

## Molecular systematics of the siphonous green algal genus *Codium* in eastern Victoria, Australia

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**Abstract** – The widely distributed genus *Codium* is a major component of the marine macroalgal flora and has its largest species diversity in warm-temperate regions. Along the South coast of Australia, *Codium* is a diverse group, but the species diversity in the easternmost region of Victoria is not well-documented. In this study, we characterized the species diversity of *Codium* from Croajingolong National Park, Gabo Island Lighthouse Reserve and Cape Howe Marine National Park. The specimens were collected from a wide range of microhabitats during an expedition of the Bush Blitz program, and *Codium* species were delimited through a combination of molecular analyses of the chloroplast markers *tufA* and *rbclL*, and morphological observations. Six species-level clusters were found, corresponding to the native *C. australicum*, *C. harveyi*, *C. lucasii*.1 and *C. fragile* subsp. *novae-zelandiae*; and the exotic *C. fragile* subsp. *fragile* and *C. tenue*. The specimens of *C. australicum* showed variability in the architecture of the thallus that was attributed to the different habitats where they were collected. The utricles of *C. harveyi* were unusual in having a thick apical cell wall that protruded inwards and had internal projections. The species found in eastern Victoria are typical of the Flindersian and Peronian biogeographic provinces, and the low diversity of *Codium* species in this region is comparable to that of New South Wales.

**Biogeography / *Codium australicum* / *Codium fragile* / *Codium harveyi* / *Codium lucasii*.1 / *Codium tenue* / morphology / southern Australia / taxonomy**

### INTRODUCTION

The southern coastline of Australia has a rich and diverse macroalgal flora with a high level of endemism, attributed to a combination of geological events, geographical isolation and climatic influence (Womersley, 1981; Phillips, 2001; Kraft, 2007). It comprises three biogeographical provinces (Peronian, Flindersian and Maugean), which were initially delimited based on the distribution of intertidal and subtidal organisms (Bennet & Pope, 1953; Womersley & Edmonds, 1958) and later validated by quantitative analysis of macroalgal distribution patterns (Waters *et al.*, 2010). The easternmost coast of Victoria includes several parks and reserves,

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the Croajingolong National Park, Cape Howe Marine National Park and Gabo Island Lighthouse Reserve (Fig. 1). The area is characterized by sandy coastal habitats with outcrops of granite and metamorphic rocks (Williams *et al.*, 2007), and from a biogeographic perspective they are situated in the overlap region of Peronian and Maugean provinces, where cold-temperate waters are influenced by the incursion of the warm East current and upwelling events (Womersley, 1981). For this reason, the south-east region of Australia is very important in terms of biodiversity.

The marine flora of this area has not been studied much, and except for the handful of records of marine macroalgae in the Australian Virtual Herbarium (AVH) and the common habitat-forming species recorded in the Parks Victoria Technical Series (Edmunds *et al.*, 2005; Williams *et al.*, 2007), very little is known about the algal species that are present. Given this gap of knowledge about the marine flora of this unique environment, an expedition was organized under the Bush Blitz program with the aim to document the biodiversity from Croajingolong National Park, Gabo Island Lighthouse Reserve and Cape Howe Marine National Park (Croajingolong National Park Victoria, 2016).

The genus *Codium* (Chlorophyta, Bryopsidales), is a frequent and abundant component of the macroalgal flora in coastal habitats and is widely distributed around the world. It is a common and diverse group in Australia, where 29 species were reported (Silva & Womersley, 1956; Womersley, 1956; Womersley, 1984; Millar & Kraft, 1994; Kraft, 2007; Silva & Chacana, 2012; Huisman, 2015). In the southern region a total of 22 species have been documented along the coasts of the Peronian, Flidersian and Maugean biogeographic provinces. Most of the *Codium* species are endemic to Australia and have wide distributions along the southern coasts, however, two introduced species – *C. fragile* subsp. *fragile* and *C. tenue* – have been reported recently (Huisman *et al.*, 2015; McDonald *et al.*, 2015; Verbruggen *et al.*, 2017), bringing the total number of *Codium* species recorded in Australia to 31.

*Codium* belongs to the Bryopsidales, an order of green algae characterized by siphonous cell architecture, meaning that their entire thallus consists of a single giant siphonous cell (Vroom & Smith, 2003). In *Codium*, the siphon branches form a more complex thallus structure composed of an inner medulla with thin siphons surrounded by a cortex of inflated utricles (Schmidt, 1923; Silva & Womersley, 1956). There are three major macro-morphological types in *Codium*: mat formers, spherical species and upright branched thalli (Schmidt, 1923; Verbruggen *et al.*, 2007). Species are classically distinguished by a combination of external features (habit, size and branching pattern of the thallus) and utricles characters (shape, size, location of plugs, thickness and ornamentations of the cell wall). Like in many other algal genera, DNA-based work has shown that morphological traits do not always correspond to species boundaries (Verbruggen *et al.*, 2007) and a combination of DNA and morphological data is often the most successful approach to species identification (e.g. Oliveira-Carvalho *et al.*, 2012; Verbruggen *et al.*, 2012; Huisman *et al.*, 2015; Verbruggen & Costa, 2015; Verbruggen *et al.*, 2017; Hoffman *et al.*, 2018).

The goal of this study is to characterize the species diversity of *Codium* collected in eastern Victoria during the Croajingolong Bush Blitz expedition, using a combination of DNA sequencing and morphological observations. We will also discuss the taxonomy and biogeography of the species.

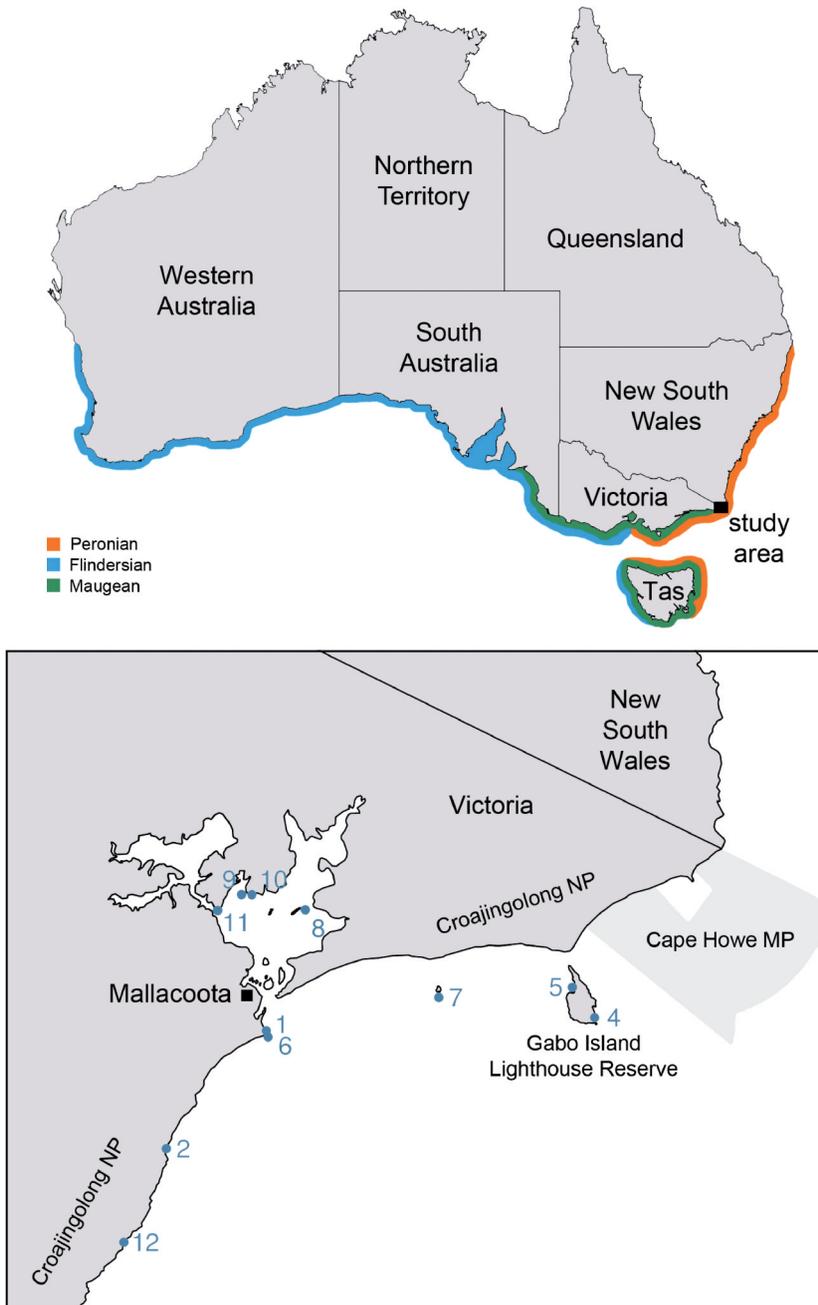


Fig. 1. Location of the study area in eastern Victoria and the collection sites (numbers 1 to 12) in Croajingolong National Park. Biogeographic provinces are indicated after Bennett & Pope (1953). 1 and 6= Mallacoota, Bastion Point; 2= Mallacoota, Pebbly Beach; 4= Gabo Island, East landing; 5= north of harbour; 7= Tullaberga Island; 8, 9, 10 and 11= Mallacoota Inlet; 12= East Gippsland, Shipwreck Creek Beach. Collection site 3 is not indicated in the figure because coordinates are unknown.

## MATERIALS AND METHODS

### Sampling

*Codium* specimens were collected in November 2016 during an expedition to eastern Victoria as part of the Bush Blitz program. The expedition covered parts of the coastlines of Croajingolong National Park, Gabo Island Lighthouse Reserve, and Cape Howe Marine National Park (Fig. 1). Algal collections were made by Kyatt Dixon, Pilar Diaz-Tapia, Guadalupe Bribiesca-Contreras and Heroen Verbruggen. All samples were collected by hand, either by walking the intertidal, snorkeling the shallow subtidal or SCUBA diving. Specimens of *Codium* were photographed in the field laboratory. A part of each sample was cleaned and dried in silica gel, another fragment was preserved in formaldehyde 4% in seawater and the remainder of each specimen was pressed in herbarium sheets and deposited in the herbarium of the Royal Botanic Gardens Victoria (MEL).

### Molecular data

Silica gel-dried samples were used for DNA extraction, amplification and sequencing of *rbcL* and *tufA* genes following previously published procedures (Verbruggen & Costa, 2015). A total of 23 sequences of *Codium* were obtained (8 *rbcL* and 15 *tufA*) and submitted to GenBank. Specimen numbers, collection sites, and GenBank accession numbers are given in Table 1.

For species delimitation and DNA-assisted identification, the *rbcL* and *tufA* sequences were aligned with published sequences of specimens from Australia and elsewhere (Huisman *et al.*, 2015; Verbruggen & Costa, 2015; Verbruggen *et al.*, 2017), yielding alignments of 173 *rbcL* sequences and 186 *tufA* sequences. The alignments were 758 and 934 nucleotides long for *rbcL* and *tufA*, respectively. Distance trees were inferred from each alignment using the UPGMA algorithm in MEGA 7.0 (1,000 bootstraps; p-distance; partial deletion; Kumar *et al.*, 2016), and species-level clusters were identified in the dendrograms as tight clusters separated by long branches. We chose to use the UPGMA method because it clearly shows identical sequences, allowing to detect species membership. To determine if the specimens of *Codium fragile* (see Results) belonged to the native or invasive clades, a UPGMA distance tree was generated using an existing alignment of *tufA* sequences of *C. fragile* (Verbruggen *et al.*, 2017) including our samples from eastern Victoria.

To obtain a species-level phylogeny of the genus, a concatenated dataset of *rbcL* and *tufA* was created, and a maximum likelihood (ML) phylogeny was inferred with the model GTR +  $\Gamma$  + I, partitioned by gene and codon position, in RAXML v.8.2.9 (1+2 positions separated from positions 3; 1,000 standard bootstraps) (Stamatakis, 2015). The ML analysis is in line with the Bayesian Inference phylogenies of *Codium* previously published (see Verbruggen *et al.*, 2007; Verbruggen & Costa, 2015). As argued in Verbruggen *et al.* (2007), we carried out the analysis without outgroups and manually rooted the resulting phylogenetic tree in the position where the molecular clock rooting method suggested it to be (see Verbruggen *et al.* (2007) for details).

Table 1. Summary of the specimens studied with collection sites, marker sequenced and species identification based on the combination of molecular and morphological data.

Sample number	Collection site	Latitude, longitude	Marker	Genbank accession	Species
HV06007	Mallacoota, Bastion Point	-37.571829; 149.765225	<i>tufA</i>	MH427846	<i>Codium australicum</i>
HV06076	Mallacoota, Bastion Point	-37.571829; 149.765225	<i>tufA</i>	MH427849	<i>Codium fragile</i> subsp. <i>novae-zelandiae</i>
HV06087	Mallacoota, Pebbly Beach	-37.615028; 149.7188	<i>tufA</i>	MH427850	<i>Codium fragile</i> subsp. <i>novae-zelandiae</i>
HV06091	Mallacoota, Pebbly Beach	-37.615028; 149.7188	<i>tufA</i>	MH427851	<i>Codium fragile</i> subsp. <i>fragile</i>
HV06098	Mallacoota, Pebbly Beach	-37.615028; 149.7188	<i>tufA</i>	MH427860	<i>Codium lucasii</i> 1
HV06161	Mallacoota, Pebbly Beach	-37.615028; 149.7188	<i>tufA</i>	MH427852	<i>Codium fragile</i> subsp. <i>novae-zelandiae</i>
HV06163	Gabo Island, West	unknown	<i>rbcl</i>	MH427838	<i>Codium australicum</i>
HV06172	Gabo Island, East landing	-37.567065; 149.917524	<i>rbcl</i>	MH427841	<i>Codium harveyi</i>
HV06199	Gabo Island, East landing	-37.567065; 149.917524	<i>tufA</i>	MH427847	<i>Codium australicum</i>
HV06216	Gabo Island, East landing	-37.567065; 149.917524	<i>tufA</i>	MH427848	<i>Codium australicum</i>
HV06221	Gabo Island, East landing	-37.567065; 149.917524	<i>rbcl</i>	MH427839	<i>Codium australicum</i>
HV06229	Gabo Island, East landing	-37.567065; 149.917524	<i>rbcl</i>	MH427842	<i>Codium harveyi</i>
HV06248	Gabo Island, East landing	-37.567065; 149.917524	<i>rbcl</i>	MH427840	<i>Codium australicum</i>
HV06251	Gabo Island, East landing	-37.567065; 149.917524	<i>rbcl</i>	MH427843	<i>Codium harveyi</i>
HV06264	Gabo Island, East landing	-37.567065; 149.917524	<i>rbcl</i>	MH427844	<i>Codium harveyi</i>
HV06309	Tullaberga Island	-37.559667; 149.845241	<i>tufA</i>	MH427853	<i>Codium fragile</i> subsp. <i>novae-zelandiae</i>
HV06404	Mallacoota, Bastion Point	-37.573466; 149.765867	<i>tufA</i>	MH427854	<i>Codium fragile</i> subsp. <i>novae-zelandiae</i>
HV06405	Mallacoota, Bastion Point	-37.573466; 149.765867	<i>tufA</i>	MH427855	<i>Codium fragile</i> subsp. <i>novae-zelandiae</i>
HV06430	Mallacoota, Inlet	-37.527591; 149.783306	<i>tufA</i>	MH427856	<i>Codium fragile</i> subsp. <i>fragile</i>
HV06437	Mallacoota, Inlet	-37.527845; 149.742689	<i>rbcl</i>	MH427845	<i>Codium tenue</i>
HV06438	Mallacoota, Inlet	-37.527591; 149.783306	<i>tufA</i>	MH427857	<i>Codium fragile</i> subsp. <i>fragile</i>
HV06464	East Gippsland, Shipwreck Creek Beach	-37.649273; 149.699128	<i>tufA</i>	MH427858	<i>Codium fragile</i> subsp. <i>novae-zelandiae</i>
HV06470	East Gippsland, Shipwreck Creek Beach	-37.649273; 149.699128	<i>tufA</i>	MH427859	<i>Codium fragile</i> subsp. <i>novae-zelandiae</i>

### Morphological observations

The pressed specimens and formalin-preserved samples were examined to get morphological identifications for the species-level clusters observed in the distance trees. Microscopic observations were made with a Leica MZ8 stereo microscope and a Leica DM750 compound microscope equipped with a Canon EOS 600D digital camera. Morphology-based identification was done with the literature available for Australian marine benthic flora (Silva & Womersley, 1956; Womersley, 1984; Kraft, 2007).

### RESULTS

The combination of morphological observations and DNA analyses of *rbcL* and *tufA* data showed that six species-level clusters were present in the study area (supplementary figures 1 and 2, see doi/10.7872/crya/v39.iss4.2018.Suppl.Mat.).

Specimen HV06437 clustered with samples of *Codium tenue* from South Africa and Western Australia based on *rbcL* data. The morphological observations of our sample confirmed its identity as *C. tenue*. The upright thallus was 15 cm long, light olive green and was covered with abundant long hairs. The branching pattern was dichotomous; however, the specimen was scarcely branched. The thallus had several short and thin proliferations irregularly distributed on the surface (Fig. 2).



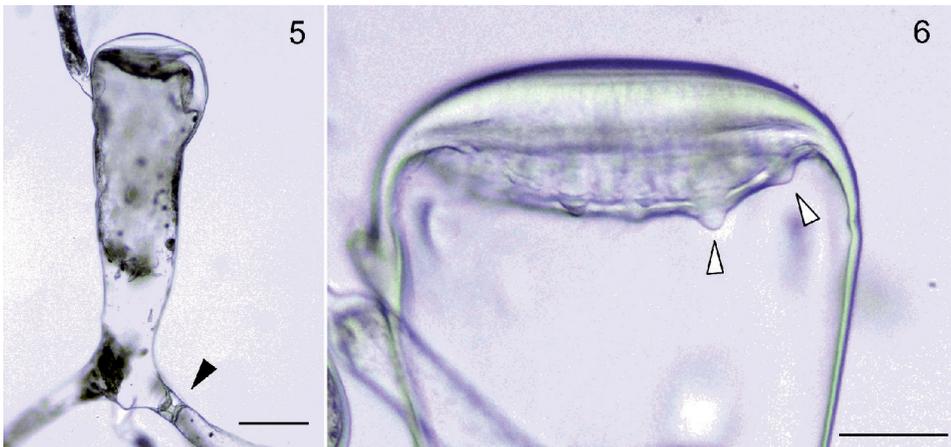
Figs 2-4. Aspect of the thallus of (2) *Codium tenue*, (3) *C. lucasii*.1, and (4) *C. harveyi*. Scale bar= 2 cm.

The axes ended in rounded apices, some being slightly attenuated. The shape of utricles was cylindrical, slightly clavate, capitate or wedged, and the apical cell wall was thin ( $< 5\mu\text{m}$ ). The plug was located close to the base of the utricles, and hairs (or scars) were abundant, up to 6 per utricle.

Specimen HV06098 (*tufA*) formed a cluster with a group of samples from Western Australia, Tasmania and Victoria that were morphologically identified as *Codium lucasii*. It is known that cryptic species exist within *C. lucasii*, and the cluster we found was clearly separated from the species-level entities *C. lucasii*.2 and *C. lucasii*.3 from Australia, as well as from the South African species *C. lucasii*.cap.1, *C. lucasii*.cap.2. and *C. lucasii*.cap.3. in the distance tree. This specimen will be referred as *C. lucasii*.1. The thallus of this specimen was prostrate and subglobose with lobes (Fig. 3). Hairs were not visible in the sample, but a few scars were present. The utricles were cylindrical with rounded or truncated apices. Plugs were connecting the base of adjacent utricles. The apical cell wall of the utricles was thin ( $10\text{--}15\mu\text{m}$ ) and had inner ornamentalions.

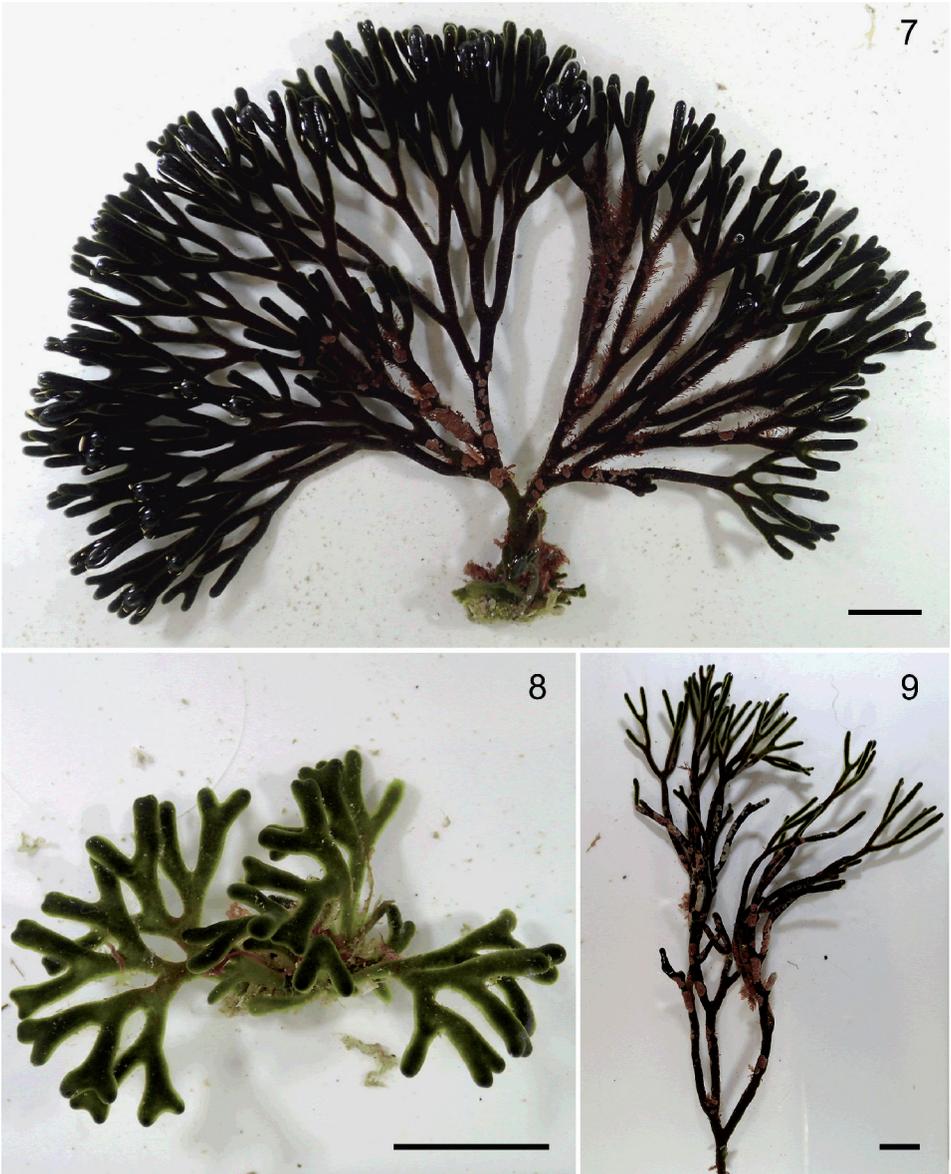
Specimens HV06172, HV06264, HV06229 and HV06251 (*rbcL*) formed a cluster with other specimens from southern Australia and were identified morphologically as *Codium harveyi*. This species formed a cluster with *C. muelleri* and an unidentified entity «*C. no.ID.46*» from Western Australia. Thalli of *C. harveyi* were erect and varied between 4 to 10 cm long (Fig. 4). The branching was dichotomous up to 7 times, and some axes were pseudomonopodial. The axes were terete except at the dichotomies where they were flattened and broad, and they become tapered at the apex. The distance between the dichotomies was between 0.4 and 2.4 cm. The utricles were cylindrical, clavate or squat,  $300\text{--}700\mu\text{m}$  long and  $100\text{--}410\mu\text{m}$  in diameter, usually with flattened apices. The plugs were located in proximity to the base of the utricle. The cell wall in the apex of utricles was moderately to highly thickened ( $60\text{ to }100\mu\text{m}$ ) and protruded inwards with short projections (Figs 5-6). Hairs were abundant, forming a bright halo on the thallus, except at the tips.

Six specimens from the study area (*rbcL*: HV06163, HV06221, HV06248; *tufA*: HV06216, HV06007, HV06199) clustered with several other samples from



Figs 5-6. Utricles of *Codium harveyi*, (5) cylindrical utricle with inwardly protruding cell wall, (6) detail of the apical cell wall with internal projections (white arrowheads). Black arrowhead indicates the position of plug. Scale bar =  $50\mu\text{m}$ .

elsewhere in southern Australia. Based on their morphology, these specimens could be assigned to *Codium australicum*. Thalli were erect, dichotomous, and the branches were terete throughout with rounded apices (Figs 7-9). The specimens showed variability in size, degree of branching, distance among dichotomies and width of axes. Some specimens were small and compact, with thin axes and short dichotomies,



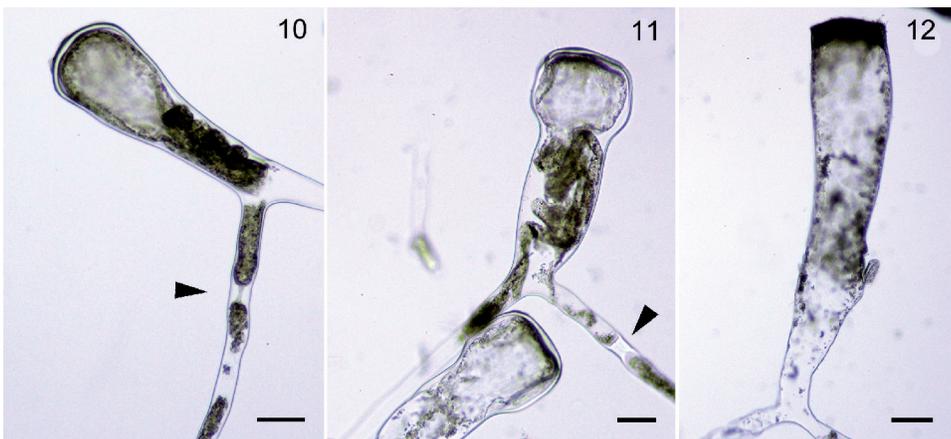
Figs 7-9. Aspect of the thallus of *Codium australicum* (7) and (8) specimens HV06216 and HV06248 collected in Gabo Island, East landing, (9) specimen HV06007 collected in Mallacoota, Bastion Point. Scale bar= 2 cm.

while others were large and profusely branched having long distances between dichotomies (Table 2). The utricles were cylindrical either with rounded or truncated apices, slightly clavate, or slightly constricted at the apex resulting in a capitate shape (Figs 10-12). The utricles were 220 to 750  $\mu\text{m}$  long and 70 to 240  $\mu\text{m}$  in diameter and plugs were located far from the base of the utricle. The apical cell wall of the utricles was thin, up to 15  $\mu\text{m}$ , and sometimes lamellate. The occurrence of hairs was variable among specimens, it was abundant in some specimens forming a bright halo throughout the thallus except at the tips and were absent in other specimens.

Eleven samples clustered with *Codium fragile* from Australia and other regions of the world in the distance tree based on *tufA* (supplementary figure 2, see doi/10.7872/crya/v39.iss4.2018.Suppl.Mat.). Among these samples, there was a separation into two groups (Figs 13-14 and Fig. 15): samples HV06438, HV06091 and HV06430 clustered with *C. fragile* subsp. *fragile* (the introduced subspecies),

Table 2. Morphological variability of the specimens of *C. australicum* collected in eastern Victoria.

	Specimen number					
	<i>HV06248</i>	<i>HV06221</i>	<i>HV06216</i>	<i>HV06163</i>	<i>HV06199</i>	<i>HV06007</i>
<b>Length (cm)</b>	3	2	14	11	11	28
<b>Width of axes (mm)</b>	1-2	0.9-2	2-3	2-3	2-3	2-6
<b>Distance between dichotomies (cm)</b>	0.4-0.97	0.3-0.5	0.7-3.6	0.7-2.3	0.6-2.6	1.4-7.1
<b>Branching degree</b>	Up to 4 times	Up to 3 times	Up to 10 times	Up to 9 times	Up to 7 times	Up to 7 times
<b>Hairs</b>	Present	Present	Not observed	Not observed	Not observed	Not observed
<b>Habitat</b>	subtidal gully, strong surge	subtidal gully, strong surge	subtidal rocky reef	subtidal	subtidal rocky reef	rocky reef, shallow subtidal



Figs 10-12. Utricles of *Codium australicum* (10) slightly clavate, (11) capitate, (12) cylindrical with truncated apex. Black arrowheads indicate the position of plug. Scale bar= 50  $\mu\text{m}$ .



Figs 13-14. Aspect of the thallus of the two subspecies of *Codium fragile*. (13) *C. fragile* subsp. *fragile* (14) *C. fragile* subsp. *novae-zelandiae*. Scale bar= 2 cm.

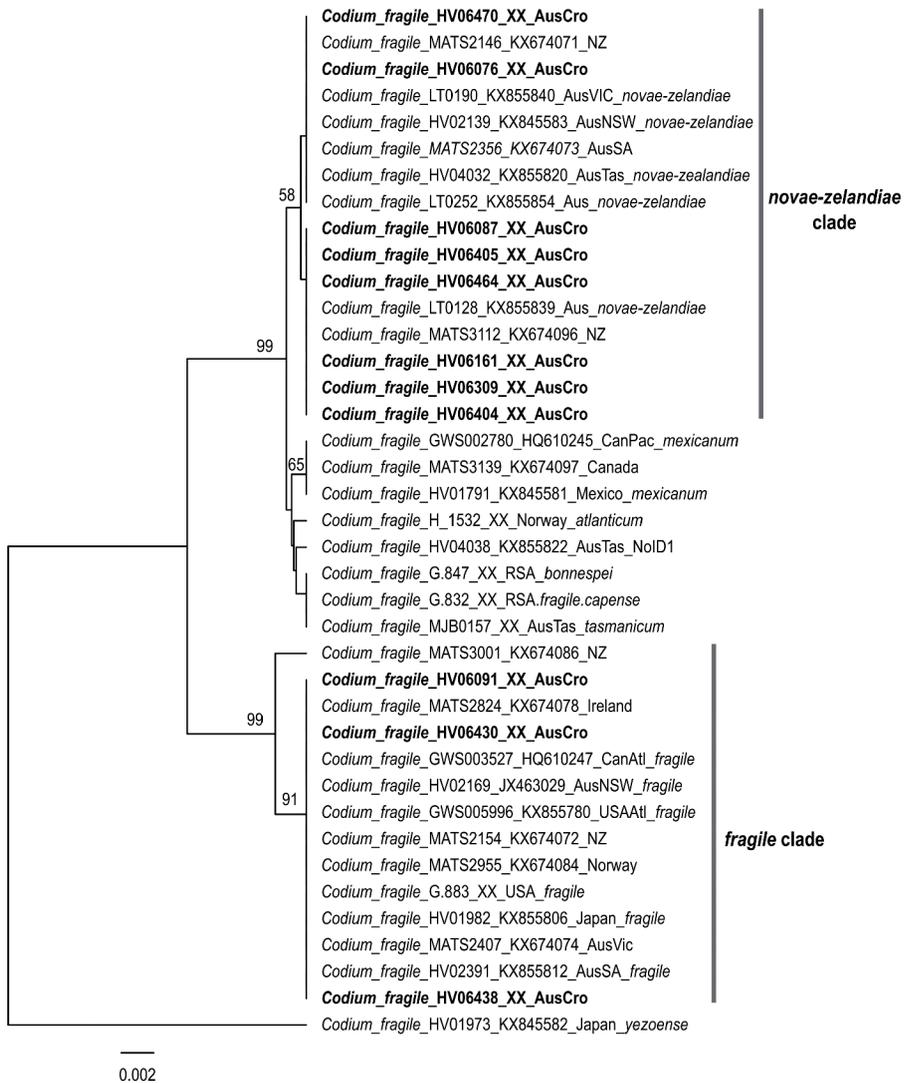


Fig. 15. UPGMA tree of *Codium fragile* based on *tufA* sequences. Specimens found in this study are in bold. Values at nodes represent bootstrap support, only shown if > 50.

whereas samples HV06470, HV06087, HV06405, HV06464, HV06309, HV06076, HV06161 and HV06404 clustered with the native clade within *C. fragile* (see Verbruggen *et al.* (2017) for more detail about *tufA* clades observed in *C. fragile*). Microscopic observations on the utricles confirmed that these two lineages were present, with longer, slender utricles in members of the native clade and more thick-set shorter utricles in the introduced lineage (cf. Verbruggen *et al.*, 2017).

The phylogeny inferred from the ML tree showed that the species of *Codium* belonged to two main clades with 100% bootstrap support (Fig. 16). Clade I consisted mostly of mat-forming species, while Clade II consisted of some mat-forming species along with all the species with spherical, erect or erect-sprawling thalli. Except for the mat-forming species *C. lucasii*.1, all the species of *Codium* found in this study belong to Clade II and were representatives of the erect architectural form (*C. australicum*, *C. harveyi*, *C. fragile* subsp. *fragile*, *C. fragile* subsp. *novae-zelandiae*, *C. tenue*). No representatives with spherical thalli were found. As shown in the phylogenetic tree, *C. harveyi* was closely related to *C. muelleri* and an unidentified entity named as “*C. no.ID.46*” collected in South Australia.

## DISCUSSION

### Diversity and biogeography of *Codium* in eastern Victoria

The specimens collected in eastern Victoria corresponded to five species of *Codium*, including two subspecies of *C. fragile*. Of the 23 specimens collected, most of them were representatives of the native species *C. australicum*, *C. harveyi*, *C. lucasii*.1 and *C. fragile* subsp. *novae-zelandiae*. Although these species are present all along the coast of southern Australia, they are more common in the south-eastern coasts, (between the east of South Australia and the north of New South Wales) than in the rest of southern Australia (Silva & Womersley, 1956; Womersley, 1984; Millar & Kraft, 1994; Huisman, 2000; Kraft, 2007; Huisman, 2015). *C. galeatum* is the only species of *Codium* reported for this area, at Cape Howe Marine National Park (Williams *et al.*, 2007). Although this species is widespread in southern Australia (Womersley, 1984; Millar & Kraft, 1994), it was not collected from the sites visited in eastern Victoria.

The diversity of *Codium* found in this study was low, especially considering that the southern coasts of Australia account for the highest richness of this genus, and that the area surveyed in this study covered diverse marine habitats. The low diversity is in line with that of the coasts of New South Wales, located within the Peronian biogeographic province (Millar & Kraft, 1994). This low richness could result from a combination of environmental conditions that prevent other species of *Codium* from reaching the easternmost part of Victoria. Among them are for example, the warmer waters from the Eastern Australian current and the absence of adequate substrate along the sandy beaches that extend from Wilsons Promontory to the eastern limit of Victoria. These environmental factors have been suggested as barriers for several southern species of macroalgae (Womersley, 1981). In terms of species composition, the species of *Codium* found in eastern Victoria are shared by both Flindersian and Peronian provinces (except for *C. tenue*).

### Taxonomic considerations

The morphology of the specimens observed in this study matched previous descriptions, except for minor variations in the shape and size of the utricles. This variability has been documented in *Codium* and can be attributed to natural variation



Fig. 16. Maximum likelihood phylogeny of *Codium* based on concatenated *rbcL* and *tufA* sequences. Species found in this study are denoted in bold. Values at the nodes represent bootstrap support, only shown if > 50.

in the population as well as the stage of development of the utricles and to their location in the thallus (Silva 1955; McDonald, *et al.*, 2015). In *C. australicum* and *C. harveyi*, we made a few observations that are relevant for taxonomic purposes and that, to our knowledge, have not been described in the literature.

The thallus architecture of *Codium australicum* was highly variable among specimens, differing in thallus size, width of the axes, degree of branching, and distance among dichotomies. Some specimens were small, with short dichotomies and thin axes, scarce branching and abundant hairs, while others were large with long dichotomies and profusely branched. Morphological plasticity of the thallus is common among macroalgae, often as a response to different microhabitat conditions. The smallest specimens of *C. australicum* found in this study were from a subtidal surge gully on Gabo Island (East landing), an habitat with strong surge selecting for compact thallus forms. These samples formed a mat over the substrate (much like mat-forming *Codium* species) from which small (<5 cm) dichotomously branched uprights grew. The larger and more profusely branched specimens were collected in areas with less influence of water currents.

The utricles of the specimens of *Codium harveyi* presented a thick apical cell wall, which protruded inwards and had small projections on the internal surface (Figs 5-6). To our knowledge, this prominent feature has not been reported in the literature of *C. harveyi*, and is relevant for identification, given that the closely related *C. muelleri* also exhibits a thickened cell wall, called *umbo*, which is used as a diagnostic character of the species (Womersley, 1984). The two structures, however, can be easily distinguished, as the *umbo* of *C. muelleri* is a narrow and pronounced projection originating from the center of the utricle apex, whereas the inward protrusion of the cell wall in *C. harveyi* utricles is broad and dome-shaped. Despite the overall morphological similarity of these two species, they can also be distinguished by the location of the plugs, in proximity to the base of the utricle in *C. harveyi*, and conspicuously more distant in *C. muelleri*.

The recently described species *Codium apiculatum* from southwestern Australia (Silva & Chacana, 2012) is morphologically similar to *C. harveyi*, but the molecular phylogenetic relationship between these two species is still unknown. Their morphological similarities include the shape of the utricles, the location of the plugs and the narrow terminal axes. *C. harveyi* can be distinguished from *C. apiculatum* by the wider terminal axes and the presence of a thick apical cell wall in the utricles, not lamellate as in *C. apiculatum*.

### **Presence of *Codium tenue* in South-east Australia**

*Codium tenue* was described by Silva (1959) from specimens collected in South Africa. Recently, *C. tenue* was found in Walpole and Nornalup Inlets Marine Park, in South-Western Australia (Huisman *et al.*, 2015). Our results clearly show that *C. tenue* is also present in an inlet in eastern Victoria, representing the second record of this species in Australia.

The overall morphology of the specimen matched the original description by Silva (1959), as well as the description of the specimens from Western Australia by Huisman *et al.* (2015), although some macroscopic characteristics such as the branching pattern and width of axes were unclear in our specimen, possible due to having only an old thallus with small branches of new growth. The utricles, however, had the typical morphology known of *Codium tenue* (Silva, 1959), and the molecular data leave no doubt that the entity found in Mallacoota Inlet was *C. tenue*.

Our sample of *Codium tenue* was collected from a shallow subtidal rock outcrop in estuarine conditions within Mallacoota Inlet (Barton *et al.*, 2008), where the seawater mixes with freshwater from the Wallagaraugh and Genoa Rivers (Reinson, 1977). Similarly, the first collection of this species in Western Australia was also in a shallow subtidal estuary (Huisman *et al.*, 2015). This is not unusual, given that *C. tenue* is known to live in estuaries and lagoons in its native range in South Africa, typically on silt substrates (Silva, 1959).

### Native and introduced subspecies of *Codium fragile*

The presence of *Codium fragile* in Australian coasts was reported several times under the synonyms of *Acanthocodium fragile*, *C. tomentosum*, *C. tomentosum* var. *australicum* and *C. mucronatum*, but it was only after recent molecular studies that the occurrence of two subspecies, the native *C. fragile* subsp. *novae-zelandiae* and the invasive *C. fragile* subsp. *fragile* was confirmed (McDonald *et al.*, 2015, Verbruggen *et al.*, 2017). Our results evidenced that the native and invasive subspecies of *C. fragile* are present in eastern Victoria. *C. fragile* subsp. *novae-zelandiae* was found in Mallacoota (Bastion Point), Tullaberga Island and Shipwreck Creek Beach, whereas the invasive subspecies was found in Mallacoota (Inlet and Pebbly Beach).

*Codium fragile* subsp. *fragile* is native from Japan and Korea and has spread in several coasts around the world (Provan *et al.*, 2005). In Australia, it was first found in Corner Inlet (Victoria) and it is currently known from New South Wales, Victoria, South Australia, Tasmania and Western Australia (Verbruggen *et al.*, 2017). In Victoria, the distribution records of this species include Corner Inlet, Phillip Bay and Western Port. Our results indicate that *C. fragile* subsp. *fragile* has also spread to the marine conservation areas in eastern Victoria. While the distribution records of this species in Victoria include Corner Inlet, Phillip Bay and Western Port, our results indicate that this species has spread eastwards from the original region of detection, reaching conservation areas located in eastern Victoria.

Although the causes of introduction of *Codium fragile* subsp. *fragile* into this region are unknown, we presume that Mallacoota inlet could have been the introduction point. The inlet is a popular place for aquatic recreational activities and has a history of aquaculture (oysters and mussels; pers. comm. Parks Victoria). Our findings suggest that the distribution of the introduced subspecies of *Codium fragile* is extensive along the coast of Victoria, including remote places like the one studied here. This highlights the success of spread of this macroalgae and denotes its potential of producing secondary introductions within Australia and elsewhere.

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## REFERENCES

- BARTON J., POPE A., QUINN G. & SHERWOOD J., 2008 — Identifying threats to the ecological condition of Victorian estuaries. Melbourne, Victoria, Department of Sustainability and the Environment Technical Report, 54 p.
- BENNETT I. & POPE E.C., 1953 — Intertidal zonation of the exposed rocky shores of Victoria, together with a rearrangement of the biogeographical provinces of temperate Australian shores. *Australian journal of marine and freshwater research* 4: 105-59.
- CROAJINGOLONG NATIONAL PARK VICTORIA., 2016 — A Bush Blitz survey report, Commonwealth of Australia 2017.
- EDMUNDS M., HART S. & INGWERSEN C., 2005 — Victorian Subtidal Reef Monitoring Program: The Reef Biota at Marine Protected Areas in the Twofold Shelf Region. Parks Victoria Technical Series No. 23. Melbourne, Parks Victoria, 70 p.
- HOFFMAN R., WYNNE M.J., SCHILS T., LOPEZ-BAUTISTA J. & VERBRUGGEN H., 2018 — *Codium pulvinatum* (Bryopsidales, Chlorophyta), a new species from the Arabian Sea, recently introduced into the Mediterranean Sea. *Phycologia* 57(1): 79-89.
- HUISMAN J.M., 2000 — *Marine plants of Australia*. Nedlands, Western Australia, University of Western Australia Press, 300 p.
- HUISMAN J.M., 2015 — *Algae of Australia. Marine Benthic Algae of North-western Australia, 1. Green and Brown Algae*. Canberra, Australian Biological Resources Study, CSIRO Publishing, 320 p.
- HUISMAN J.M., DIXON R.R.M., HART F.N., VERBRUGGEN H. & ANDERSON R.J., 2015 — The South African estuarine specialist *Codium tenue* (Bryopsidales, Chlorophyta) discovered in a south-western Australian estuary. *Botanica marina* 58(6): 511-521.
- KRAFT G.T., 2007 — *Algae of Australia. Marine Benthic Algae of Lord Howe Island and the Southern Great Barrier Reef, 1. Green Algae*. Canberra, Australian Biological Resources Study, CSIRO Publishing, 347 p.
- KUMAR S., STECHER G. & TAMURA K., 2016 — MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular biology and evolution* 33(7): 1870-1874.
- MCDONALD J.I., HUISMAN J.M., HART F.N., DIXON R.R.M. & LEWIS J.A., 2015 — The first detection of the invasive macroalga *Codium fragile* subsp. *fragile* (Suringar) Hariot in Western Australia. *BioInvasions records* 4(2): 75-80.
- MILLAR A.J.K. & KRAFT G.T., 1994 — Catalogue of marine benthic green algae (Chlorophyta) of New South Wales, including Lord Howe Island, South-western Pacific. *Australian systematic botany* 7: 419-453.
- OLIVEIRA-CARVALHO M.F., OLIVEIRA M.C., BARRETO PEREIRA S.M., & VERBRUGGEN H., 2012 — Phylogenetic analysis of *Codium* species from Brazil, with the description of the new species *C. pernambucensis* (Bryopsidales, Chlorophyta). *European journal of phycology* 47(4): 355-365.
- PHILLIPS J.A., 2001 — Marine macroalgal biodiversity hotspots: why is there high species richness and endemism in southern Australian marine benthic flora? *Biodiversity and conservation* 10: 1555-1577.
- PROVAN J., MURPHY S. & MAGGS C.A., 2005 — Tracking the invasive history of the green alga *Codium fragile* ssp. *tomentosoides*. *Molecular ecology* 14: 189-194.
- REINSON G.E., 1977 — *Hydrology and sediments of a temperate estuary – Mallacoota Inlet, Victoria*. Canberra, Australian Government Publishing Service, 91 p.
- SCHMIDT O.C., 1923 — Beitrage zur Kenntnis der Gattung *Codium* Stackh. *Bibliotheca botanica* 91, 67 p.
- SILVA P.C., 1955 — The dichotomous species of *Codium* in Britain. *Journal of the marine biological association of the United Kingdom* 34: 565-577.
- SILVA P.C. & WOMERSLEY H.B.S., 1956 — The genus *Codium* (Chlorophyta) in southern Australia. *Australian journal of botany* 4(3): 261-289.
- SILVA P.C., 1959 — The genus *Codium* (Chlorophyta) in South Africa. *Journal of South African botany* 25: 103-165.
- SILVA P.C. & CHACANA M.E., 2012 — *Codium apiculatum* (Codiaceae, Chlorophyta), a new species from southern and southwestern Australia. *Cryptogamie, Algologie* 33(1): 35-40.
- STAMATAKIS A., 2006 — RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688-2690.

- VERBRUGGEN H., LELIAERT F., MAGGS C.A., SHIMADA S., SCHILS T., PROVAN J., BOOTH D., MURPHY S., DE CLERCK O., LITTLER D.S., LITTLER M.M. & COPPEJANS E., 2007 — Species boundaries and phylogenetic relationships within the green algal genus *Codium* (Bryopsidales) based on plastid DNA sequences. *Molecular phylogenetics and evolution* 44: 240-254.
- VERBRUGGEN H., PAULY K. & DE CLERCK O., 2012 — The new species *Codium recurvatum* from Tanzania. *European journal of phycology* 47(3), 216-222.
- VERBRUGGEN H. & COSTA J.F., 2015 — Molecular survey of *Codium* species diversity in southern Madagascar. *Cryptogamie, Algologie* 36: 171-187.
- VERBRUGGEN H., BROOKES M.J.L. & COSTA J.F., 2017 — DNA barcodes and morphometric data indicate that *Codium fragile* may consist of two species (Bryopsidales, Chlorophyta). *Phycologia* 56: 54-62.
- VROOM P.S. & SMITH C., 2003 — Life without cells. *Biologist* 50(5): 222-226.
- WATERS M.J., WERNBERG T., CONNELL S.D., THOMSEN M.S., ZUCCARELLO G.C., KRAFT G.T., SANDERSON J.C., WEST J.A. & GURGEL C.F.D., 2010 — Australia's marine biogeography revisited: Back to the future? *Austral ecology* 35: 988-992.
- WILLIAMS J., GILMOUR P. & EDMUNDS M., 2007 — *Victorian Subtidal Reef Monitoring Program: The Reef Biota within the Twofold Shelf Bioregion, (volume 2)*. Parks Victoria Technical Series No. 45. Melbourne, Parks Victoria, 62 p.
- WILLIAMS S.L. & SMITH J.E., 2007 — A global review of the distribution, taxonomy and impacts of introduced seaweeds. *The annual review of ecology, evolution and systematics* 38: 327-359.
- WOMERSLEY H.B.S. & EDMONDS S.J., 1958 — A general account of the intertidal ecology of south Australian coasts. *Australian journal of marine and freshwater research* 9: 217-60.
- WOMERSLEY H.B.S., 1956 — A critical survey of the marine algae of Southern Australia, I Chlorophyta. *Australian journal of marine and freshwater research* 7(3): 343-383.
- WOMERSLEY H.B.S., 1981 — Biogeography of Australasian marine macroalgae, in: Clayton M.N. and King R.J. (eds), *Marine Botany: an Australian Perspective*, Longman Chesire, Melbourne. Pp 292-307.
- WOMERSLEY H.B.S., 1984 — *The marine benthic flora of Southern Australia, Part I*. South Australia, Government Printer, 329 p.