gene regions. Two COI sequence fragments were obtained by cloning into a vector, explained in the molecular analysis section. The final taxonomic assignment was made based on the molecular results.

	Sample ID	Morphotype	COI Haplotype	Species identification	Sampling station	Coordinates	Sampling date (d/m/y)	COI	H3	18S	28S
1	1dunedin		H7	*B. diegensis*	Dunedin	45°52′17″S, 170°31′43″E	11/6/20	yes	yes	no	no
2	1adunedin		-	*B. diegensis*	Dunedin	45°52′17″S, 170°31′43″E	11/6/20	no	yes	no	no
3	3adunedin		-	*B. diegensis*	Dunedin	45°52′17″S, 170°31′43″E	11/6/20	no	no	yes	no
4	4adunedin		-	*B. diegensis*	Dunedin	45°52′17″S, 170°31′43″E	11/6/20	no	no	yes	yes
5	6adunedin		-	*B. diegensis*	Dunedin	45°52′17″S, 170°31′43″E	11/6/20	no	no	yes	yes
6	S4	purple	-	*B. jacksonianum*	Dunedin	45°52′17″S, 170°31′43″E	12/9/20	yes/ cloned	yes	no	no
7	S5		H4	*B. diegensis*	Dunedin	45°52′17″S, 170°31′43″E	12/9/20	yes	yes	no	yes
8	S6		-	*B. diegensis*	Dunedin	45°52′17″S, 170°31′43″E	12/9/20	no	no	yes	no
9	6nc		H3	*B. diegensis*	Nelson	41°15′54.5"S, 173°16′44.8"E	11/6/20	yes	no	no	no
10	7n		-	*B. diegensis*	Nelson	41°15′54.5"S, 173°16′44.8"E	11/6/20	no	yes	no	no
11	13		-	*B. diegensis*	Nelson	41°15′54.5"S, 173°16′44.8"E	11/6/20	no	yes	no	no
12	14		-	*B. diegensis*	Nelson	41°15′54.5"S, 173°16′44.8"E	11/6/20	no	yes	no	no
13	1pict	orange	H1	*B. diegensis*	Picton	41°17′21.5"S, 174°00′32.9"E	20/7/20	yes	yes	no	no
14	1cpict		-	*B. diegensis*	Picton	41°17′21.5"S, 174°00′32.9"E	20/7/20	no	yes	yes	yes
15	2pict	orange	H3	*B. diegensis*	Picton	41°17′21.5"S, 174°00′32.9"E	20/7/20	yes	no	no	no
16	5pict	orange	-	*B. diegensis*	Picton	41°17′21.5"S, 174°00′32.9"E	20/7/20	no	yes	yes	yes
17	6pict		-	*B. diegensis*	Picton	41°17′21.5"S, 174°00′32.9"E	20/7/20	no	no	yes	yes
18	7pict		-	*B. diegensis*	Picton	41°17′21.5"S, 174°00′32.9"E	20/7/20	no	no	yes	yes
19	8pict		H3	*B. diegensis*	Picton	41°17′21.5"S, 174°00′32.9"E	20/7/20	yes	no	no	no
20	9pict	orange-brown	H1	*B. diegensis*	Picton	41°17′21.5"S, 174°00′32.9"E	20/7/20	yes	no	no	no
21	10pict		H3	*B. diegensis*	Picton	41°17′21.5"S, 174°00′32.9"E	20/7/20	yes	no	no	no
22	11pict		H3	*B. diegensis*	Picton	41°17′21.5"S, 174°00′32.9"E	20/7/20	yes	no	no	no
23	12pict		H3	*B. diegensis*	Picton	41°17′21.5"S, 174°00′32.9"E	20/7/20	yes	no	no	no
24	13pict	brown-white	H1	*B. diegensis*	Picton	41°17′21.5"S, 174°00′32.9"E	20/7/20	yes	no	yes	no
25	14pict	orange-brown	H3	*B. diegensis*	Picton	41°17′21.5"S, 174°00′32.9"E	20/7/20	yes	no	no	no
26	2chch	orange-brown	H1	*B. diegensis*	Christchurch	43°36′17.89"S, 172° 42′44.71"E	14/7/20	yes	no	no	no
27	2achch		-	*B. diegensis*	Christchurch	43°36′17.89"S, 172° 42′44.71"E	14/7/20	no	yes	no	no
28	3chch	orange	H5	*B. diegensis*	Christchurch	43°36′17.89"S, 172° 42′44.71"E	14/7/20	yes	no	no	no
29	4chch	orange	H1	*B. diegensis*	Christchurch	43°36′17.89"S, 172° 42′44.71"E	14/7/20	yes	no	no	no

(*Continued*)

Table 1. Continued.

	Sample ID	Morphotype	COI Haplotype	Species identification	Sampling station	Coordinates	Sampling date (d/m/y)	COI	H3	18S	28S
30	5chch	orange-brown	H1	*B. diegensis*	Christchurch	43°36′17.89"S, 172° 42′44.71"E	14/7/20	yes	no	yes	yes
31	6chch	brown-white	H5	*B. diegensis*	Christchurch	43°36′17.89"S, 172° 42′44.71"E	14/7/20	yes	yes	no	no
32	PW1		H2	*B. diegensis*	Christchurch	43°36′17.89"S, 172° 42′44.71"E	14/7/20	yes	no	no	no
33	F1		H1	*B. diegensis*	Invercargill	46°35′45.0"S 168°20′08.0"E	2/2/2021	yes	no	no	no
34	F2	purple-white wheel	-	*B. aff. anceps*	Invercargill	46°35′45.0"S 168°20′08.0"E	2/2/2021	yes	no	yes	no
35	WH1	purple-gold wheel	-	*B. diegensis*	Whangateau	36°19′11.03"S, 174° 46′55.36"E	23/9/20	no	yes	no	no
36	WH2	purple	-	*B. aff. anceps*	Whangateau	36°19′11.03"S, 174° 46′55.36"E	23/9/20	yes/ cloned	no	no	yes
37	WH3	orange	H1	*B. diegensis*	Whangateau	36°19′11.03"S, 174° 46′55.36"E	23/9/20	yes	no	no	yes
38	NP1	orange	-	*B. diegensis*	New Plymouth	39° 3′17.91"S, 174°3′20.85"E	8/9/2020	no	yes	yes	yes
39	NP2		-	*B. diegensis*	New Plymouth	39° 3′17.91"S, 174°3′20.85"E	8/9/2020	no	yes	yes	yes
40	NP3	orange-brown	H6	*B. diegensis*	New Plymouth	39° 3′17.91"S, 174°3′20.85"E	8/9/2020	yes	yes	yes	yes

used in this study are listed in Table S1. The PCR products were cleaned with the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher). Five microlitres of the post-PCR product were mixed with 2 µl of ExoSAP reagent and incubated at 37 °C for 15 min and then at 80 °C for 15 min. After the clean-up, the fragments were sent to the sequencing facility.

Two samples (*B. aff. anceps* and *B. jacksonianum*) were cloned into the vector for sequencing (Table 1). This was data could not be obtained by sequencing these PCR products directly (generated poor sequencing data). Thus, the fragments were inserted into the vector and then sequenced. First, the DNA fragments were amplified from gDNA using primers. The fragments were then ligated into the pCRII-TOPO vector (Life Technologies). Competent *E. coli* cells were transformed with the plasmid and selected by blue-white screening. Finally, plasmids were isolated and sequenced. The Otago University Sequencing Facility sequenced all the samples using Sanger sequencing. The tubes were prepared with 3.2 pmol forward primer and 4 µl of cleaned post-PCR template per sample.

All the forward sequences were checked based on their chromatogram data and trimmed if required so that only confident base calls were used for alignment. For comparative analysis, *Botrylloides* COI sequences from GenBank (including the misidentified *B. leachii* sequences) were added to the network and phylogenetic analyses (sequences were retrieved on 29/11/22) (Table S3). The outgroup species of the COI, H3, 18S, and 28S phylogenies are *Symplegma brakenhielmi* (Accession no: LS992554), *Styela clava* (Accession no: XR_005568549), *Symplegma viride* (Accession no: DQ346655), and *Ciona intestinalis* (Accession no: AF212177), respectively.

All sequences were aligned, edited, and trimmed using BioEdit version 7 per marker for multiple alignments (Hall 1999) and ClustalX (Thompson et al. 1997). Finally, COI haplotypes were identified using NETWORK version 10 with a median-joining algorithm (Bandelt et al. 1999). The unique sequences obtained in the current study were uploaded to GenBank (Table S2).

Bayesian probabilities and branch lengths were calculated for all loci by using MrBayes 3.2 (Figure S1) (Ronquist et al. 2012). We used the HKY + I + G model for COI, HKY + G for H3, K80 + G for 18S and GTR + G models for 28S phylogenies (Kimura 1980; Hasegawa et al. 1985; Tavaré 1986). The best model selection calculations were computed via jmodeltest2 using the AICs and BIC methods for the Bayesian phylogenies (Darriba et al. 2012). The posterior probabilities of mitochondrial and nuclear sequences were analysed using two independent runs of the Monte Carlo Markov model. At least one million generations were calculated with a sampling frequency of 1000 for each generation of nuclear genes, while over 15 million generations were generated for the COI tree. The split frequencies of all phylogenetic calculations were lower than 0.01. Bayesian trees were optimised using FigTree 1.4.4 (Rambaut 2018).

Operational taxonomic units (OTU) and species delimitation were selected based on analyses using the Automatic Barcode Gap Discovery (ABGD) method of assembling species by automatic partitioning (ASAP) (Puillandre et al. 2021) and bPTP using Bayesian support values (Zhang et al. 2013). Jukes-Cantor and Kimura 2 parameter-based estimations were calculated using the ASAP (Jukes and Cantor 1969; Kimura 1980). The Poisson tree processes (bPTP) were implemented using the Bayesian tree model with Python package version.

Results

Morphological observations

Botrylloides zooids are organized side by side as branching double-row systems, also called '*leachii*' type, with their dorsal lamina facing the surrounding environment. Different morphs of *B. diegensis* from the Aotearoa New Zealand coasts were observed, including orange, orange-brown, brown, brown-white, and purple with gold wheels (Figure 1 and S2). All zooids share similar zooidal features and are equal in tentacle number (four large, four smaller, and eight petite) (Figure S3)

Phylogeny and haplotype diversity

A phylogeny based on the COI locus was constructed to evaluate taxonomy (Figure 2). Most of the COI sequences (Table 1) were grouped with the *B. diegensis* sequences from GenBank, and with the ones that were misidentified as *B. leachii* but are now recognised as *B. diegensis* (Figure 2) (Viard et al. 2019). Therefore, these colonies were taxonomically assigned to *B. diegensis* (Table 1). Sequences of two colonies, one from Whangateau (WH2) and the other from Invercargill (F2), clustered with *B. cf. anceps* COI barcode sequence from Australia (Accession no: MT873573) and one Dunedin sample (S4) 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 colony (S4) from Dunedin is nominated as *B. jacksonianum* due to the low mismatch ratio (<1%).

Using ASAP analysis to partition the species, 23 operational taxonomic units (OTUs) were identified (Figure 3; 153 sequences). All *B. diegensis* samples from this study

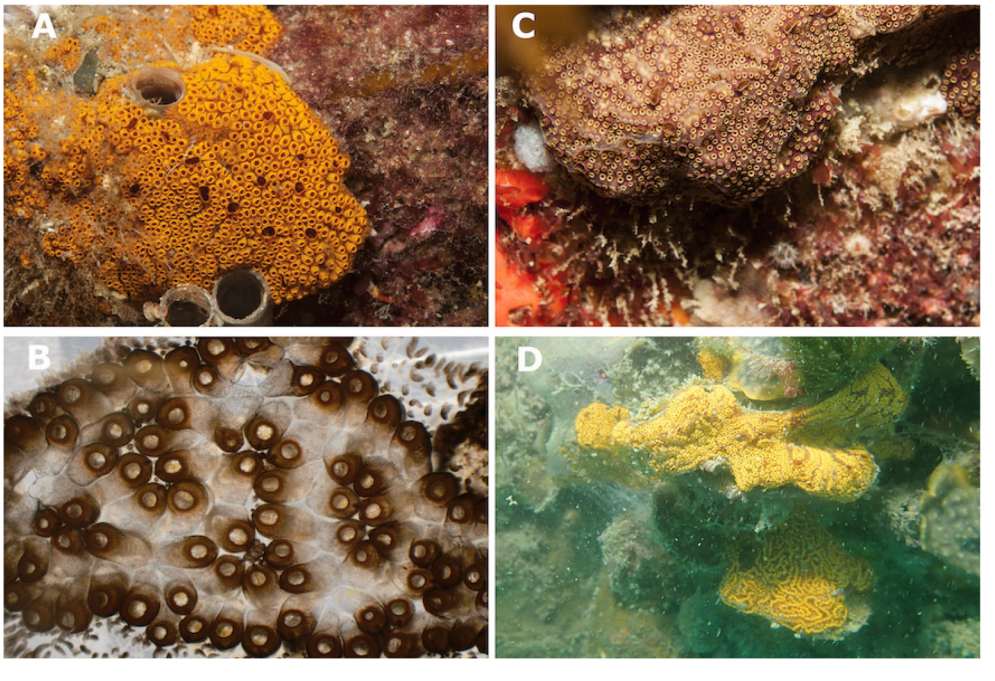

Figure 1. Several colour morphs of *B. diegensis* from Aotearoa, New Zealand. **A**, Common orange morph. **B**, A brown *B. diegensis* colony. **C**, Purple with gold wheel morphotype. **D**, Brown-orange colony.

Figure 2. Bayesian tree of *Botrylloides* based on the mitochondrial COI locus. The current study sequences and all *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 at a sampling frequency of 1000 for each generation. The split frequencies were less than 0.01. The red sequences indicate the current study sequences with sampling location, haplotype information and GenBank accession numbers. The scale indicates the evolutionary distance.

clustered with *B. diegensis* samples from the database in the same OTU, along with some of the sequences from the database previously misidentified as *B. leachii* (71 sequences in total). Confirmed *B. leachii* sequences formed a separate OTU (Figure 3).

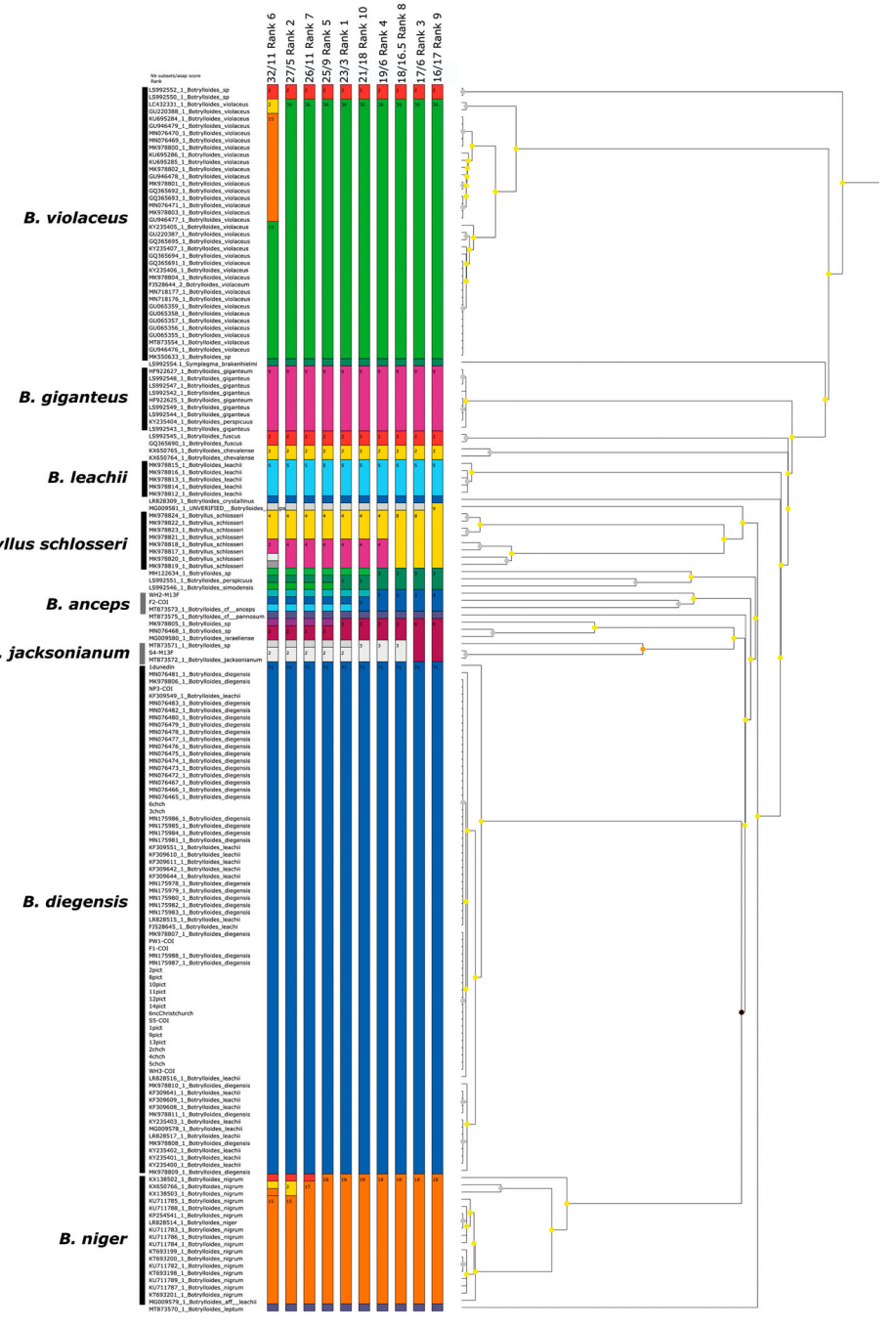

Figure 3. Molecular species delimitation using ASAP. ASAP scores were measured for *Botrylloides* species based on the Jukes-Cantor and kimura 2-parameter model selection. The colours illustrate the OTUs, while the numbers within each OTU show the sample numbers belonging to that unit. Each column represents a partition with the highest ASAP score. The column headers are OTU numbers and ASAP scores. The lowest ASAP score was considered for OTU selection. The ultrametric clustering tree is shown on the right and the node colour indicates the *p*-value. *Symplegma brakenhielmi* (Accession no: LS992554) as an outgroup.

B. jacksonianum was another OTU. *B. aff. anceps* samples of this study were two distinct OTUs closely aligned with the *B. cf. anceps* database OTU (MT873573). These results are consistent with the bPTP analysis (Figure S4).

In addition to the mitochondrial marker COI, three other nuclear regions, H3, 18S, and 28S, were analysed. Bayesian trees were constructed based on H3, 18S, and 28S sequences (Figure 4). The H3 tree (Figure 4A) confirmed the clustering of the Aotearoa New Zealand samples (except S4, F2 and WH2) with a database sequence labelled (misidentified) as *B. leachii* (Accession no: MG009592*)*. The low evolutionary distance of the database *B. leachii* sequences based on 18S (MG009583) and 28S (MG009588) barcodes further confirmed the identification of the current study sequences as *B. diegensis* (Figure 4B and C). This *B. leachii* database sample is *B. diegensis* owing to its COI barcode (MG009578) clustering with the *B. diegensis* sequences in the Bayesian phylogeny, ASAP and bPTP analyses as all the database sequences belong to *B. leachii* are assigned as *B. diegensis* by Viard et al. (2019) which is consistent with the current study molecular analyses.

Based on 18S, *B. aff. anceps* from Invercargill was separated from *Botrylloides anceps* from Israel (indicated as unverified in GenBank) (Figure 4B). The lack of 18S sequences in the database resulted in insufficient separation of the outgroup (*Symplegma viride*) from *Botrylloides* sequences. In contrast, the H3 and 28S outgroups could be separated in the tree further from the compared sequences. Phylogeny based on 28S assigned *B. aff. anceps* from Whangateau to be more closely related to *B. anceps* from Israel (Accession no: MG009591) (Figure 4C).

Seven haplotypes were identified based on clustering of the COI Network from the seven sampling locations (Figure 5). Distance values (0.2–1%) of the sequences supported their identification as *B. diegensis* based on the COI species delineation threshold (< 2%) (Hebert et al. 2003) compared to database sequences based on the BLAST mismatch

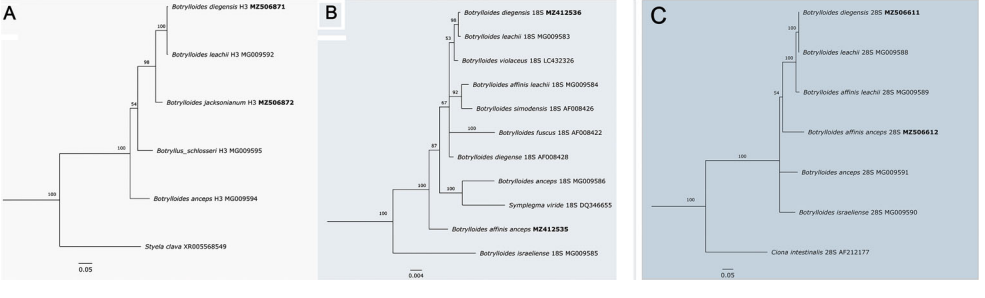

Figure 4. Bayesian trees based on nuclear markers. **A**, Tree based on Histone 3 based on the Hasegawa-Kishino-Yano (HKY + G)) substitution model (Hasegawa et al. 1985). The outgroup was *Styela clava* (Accession no: XR_005568549). **B**, Phylogeny based on the 18S ribosomal subunit gene was constructed based on the 2-parameter kimura with a gamma substitution rate (K80 + G) (Kimura 1980). The outgroup is *Symplegma viride* (Accession no: DQ346655). **C**, The 28S ribosomal subunit tree was calculated using the general reversible time with the gamma (GTR + G) model (Tavaré 1986). *Ciona intestinalis* (AF212177) was used as the outgroup. Phylogenies were constructed with the study sequences and all *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. The split frequencies were less than 0.01. The numbers in the tree indicate bootstrap frequencies. Accession numbers of the sequences used in this study are shown in bold.

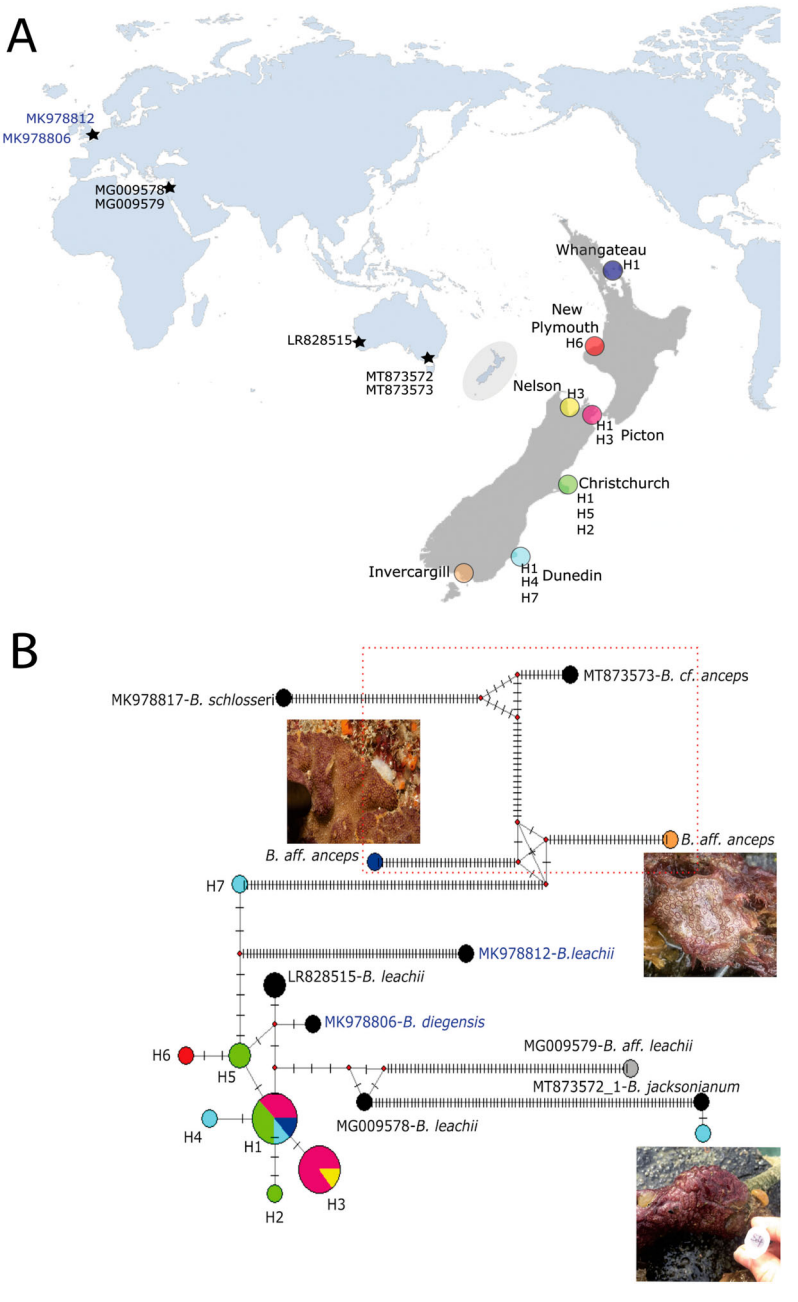

Figure 5. Phylogeography of Aotearoa New Zealand *Botrylloides* ascidians. **A**, Location of sample sites in Aotearoa New Zealand (Turquoise: Dunedin, Green: Christchurch, Orange: Invercargill, Yellow: Nelson, Pink: Picton, Blue: Whangateau, Red: New Plymouth). The locations of the database sequences used in the analysis are also shown on a world map. **B**, The stripes on the lines indicate the mutation steps between the haplotypes. Circle size represents haplotype frequency. Colours mark the regions where the samples were collected, as shown on the map in panel A. The analysed partial COI sequences were 647 bp. Grey and black circles indicate the database haplotypes. Black represents *B. diegensis/leachii*, and gray represents *B. aff. leachii* (GenBank sequences). Blue text indicates *B. leachii* MK978812), and *B. diegensis* reference sequences (MK978806) (Viard et al. 2019). *The B. anceps* cluster is highlighted with a red dashed line box.

ratio. Haplotype H1 appears to be widely distributed, found in both North and South Islands (Figure 5). Additionally, three samples from Whangateau (WH2 – *B. aff. anceps*), Dunedin (S4 – *B. jacksonianum*), and Invercargill (F2 – *B. aff. anceps*) were identified as distinct species based on their evolutionary distance (Figure 5), consistent with the phylogeny and species delineation analyses (Figure 2–4).

Discussion

Botrylloides colonies were collected from seven different regions of the North and South Island of Aotearoa New Zealand, and phylogenetic analysis was based on mitochondrial and nuclear markers. Because of the absence of spoked-wheel morphology and based on comparative molecular results, most of the samples collected from Aotearoa New Zealand were identified as *B. diegensis* (Viard et al. 2019). For this reason, previously reported studies from our lab that denoted our model organism as *Botrylloides leachii* is *Botrylloides diegensis* (Zondag et al. 2016; Blanchoud et al. 2017; Blanchoud et al. 2018; Blanchoud et al. 2018; Zondag et al. 2019). This kind of revision in the annotation of species taxonomy is not uncommon among tunicates. (Monniot and Dettai 2015; Gissi et al. 2017; Ishak et al. 2018). An example of this is the incorrect assignment of *C. robusta* as *C. intestinalis* (Gissi et al. 2017). The first published genome (Dehal et al. 2002) was, in fact, that of *C. robusta* and not *C. intestinalis*. In addition, these errors are not new (Vilgalys 2003) but still common and with important implications nowadays with metabarcoding development (Keck et al. 2022).

Two other *Botrylloides* species collected are the first molecular records from Aotearoa New Zealand: *Botrylloides jacksonianum* and *Botrylloides affinis anceps*. The Whangateau species was assigned as *B. aff. anceps* because of its high molecular affinity to the record from Australia (MT873573) (Salonna et al. 2021). Although we were unable to obtain a detailed anatomical comparison for both records, our results are consistent, particularly with respect to *B. anceps*, which has previously been stated to be indigenous to the Indo-Pacific region (Brunetti 2009). ASAP analyses resulted in three separate OTUs for *the B. anceps* samples (Figure 4). Because of the high molecular affinity of these samples, we hypothesised that *B. anceps* might be a species complex. Similarly, *B. schlosseri*, a sister genus, has recently been reported to be a species complex containing divergent clades with high evolutionary distances (Brunetti et al. 2017; Nydam et al. 2017; Brunetti et al. 2020; Ben-Hamo and Rinkevich 2021; Reem et al. 2021). A larger number of *B. anceps* samples would be needed to resolve this hypothesis.

In addition to a recent study (Salonna et al. 2021), little is known about *B. jacksonianum.* Salonna et al. (2021) concluded that *B. jacksonianum* (Herdman 1886), which was synonymised with *B. leachii* by Kott (Kott 1985, 2005), is a distinct species. Our network construction, phylogenetic tree, and ABGD analyses also support the conclusion of Salonna et al. (2021)'s paper that it is a separate species. We found that the zooid sizes of *B. diegensis* and *B. aff. anceps* were similar, whereas *B. jacksonianum* was larger. As a general observation, we commonly found *B. diegensis* in summer. However, *B. jacksonianum* was observed more often during winter during our regular samplings in Dunedin.

As a single monophyletic cluster, seven *B. diegensis* haplotypes were observed from the Aotearoa New Zealand coast based on COI sequences. Although a small number of specimens were barcoded, a relatively higher number of haplotypic variations were recorded.

Globally sampled *B. diegensis* colonies revealed eight haplotypes (Viard et al. 2019) which might be due to the low sampling number from each area. The number of successfully sequenced barcodes is likely due to multiple factors such as DNA quality, primer efficiency, contamination, and sampling site. The reason for this is that the colonies being sampled contain DNA contaminants from other organisms that they consume, such as algae, bacteria, and the surfaces of mollusc shells and other tunicates to which they were attached (Blanchoud et al. 2018; Dias et al. 2021; Paz and Rinkevich 2021; Temiz et al. 2023). Therefore, instead of a multi-barcode approach, multiple markers were needed for barcoding the colonies of this study. Identification of *B. diegensis* was well-resolved with all the markers of the study while the highest resolution was with COI. This was because of the higher abundance of COI reference sequences in the database. However, nuclear markers showed weaker resolution due to the low number of database reference sequences for nuclear markers particularly for *B. anceps* and *B. jacksonianum*. It is known that COI is efficient for the identification of ascidians which is consistent with our findings. (Viard et al. 2019; Nydam et al. 2021; Salonna et al. 2021; Karahan et al. 2023; Temiz et al. 2023).

In conclusion, we identified our samples using the COI, H3,18S, and 28S markers. As one of the first barcoding studies of *Botrylloides* ascidians in Aotearoa New Zealand, this study provides valuable insights into the diversity of *Botrylloides*. We found three species of *Botrylloides*, *Botrylloides diegensis, Botrylloides jacksonianum,* and *Botrylloides affinis anceps,* in the marinas of Aotearoa New Zealand. In our previous publications, we checked our COI barcodes, which matched the time with the *B. leachii* sequences in the GenBank database and the morphological information in the associated publications. However, since then, these sequences have been incorrectly annotated and belong to *B. diegensis*. Therefore, this study confirms that the species in our previous publications was not *B. leachii* but *B. diegensis*.

Acknowledgements

We would like to thank Richard Taylor from the University of Auckland, who kindly provided the samples from Whangateau. The authors also thank Dr Michael Meier for assistance during sampling. Finally, we thank Stephanie Workman for her comments on the earlier drafts. This study was supported by a Royal Society of New Zealand Marsden Fund Grant (UOO1713). B Temiz was supported by an Anatomy Department PhD scholarship from the University of Otago.

Data availability statement

Reference sequences were uploaded to the GenBank nucleotide database. The accession numbers are (MZ412536, MZ506611, MZ533117, MZ533119, MZ506871, MZ506872, MZ412535, MZ506612, MZ533118, MZ533120) (Table S2). The phylogenies were uploaded to the Open Tree of Life database (https://tree.opentreeoflife.org/curator/study/view/ot_2094). The accession numbers of the COI sequences and outgroups can be found in Supplementary Information, Table S2 (current study sequences), and Table S3 (database sequences). Data generated and/or analysed during the current study are available from the corresponding author upon request.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by Royal Society of New Zealand Marsden Fund [grant no UOO1713].

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