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Cryptic diversity in coastal Australasia: a morphological and mitonuclear genetic analysis of habitat-forming sibling species

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Cryptic diversity represents a major challenge to the accurate assessment of biodiversity, but the combined use of genetic and morphological analyses has proven to be a powerful approach to detect it. This is especially important for groups for which genetic information is not yet available. Here, we studied the highly conspicuous habitat-forming *Pyura stolonifera* species complex (Tunicata), which, as has recently been revealed, shows surprising levels of cryptic diversity, but whose systematics and biogeographical patterns in Australasia nonetheless remain poorly understood. We first present detailed taxonomic information of all the species associated with the *P. stolonifera* species complex. We then proceed to describe the results of an exhaustive survey that included south-east Australia, Tasmania, and New Zealand. Subsequently, we present morphological and mitonuclear genetic analysis of two unresolved lineages that comprise the species *Pyura praeputialis* and a species that is formally described here (***Pyura doppelgangera* sp. nov.**). Although the ranges of these two species overlap on mainland Australia, we found no sites at which both species live in sympatry, and there was no morphological or genetic evidence of hybridization. Taken together, the present study illustrates the usefulness of a combined morphogenetic approach in unravelling overlooked marine diversity in a relatively well-studied region.

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INTRODUCTION

Cryptic species are two or more discrete species that, despite being divergent and often reproductively isolated (Gómez *et al*., 2007), are difficult or impossible to distinguish morphologically and have therefore been classified as a single species (Beheregaray & Caccone, 2007; Pfenninger & Schwenk, 2007). Many cryptic species are differentiated by nonvisual mating signals and/or are under selection that promotes morphological stasis (Bickford *et al*., 2007). In addition, phenotypic plasticity might obscure species-specific morphological characters that are used to delimit species (Mickevich & Johnson, 1976). All this has resulted in the perception that a large number of cryptic species has been historically overlooked (Dirzo & Raven, 2003; Appeltans *et al*., 2012), an issue that is particularly relevant for marine invertebrates (Knowlton, 1993; López-Legentil & Turon, 2005).

With the advent of PCR-based genetic methods two decades ago, the discovery of cryptic species has increased exponentially, suggesting that global

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biodiversity has been vastly underestimated. As discoveries of cryptic species based on genetic methods have often been subsequently confirmed with morphological and/or ecological data (Bickford *et al*., 2007), the combined use of genetic and morphological methods is often considered the most powerful approach for unravelling cryptic biodiversity. In an effort to standardize genetic species identifications and compare different taxa, it has been proposed that species be characterized using the same genetic marker (Blaxter, 2004). The mitochondrial DNA (mtDNA) cytochrome c oxidase subunit I (COI) is now routinely used as the universal barcoding marker for animals, and it has proven very useful in the detection of cryptic biodiversity (Hebert *et al*., 2004). However, there are numerous problems with mtDNA sequences that can result in mismatches with morphological data. These include the amplifications of pseudogenes (mitochondrial genes integrated into the nuclear genome, Bensasson *et al*., 2001), effects on mtDNA evolution by inherited microbial symbionts (Hurst & Jiggins, 2005), and bias in the rate of introgression across hybrid zones towards mtDNA (Chan & Levin, 2005) that can result in the complete replacement of the mitochondrial genome of one species with that of another (Irwin, Rubtsov & Panov, 2009). The generation of additional genetic data from one or more nuclear loci not only serves to confirm the results of the mtDNA data, but in addition, such biparentally inherited markers can provide information on reproductive isolation between cryptic species. Compliance with the classical definition of species through reproductive incompatibility (the biological species concept of Mayr, 1942) provides a particularly strong justification for treating genetically distinct evolutionary lineages as distinct species.

In the present study, we used a combined morphological and mitonuclear genetic approach to resolve cryptic speciation within the *Pyura stolonifera* species complex, which has recently revealed surprising levels of cryptic diversity (Rius & Teske, 2011; Teske *et al*., 2011), but whose systematics and biogeographical patterns in Australasia remain poorly understood. The species within this complex are highly conspicuous components of the rocky shore faunas of coastal regions across three different continents (see Rius & Teske, 2011), with the intertidal zone of temperate Australasia generally dominated by the large and highly conspicuous ascidian *Pyura praeputialis* (Heller, 1878). The existence of a small form of *P. praeputialis*, whose distribution is centred around Tasmania, and which has recently been reported along the north coast of New Zealand (Hayward & Morley, 2009; Page, Kelly & Herr, 2012), has long been known (e.g. Kott, 1985). However, both forms have traditionally been treated as a single species, mainly because of their morphological similarity. Consequently, prior to the finding that it represents a distinct evolutionary lineage (Teske *et al*., 2011), no taxonomic (Kott, 1985, 2006), biogeographical (Bennett & Pope, 1960) or genetic (Astorga, Guiñez & Castilla, 2009) study has proposed that it might represent a distinct species. Here, we provide a detailed report of the morphology of the two lineages that comprise *P. praeputialis* (i.e. small and large forms), and compare them with all other species within the *P. stolonifera* species complex. We also reconstruct phylogenetic relationships of the two *P. praeputialis* lineages and provide detailed information on their geographical ranges. Based on the analyses conducted in this study, we then proceed to formally describe the small form as *Pyura doppelgangera* sp. nov.

MATERIAL AND METHODS

Taxonomic revision

We examined the morphology of specimens that were preserved in 4% formaldehyde solution on collection. Taxonomic identification was carried out by removing the tunic, dissecting the body of the animal, and identifying all internal and external morphological characters. When necessary, specimens were stained with Nile blue sulphate. The specimens examined included members of all the species associated with the *P. stolonifera* species complex (Table 1), except *Pyura dalbyi* Rius & Teske, 2011, whose morphology was described in detail in Rius & Teske (2011). Representative specimens of all species have been deposited in the Iziko South African Museum, Cape Town.

In addition to general observations of the morphological characters through dissection, sections of the siphonal spines from specimens of all species within the species complex were fixed, critical-point dried, sputter-coated with gold, and observed with a Hitachi H2300 scanning electron microscope (SEM). We obtained SEM photographs of all species within the species complex, including *P. dalbyi*.

Phylogenetic analyses

Previous genetic evidence showed strong distinctness between the large and small morphs of *P. praeputialis* based on DNA sequence data from four loci (Teske *et al*., 2011), but sampling records only included a few sites from Victoria (where three evolutionary lineages within the species complex are present) and Tasmania. In addition, the genetic markers used did not amplify for all specimens. Because of these limitations, the existence of genetically intermediate forms or hybrid populations could not be ruled out.

We analysed genetic differentiation between the two lineages within *P. praeputialis* using DNA

Table 1. Species, sample sites, and geographical details where the specimens of *Pyura* spp. used for morphological examination were collected. The small morph of *Pyura praeputialis* is referred to as ***Pyura doppelgangera* sp. nov.** and the large morph as *Pyura praeputialis*

Species	Sampled sites	Site no.	Latitude (S), longitude (E)	Geographical location	No. individuals examined
Pyura stolonifera	Yzerfontein*	1	33°20′49″, 18°09′06″	Western Cape, South Africa	2
Pyura herdmani	Mossel Bay*	2	34°10′42″, 22°08′41″	Western Cape, South Africa	1
	Park Rynie*	3	30°19′07″, 30°44′32″	KwaZulu-Natal, South Africa	2
Pyura praeputialis	Balmoral Beach	27	33°49′37″, 151°15′22″	New South Wales, Australia	4
***Pyura doppelgangera* sp. nov.**	Henley Beach†	4a	34°55′11″, 138°29′31″	South Australia, Australia	9
	Semaphore Beach	4b	34°51′00″, 138°28′79″	South Australia, Australia	3
	Glenelg†	4d	34°58′50″, 138°30′35″	South Australia, Australia	2
	Port Welshpool†	24	38°41′10″, 146°27′51″	Victoria, Australia	1
	Trial Harbour†	12	41°55′52″, 145°10′18″	Tasmania, Australia	1
	Bridport†	15	41°00′17″, 147°23′38″	Tasmania, Australia	10
	Bicheno	17	41°52′12″, 148°18′12″	Tasmania, Australia	3
	Taroona Beach†	20	42°57′06″, 147°21′20″	Tasmania, Australia	15
	North Herekino – Waikiri Stream	32	35°16′19″, 173°08′21″	New Zealand	4
	North Herekino – Hunahuna Stream*	33	35°15′13″, 173°07′11″	New Zealand	4
	The Bluff*	34	34°41′06″, 172°53′23″	New Zealand	6
	North Twilight Beach*	35	34°29′22″, 172°40′56″	New Zealand	2
	Te Werahi Beach*	36	34°28′10″, 172°39′26″	New Zealand	2
	Parengarenga Harbour	37	34°31′08″, 172°59′26″	New Zealand	4
	Rangiputa	38	34°52′37″, 173°23′03″	New Zealand	6

*Specimens sequenced in Teske *et al.* (2011).
†Specimens sequenced in this study (see Table 2).

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Table 2. Locations of samples used for genetic analyses of *Pyura praeputialis* (A) and ***Pyura doppelgangera* sp. nov.** (B), and number of mitochondrial cytochrome c oxidase subunit I (COI) and nuclear adenine nucleotide transporter (ANT) intron sequences generated. Sequences generated specifically for this study are marked with asterisks

Region	Site name	Site no	Latitude (S), longitude (E)	Species	COI	ANT
Australia						
SA	Henley Beach	4a	34°55′11″, 138°29′31″	B	8	6
	Largs Bay	4c	34°47′48″, 138°29′16″	B	4	0
	Glenelg	4d	34°58′50″, 138°30′35″	B	4	8
	Brighton Beach	4e	35°01′03″, 138°30′46″	B	4	0
VIC	Marengo Bay	5	38°46′41″, 143°39′60″	A	3 + 8*	16*
	Portsea	6	38°19′07″, 144°42′44″	A	8 + 8*	14*
	Cowes	7	38°26′48″, 145°14′23″	A	8*	16*
	Kilcunda	8	38°33′23″, 145°28′50″	A	30	30
	Walkerville	9	38°51′49″, 146°00′08″	A	5	0
	Port Welshpool	10	38°41′10” 146°27′51”	B	5	20
	Port Albert	11	38°40′24″, 146°41′43″	B	5 + 8*	16*
	Cape Conran	22	37°48′52″, 148°43′36″	A	21	28
	Mallacoota	23	37°34′14″, 149°45′52″	A	18	0
TAS	Trial Harbour	12	41°55′52″, 145°10′18″	B	9*	6*
	Couta Rocks	13	41°10′29″, 144°40′53″	B	3*	4*
	Beauty Point	14	41°09′01″, 146°49′24″	B	8	22
	Bridport	15	41°00′17” 147°23′38”	B	7*	10*
	The Gardens	16	41°10′25″, 148°16′52″	B	7*	10*
	Coles Bay	18	42°07′21″, 148°16′57″	B	3*	6*
	Pirates Bay	19	43°01′53″, 147°56′42″	B	5*	6*
	Taroona Beach	20	42°57′06″, 147°21′20″	B	0	4
	Two Tree Point	21	43°20′02″, 147°19′32″	B	2	8
NSW	Eden	24	37°04′01″, 149°54′47″	A	16	0
	Ulladulla	25	35°21′35″, 150°29′11″	A	17	0
	Kiama	26	34°40′31″, 150°51′30″	A	14	0
	Black Head	28	32°04′15″, 152°32′55″	A	20	0
	Port Macquarie	29	31°25′47″, 152°55′24″	A	21	0
	Ballina	30	28°52′05″, 153°35′36″	A	19	0
	Fingal Head	31	28°11′56″, 153°34′16″	A	20	38
New Zealand	North Island		Various sites, see Teske *et al*., 2011	B	8*	84
Chile	Antofagasta	39	23°42′25″, 70°25′51″	A	20	52
			Total no. sequences		335	404

Acronyms: NSW, New South Wales; SA, South Australia (sites a, c, d and e represent different jetties in Adelaide); TAS, Tasmania; VIC, Victoria. Sites at which neither species was found (A–H in Fig. 4), indicating possible distribution gaps in South Australia and Victoria: A, Coffin Bay National Park (34°37′16″S, 135°28′09″E); B, Port Lincoln (34°43′09″S, 135°52′11″E); C, Whyalla (33°02′24″S, 137°35′34″E); D, Point Souttar (34°53′44″S, 137°16′58″E); E, Edithburgh (35°05′05″S, 137°44′56″E); F, Robe (37°09′28″S, 139°44′59″E); G, Portland (38°20′37″S,141°36′33″E); H, Lakes Entrance (37°52′51″S, 147°59′23″E).

sequence data from COI and nuclear adenine nucleotide transporter (ANT) intron sequences. Details of the sampling sites are listed in Table 2, including sites at which neither species was found despite intensive sampling. We strived to obtain both genetic and morphological data from as many specimens as possible (see details in Table 1).

As amplification of the mitochondrial COI gene was unreliable using the universal primers LCO1490 and HCO2198 designed by Folmer *et al*. (1994; see Teske *et al*., 2011), we designed internal primers to amplify this marker in the two lineages of *P. praeputialis*. Primers PyCOI-F (5′-GAA TTG TCT CAA GTA RGG CAG GT-3′) and PyCOI-R (5′-GAC CCY AGC TAA ATG CAA AG-3′) were run under the same PCR conditions described previously for the universal primers (Teske *et al*., 2011), except that the annealing temperature was increased to 55 °C and the $MgCl_2$ concentration reduced to 3 mM. Samples amplified successfully with these primers, and in specimens for

which sequences had previously been generated with the universal primers, the overlapping sequence sections were identical. Sequences of the nuclear ANT gene were generated using the primers StolidoANT-F (5′-CAG GGT ATC ATT GTR TAC MGA G-3′; Teske *et al*., 2011) and *ANTr1* (5′-CCA GAC TGC ATC ATC ATK CGR CGD C-3′; Jarman, Ward & Elliott, 2002). These were amplified and phased as described in Teske *et al*. (2011).

Phylogenetic trees were reconstructed to determine distinct evolutionary lineages. We reconstructed maximum likelihood trees in MEGA v.5 (Tamura *et al*., 2011) using sequences that were aligned with ClustalW (Thompson *et al*., 1994) and specified default settings. Each allele was represented once (47 COI alleles and 55 ANT alleles). The most suitable evolutionary model for each data set was determined based on the lowest Bayesian information criterion (BIC) scores using the model selection option in MEGA. For the COI sequences, the Kimura two-parameter model (Kimura, 1980) was chosen, and for the ANT sequences, the BIC score was lowest for the Tamura–Nei model (Tamura & Nei, 1993) and included a Γ distribution parameter (0.2326) with five rate categories. Support for nodes was determined by specifying 1000 bootstrap replications (Felsenstein, 1985). For each species and locus we measured allelic richness with the programme HP-RARE v.1.1 (Kalinowski, 2005), which uses rarefaction to correct for bias in sample size. The number of individuals from the species for which the lower number of sequences had been generated was used for rarefaction. Mean p-distances between species were calculated for each locus in MEGA v.5.

RESULTS

Taxonomic revision

We examined the morphology of specimens within the *P. stolonifera* species complex in detail (see Table 3 for all taxonomic details). They all shared certain characters such as the shape of the siphons with a cross-shaped opening, number of branchial folds (six) on both the left and right sides, and three different sizes of oral tentacles. In addition, the ramification complexity (third order branches) and the absence of atrial tentacles at the entrance of the exhalant siphon were consistent characters found in all members of the species complex. The main differences amongst species concern the disposition of the gut and gonads, the shape of the dorsal tubercle, and the morphology of the tunic and the siphonal spines (Fig. 1). Representative specimens were deposited in the Iziko South African Museum, accession numbers: 25990 (*P. praeputialis*, Balmoral Beach, Sydney, Australia), 26004 (*Pyura herdmani*, Mossel Bay, South Africa), 26005 (*P. herdmani*, Park Rynie, South Africa), 26006 (*P. praeputialis*, Henley Beach, Australia), and 25995 (*P. stolonifera*, Yzerfontein, South Africa) (see Table 1 for details).

Species description

Pyura doppelgangera sp. nov.

Material examined: We examined specimens from a variety of locations (Table 1) and a representative suite of these was deposited in the Iziko South African Museum (accession numbers: 25991 – Port Welshpool, 26008 holotype – Bridport, 26009 paratype – Bridport). Samples from the type locality (Bridport) were either used for morphological or genetic analyses (Tables 1, 2).

Description (holotype and paratype): The specimens (measured in contracted individuals) are 26 and 30 mm in maximum dorsal length, their height (measured from the base to the top of the siphonal area) 34 to 45 mm, and their width 18 to 20 mm. The specimens show a short peduncle that appears from the base. The tunic has sand embedded in it and has no pointed papillae (Fig. 2D). The tunic thickness is thin compared to other representatives of the *P. stolonifera* species complex. Siphonal spines are long and pointed and their bases are slightly expanded (Fig. 1A).

The body wall is orange, but dark purple around the siphons. The body size is 25 to 26 mm in length, 20 to 21 mm in height, and 12 to 15 mm in width. There are anterior longitudinal muscular bands across the body, with circular muscular bands around the siphons. There are 33 to 34 ramified oral tentacles of different sizes (alternating between large and small).

The branchial formula is: right side of the specimen, counting from the endostyle (RE) 3 (12) 1 (12) 2 (13) 3 (12) 2 (16) 2 (15) 2 dorsal lamina (DL) 3 (14) 2 (11) 2 (14) 2 (12) 2 (10) 2 (9) 5 left side, to the endostyle (EL) and RE 5 (11) 2 (10) 3 (10) 2 (14) 3 (12) 1 (15) 2 DL 3 (14) 2 (11) 3 (14) 2 (13) 2 (10) 2 (8) 3 EL. Branchial stigmata are straight and between 9 and 11 are present per mesh. The dorsal tubercle is arranged as a double spiral and is not granulated (Fig. 2F). The dorsal lamina is short and contains small languets (Fig. 2G). The dorsal tubercle shows a characteristic orientation in relation to the line delineated by the dorsal lamina. In this case it is between 40 and 80° to the left (Fig. 2F, G).

The right gonad show the same positioning as in other Australasian forms, forming two rows of lobes. The gonoduct of the right gonad opens 2–3 mm away from the gonad and is perpendicular to the vertical

Table 3. Taxonomic details of the examined *Pyura stolonifera* species complex specimens, except for the species ***Pyura doppelgangera* sp. nov.** (see main text) and *Pyura dalbyi* (see Rius & Teske, 2011)

Species	Body wall colour*	No. oral tentacles†	Branchial formula (small specimen)	Branchial formula (large specimen)‡	No. stigmata per mesh†	Dorsal tubercle shape	Dorsal lamina	No. of rows		Gonoduct longitude (mm)		Digestive secondary gut loop	Anal border lobes
								Right gonad	Left gonad	Right gonad	Left gonad		
Pyura praeputialis	Pale orange to violet	26–33	RE 4 (12) 2 (11) 1 (13) 2 (14) 2 (14) 1 (14) 3 DL 3 (16) 2 (12) 1 (14) 2 (13) 1 (11) 1 (9) 2 EL	RE 5 (12) 2 (14) 2 (17) 2 (20) 2 (21) 2 (21) 4 DL 5 (24) 3 (23) 2 (19) 2 (18) 2 (16) 2 (13) 2 EL	6–16	Double spiral cone	Absent or short with few languets	2	2	0.5–4	10–22	Medium	Yes
Pyura stolonifera	Yellow	22–24	RE 8 (12) 2 (15) 2 (17) 2 (18) 2 (21) 2 (17) 2 DL 3 (16) 2 (16) 3 (16) 3 (14) 4 (12) 3 (8) 3 EL	RE 4 (10) 5 (13) 5 (15) 4 (18) 3 (16) 3 (18) 10 DL 8 (22) 3 (16) 3 (19) 5 (15) 5 (13) 5 (10) 5 EL	5–7	Double spiral cone	Absent	1	1	1.5–3	18–20	Long	Yes
Pyura herdmani	Pale brown to orange	18–34	RE 4 (11) 3 (12) 4 (14) 3 (15) 3 (15) 3 (15) 6 DL 5 (15) 3 (15) 3 (15) 3 (14) 3 (13) 3 (12) 5 EL	RE 6 (13) 4 (14) 4 (18) 3 (22) 3 (23) 3 (23) 6 DL 6 (20) 3 (19) 4 (18) 3 (19) 4 (17) 3 (16) 13 EL	6–8	Sponge-like	Short with no languets	1	1	5–6	10–11	Short	Yes

*Some colour variation is expected owing to a different concentration of, and exposure time to, formalin.
†This quantitative character is positively correlated with body size in all species.
‡Counts are made from the endostyle on the right side of the body (RE) to the dorsal lamina (DL) and from the DL to the endostyle on the left side (EL).

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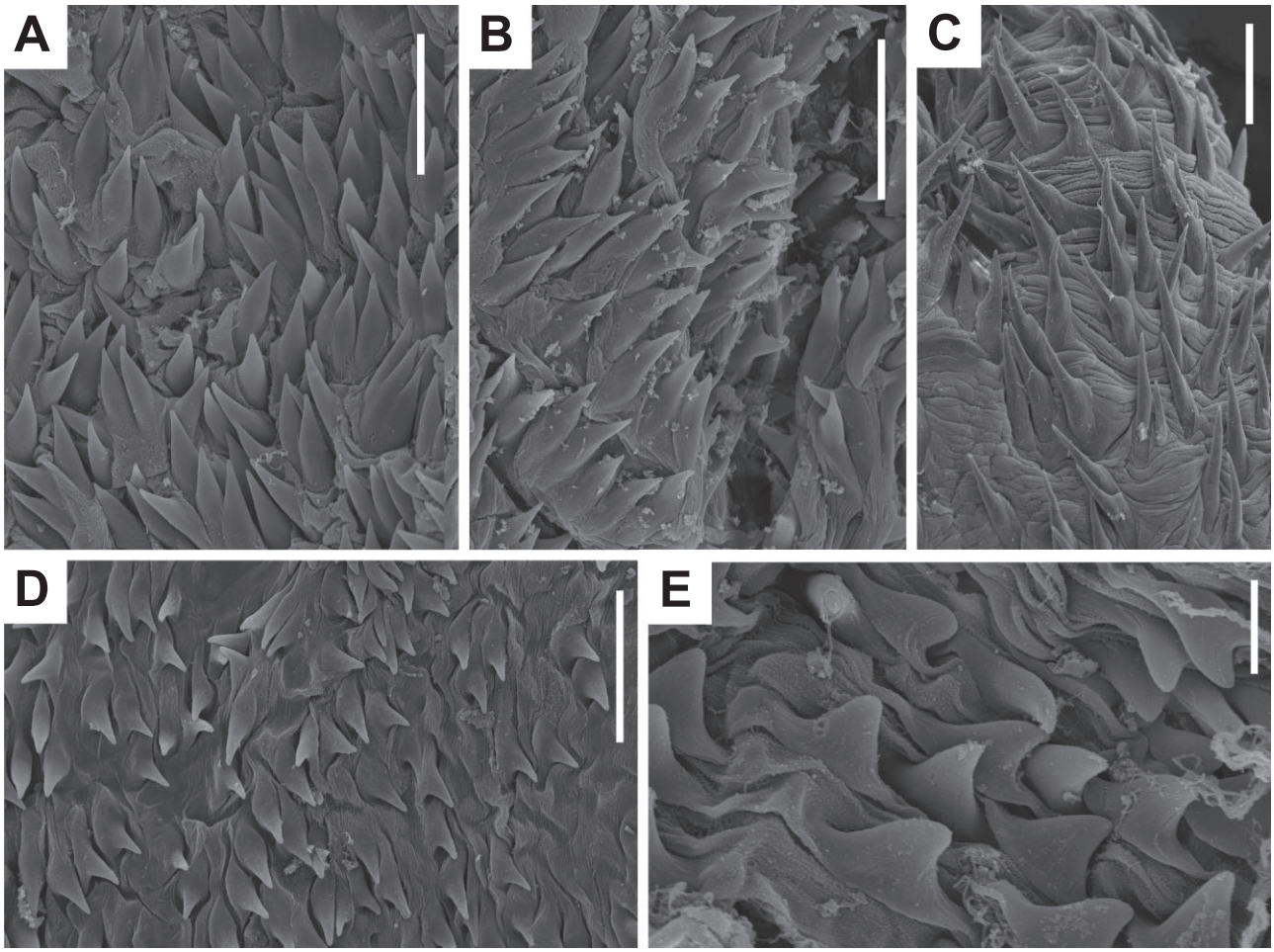

Figure 1. Scanning electron microscope photographs of the siphonal spines of the species comprising the *Pyura stolonifera* species complex. A, ***Pyura doppelgangera* sp. nov.**, B, *Pyura praeputialis*, C, *Pyura dalbyi*, D, *Pyura herdmani*, E, *P. stolonifera*. Scale bars: A = 100 μm; B, D = 40 μm; C = 50 μm; E = 10 μm.

position of the gonad. The left gonad is inside the gut loop and forms a single row of lobes (Fig. 2G). The gonoduct on the left side of the body opens 7–12 mm away from the first gonadic block and is located next to, but separated from, the anal aperture (Fig. 2C). The gut is located on the left side of the body and forms a sharply curved loop that is followed by an extremely short secondary loop just before the anus (Fig. 2G). The hepatic gland is large and branched and contains two bifurcated hepatic lobes. The anal border has fine edges and no lobes (Fig. 2A, B).

Morphological variation: The examination of *P. doppelgangera* specimens (see Table 1) revealed some morphological variation. The maximum dorsal length of the specimen (with tunic) is generally no larger than 55 mm, with the height ranging from 25 to 50 mm and the width from 15 to 35 mm. The tunic thickness is variable and can be as tough as that of other representatives of the *P. stolonifera* species complex, but some of the specimens examined had an extremely thin and flimsy tunic. Most individuals are aggregated in clumps and some show a short peduncle that appears from the base. The body wall is generally dark purple but it can be orange. The siphons are always dark violet, and there is normally a gradient of colours ranging from orange to purple around the siphons. The body size ranges from 23 to 45 mm in length, 15 to 32 mm in height, and 7 to 21 mm in width (as above, size of contracted specimens). There can be from 18 to 33 ramified oral tentacles (with more tentacles in larger individuals than in smaller ones). The branchial formula of the smallest individual found is: RE 5 (9) 2 (10) 2 (11) 2 (15) 2 (14) 1 (13) 2 DL 2 (14) 1 (12) 1 (14) 2 (13) 2 (11) 1 (10) 1 EL, and that of the largest individual is: RE 6 (12) 2 (12) 3 (14) 2 (16) 2 (12) 1 (17) 3 DL 3 (19) 2 (16) 2 (18) 3 (17) 2 (17) 2 (13) 4 EL. Branchial

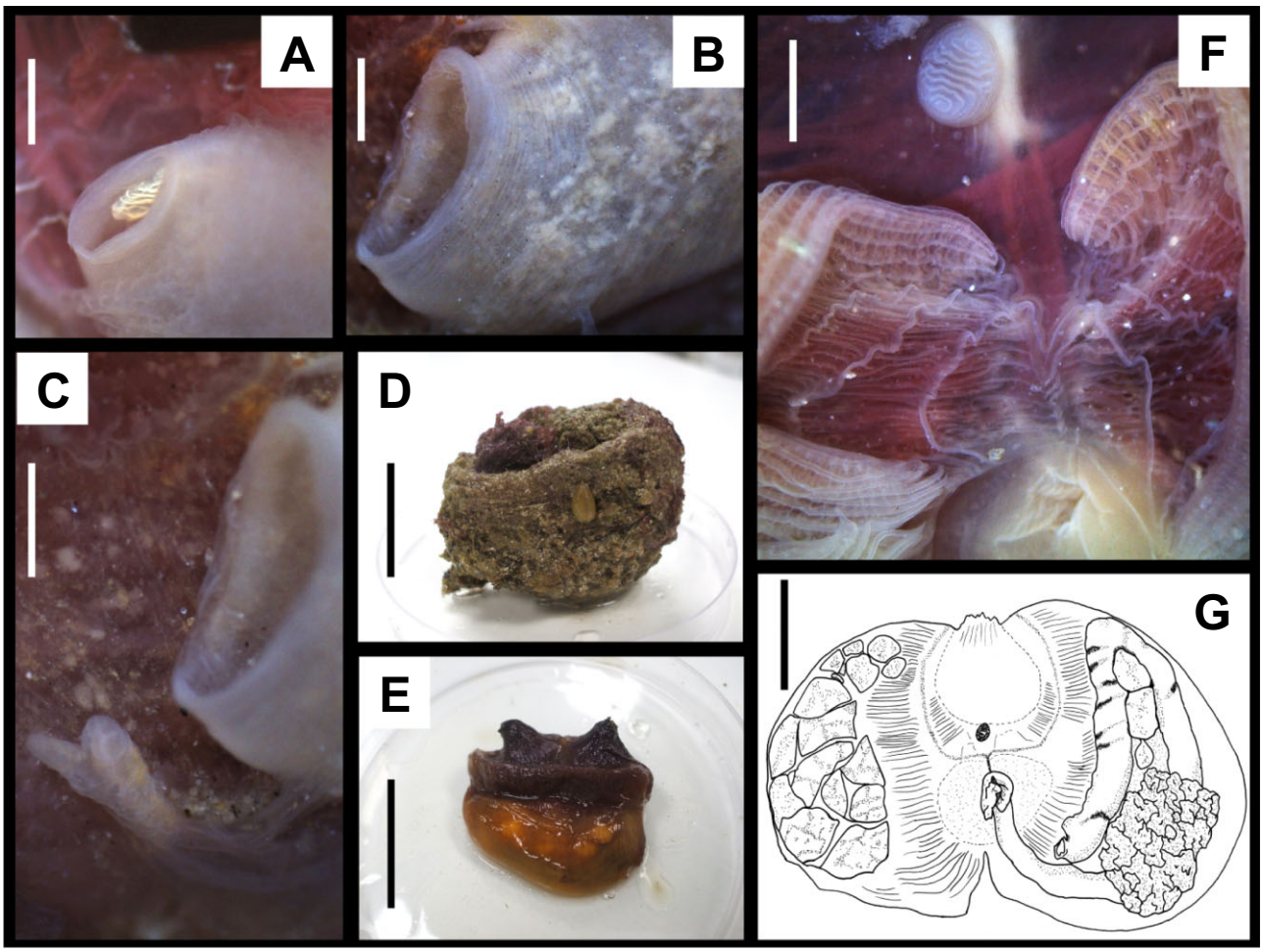

Figure 2. ***Pyura doppelgangera*** **sp. nov.** A, anal border (view from above); B, anal border (lateral view); C, gonoduct left side; D, an individual specimen collected in Tasmania. Photograph: Carmen Primo; E, the same individual without tunic. Photograph: Carmen Primo; F, dorsal tubercle and lamina with languets; G, internal structure detailing the position of the hepatic gland, dorsal tubercle, gonad on the right side of the body and the gut and gonad on the left side. Scale bars: A–C = 1 mm; D, E = 20 mm; F = 2 mm; G = 10 mm.

stigmata range from seven to 12 per mesh. The dorsal tubercle is always arranged as a double spiral and in most cases is not granulated. In the few individuals that show granulation, it is considerably less than in *P. praeputialis*. The dorsal lamina is short (containing small languets) or non-existent. The dorsal tubercle is between 20 and 90° to the left of the line delineated by the dorsal lamina, but in a few instances it is found 20° to the right. The gonoduct of the right gonad opens 1 to 6 mm away from the gonad, whereas the gonoduct on the left side of the body is longer (opening approximately 8–12 mm away from the first gonadic block).

Remarks: There are a number of distinctive internal characters that are unique to this species (Fig. 2). The four main ones are: (1) the dorsal tubercle is smooth; (2) the anus has no defined lobes; (3) the digestive track does not have a secondary gut loop or it is short and never curves sharply; (4) the gonoduct on the left side is clearly separated from the anal aperture.

Distribution: This species has been found on lower intertidal shores along the entire Tasmanian coast and in northern New Zealand, and exclusively on artificial structures in Victoria and South Australia.

Etymology: The species is named *P. doppelgangera* after the German word 'Doppelgänger', which in its narrowest sense means look-alike (i.e. somebody who closely resembles somebody else). The ending 'a' is used to indicate a feminine noun in the nominative singular. The meaning of the name reflects the difficulty of distinguishing this species from *P. praeputialis* because of its similar morphology.

Morphological differences between *Pyura praeputialis* and *Pyura doppelgangera* sp. nov.

Although a difference exists regarding the maximum body size of these closely related species, they are indistinguishable externally, and it is therefore necessary to dissect them and compare internal structures. The morphological characters that distinguish these species were subtle and required the dissection of a large number of individuals to define boundaries of variability of each morphological character. The dorsal tubercle is different – smooth in *P. doppelgangera* (Fig. 3A, B) and 'granulated' in *P. praeputialis* (Fig. 3F, G). The anus has no defined lobes (and no protruded 'lip') in *P. doppelgangera* (Fig. 3D, E), whereas *P. praeputialis* always has lobes (and a protruded 'lip') (Fig. 3I, J). The gut of *P. praeputialis* has a characteristic long secondary loop that generally curves sharply (Fig. 3H), whereas *P. doppelgangera* has a short secondary loop that never curves sharply, if it is present at all (Fig. 3C). The gonoduct on the left side is clearly separated from the anal aperture in *P. doppelgangera* (Fig. 3E), whereas in *P. praeputialis*, the gonoduct aperture is below the anus.

Heller (1878) described two similar species: *Cynthia stolonifera* from South Africa and *C. praeputialis* from Australia, which were later both considered to be of the genus *Pyura* (Millar, 1966). Subsequently, Monniot & Bitar (1983) compared specimens from Morocco, Chile, and Australia. The first one corresponded to *P. herdmani*, but the other two were *P. praeputialis*, as is evident from the characteristic shape of *P. praeputialis*' digestive track with a secondary gut loop that curves sharply, something that is not found in *P. doppelgangera*. As previous taxonomists have described in detail specimens of the large form (*P. praeputialis*), we considered it appropriate to describe the small form as *P. doppelgangera*.

Biogeography

The distribution of the five species within the *Pyura stolonifera* species complex is shown in Fig. 4. There are two regions – southern Africa and south-eastern Australia – where two and three species coexist, respectively. The ranges of *P. praeputialis* and *P. doppelgangera* overlap along the south-eastern coast of Australia. *Pyura doppelgangera* was found both on natural and artificial substrates in Tasmania and New Zealand, and exclusively on artificial structures in Victoria and South Australia, whereas *P. praeputialis* was found on both natural and artificial substrata throughout its range.

Phylogenetic analyses

We generated a total of 74 COI sequences and 104 ANT sequences specifically for this study, and combined this data set with all previously published DNA sequences (Table 1). Trimmed COI sequences were 208 bp in length and contained 47 polymorphic positions. Although ANT sequences of the two lineages could be aligned, the trimmed segments were not identical, with the *P. doppelgangera* sequences being 253 bp in length and having 92 additional nucleotides at the 5′ end, and the *P. praeputialis* sequences being 175 bp in length and having 14 additional nucleotides at the 3′ end. The aligned data set of 267 bp contained 39 variable nucleotide positions and four indels.

The phylogenetic trees with the highest likelihood values reconstructed using COI and ANT sequences (Fig. 5) both recovered *P. praeputialis* and *P. doppelgangera* as distinct evolutionary lineages. *Pyura praeputialis* was found in south-east Australia [east and west of Wilson's Promontory, a location where phylogeographical breaks have been reported in other marine invertebrates (Ayre, Minchinton & Perrin, 2009)] and in Chile. *Pyura doppelgangera* was found in south-east Australia (east of Wilson's Promontory), South Australia, Tasmania, and New Zealand. In the region of overlap in south-east Australia, the two species were never found at the same site (Table 2, Fig. 4), and we did not find a single individual that had ANT intron sequences from both species that would have indicated that they can interbreed. For both loci, *P. praeputialis* had greater allelic richness than *P. doppelgangera* (COI: *P. praeputialis*: 38, *P. doppelgangera*: 5; ANT: *P. praeputialis*: 38, *P. doppelgangera*: 15; GenBank accession numbers: see details in Supporting Information, Appendix S1-S4). Mean p-distances between *P. praeputialis* and *P. doppelgangera* were 0.10 for COI and 0.09 for ANT.

DISCUSSION

The alpha taxonomy of ascidians is characterized by excessive lumping (Knowlton, 2000), and numerous recent studies have identified high levels of cryptic diversity in this group (e.g. Tarjuelo *et al*., 2001; Pérez-Portela *et al*., 2007; Teske *et al*., 2011). Many ascidians are habitat-forming species that can strongly influence community structure of benthic ecosystems, and can be aggressive invasive species (Castilla *et al*., 2004; Lambert, 2007) with large geographic ranges (Pineda *et al*., 2012; Rius *et al*., 2012). Thus, resolving the taxonomy of ascidians is important not only for biodiversity management but also to understand ecosystem functioning. Our study presents an example of how the combined use of morphological, biogeographical, and

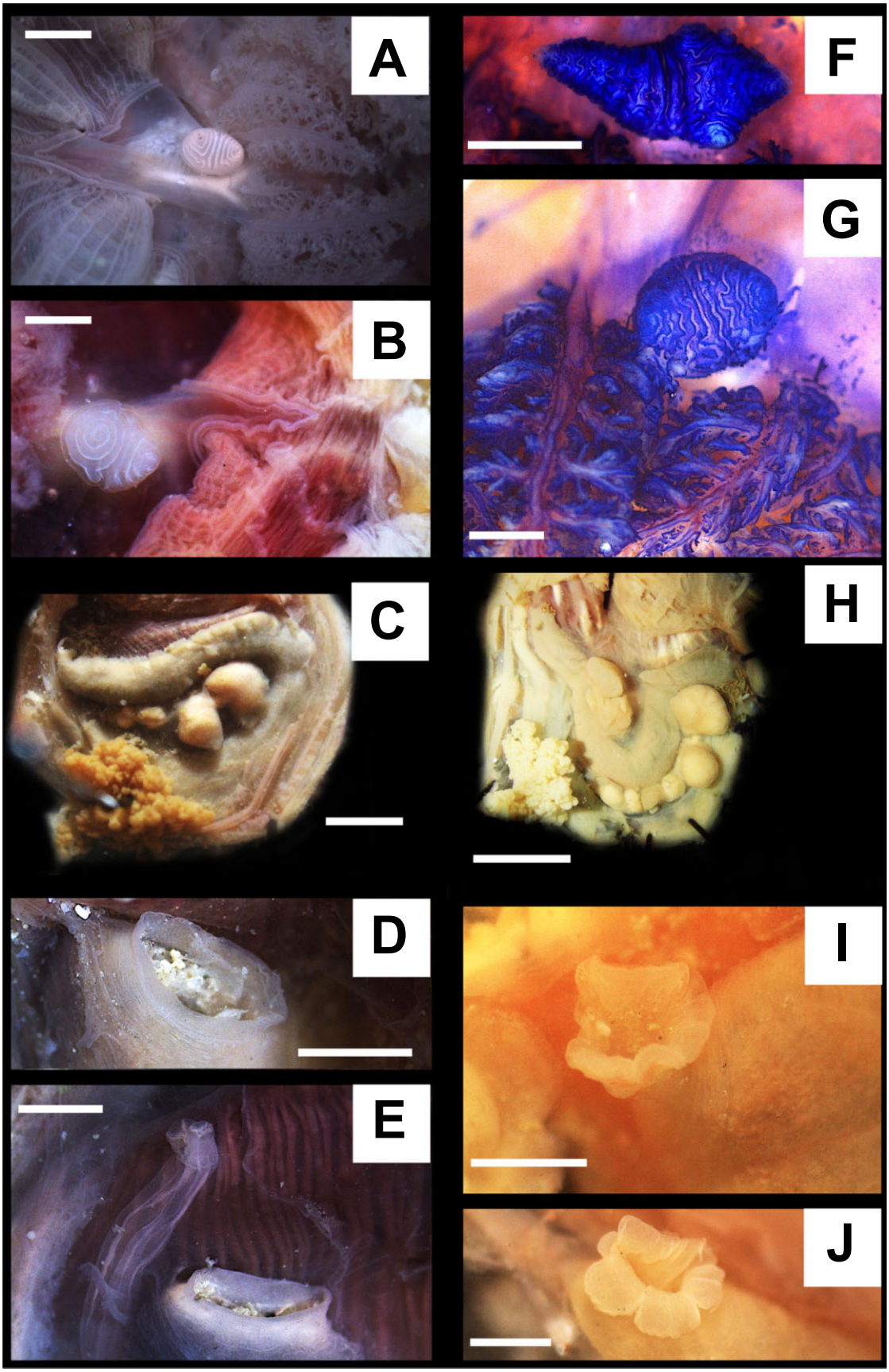
A
B
C
D
E
F
G
H
I
J

Figure 3. Morphological differences between ***Pyura doppelgangera* sp. nov.** (A–E) and *Pyura praeputialis* (F–J). A, dorsal tubercle; B, dorsal tubercle and lamina with languets; C, digestive tract; D, anal border; E, anal border with the gonoduct left side; F, dorsal tubercle (stained with Nile blue sulphate); G, dorsal tubercle and branchial tentacles (stained with Nile blue sulphate); H, digestive tract; I, anal border; J, anal border. Scale bars: A, D–F = 2 mm; B, G, I, J = 1 mm; C = 10 mm; H = 20 mm.

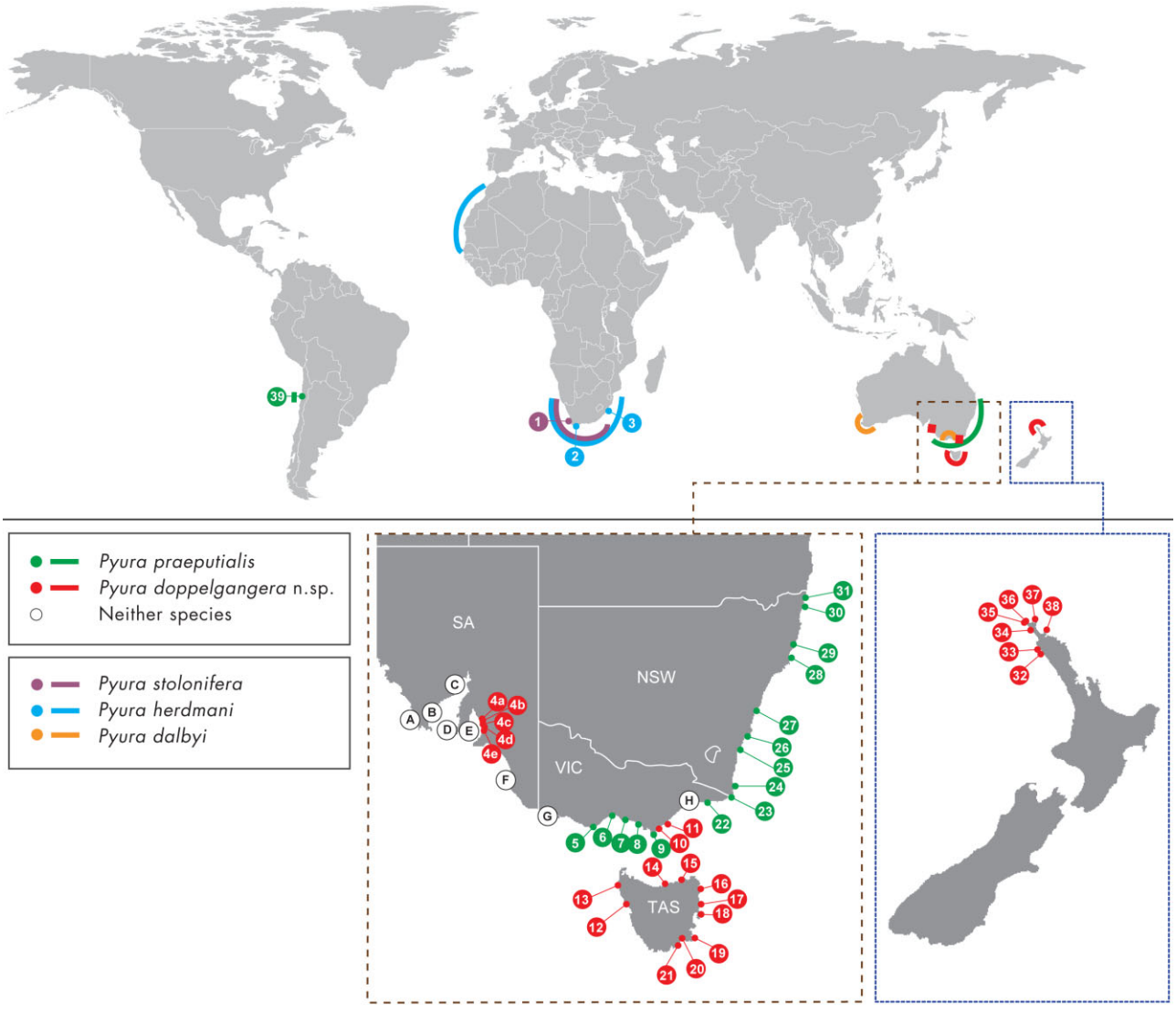

Figure 4. World map indicating the known distribution of the different species of the *Pyura stolonifera* species complex. The magnified maps show the sites in south-east Australia and New Zealand at which samples of *Pyura praeputialis* and ***Pyura doppelgangera* sp. nov.** were collected. Site numbers correspond to those used in Tables 1 and 2. Neither lineage was present at sites A–H, indicating possible distribution gaps at sites in South Australia (SA; A–F) and Victoria (Vic; G, H) (see details in Table 2). NSW, New South Wales; TAS, Tasmania.

genetic approaches provides a clear understanding of overlooked ascidian biodiversity in a region (temperate Australasia) that has been comparatively well studied.

The existence of a large and small form of the ascidian *P. praeputialis* (often referred to as *P. stolonifera* in the literature) has long been known (Kott, 1985). A previous genetic study suggested that

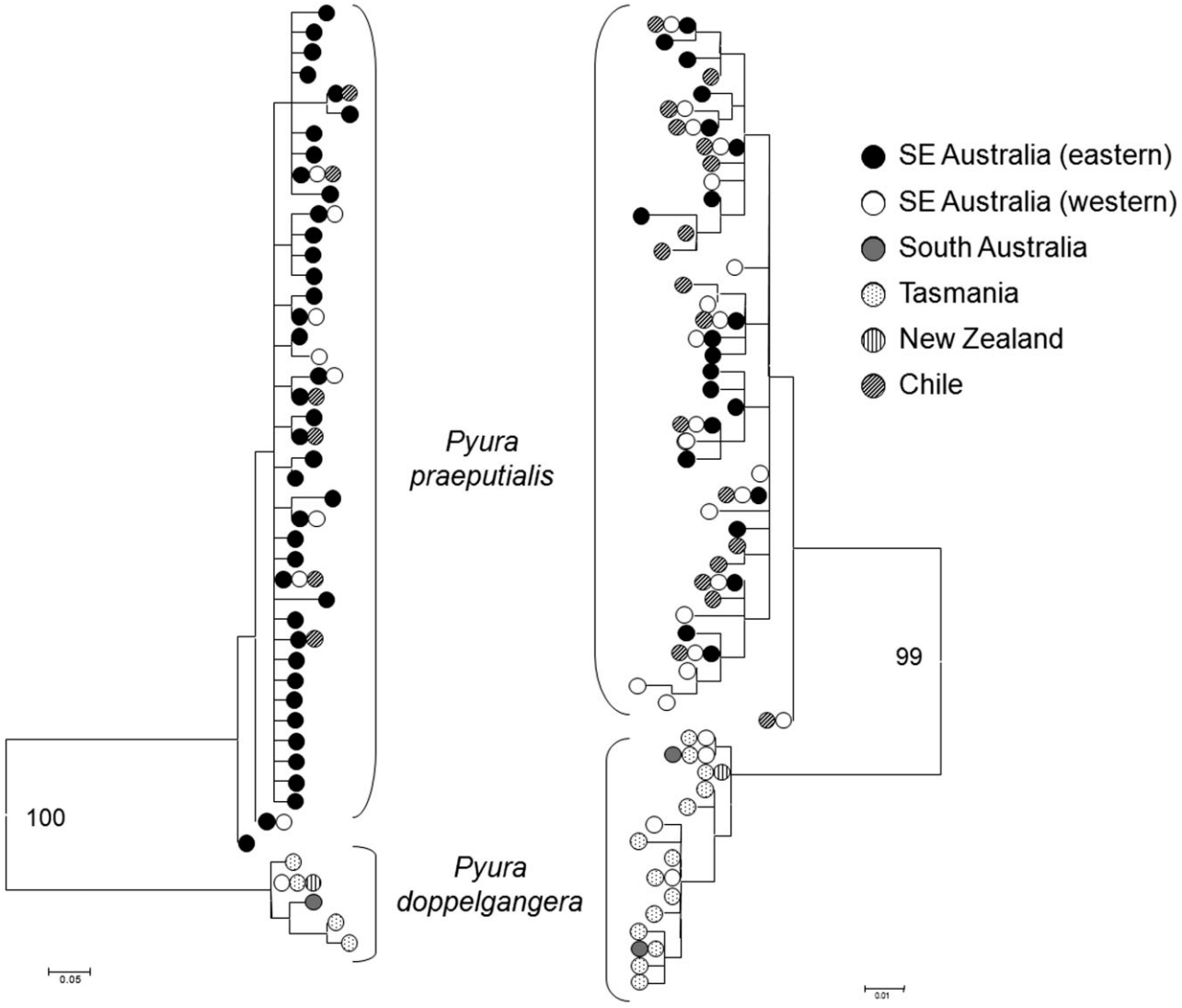

Figure 5. Maximum likelihood trees constructed from mitochondrial cytochrome c oxidase subunit I (left; log likelihood: –399.5730) and nuclear adenine nucleotide transporter intron (right; log likelihood: –12170.8682) sequences of *Pyura praeputialis* and ***Pyura doppelgangera* sp. nov.** Nodal support from 1000 bootstrap replications (> 75%) is indicated next to some branches. Circles indicate regions in which a particular allele was present. For simplicity, allele frequencies are not indicated.

the two were geographically isolated with separate ranges in mainland Australia and Tasmania, respectively, which may have driven genetic divergence (Astorga *et al*., 2009). The present study provides conclusive evidence that the two forms represent two morphologically and genetically distinct species. The support for this is particularly strong on the basis of mitonuclear genetic data, where both loci recovered congruent, highly divergent evolutionary lineages that shared no alleles. Although the two species are indistinguishable on the basis of their external morphology, numerous internal morphological characters were identified that can be used to distinguish them unequivocally from all other species that were traditionally referred to as *P. stolonifera*.

Biogeography and reproductive isolation

Exhaustive sampling in Australasia revealed that although the distribution of both species is centred on one particular region (the south-east Australian mainland for *P. praeputialis* and Tasmania for *P. doppelgangera*), their ranges overlap in the Australian province of Victoria. Despite this overlap, the two

species have so far never been found in sympatry. This, together with the fact that no genetic hybrids were found in this study, may be an indication of reproductive isolation between biological species (Mayr, 1942). However, specific *in vitro* experiments are required to confirm this conclusively, especially as hybridization is possible between other members of the *P. stolonifera* species complex (M. Rius, pers. observ.). Interestingly, whereas *P. doppelgangera* was found mostly on natural substrates in Tasmania, it was exclusively found on artificial structures in Victoria and South Australia. This could be indicative that this species has recently been introduced to mainland Australia, where it has established itself in habitats where *P. praeputialis* is absent. To date this species has only been found in areas where *P. praeputialis* is not present. We have shown that all reports of *P. praeputialis* as an introduced species in New Zealand correspond to *P. doppelgangera* (Hayward & Morley, 2009; Page *et al*., 2012). However, the introduced/native status and the invasion routes that might have shaped the current distribution remain to be tested using multilocus genetic data.

Pyura praeputialis can strongly alter coastal biotas because of its tendency to form dense aggregates (Castilla *et al*., 2004). Regarding *P. doppelgangera*, aggregations of this type have been reported on artificial structures in South Australia and Victoria (south-east Australia, east of Wilson's Promontory; P. R. Teske, pers. observ.) while the species only forms sporadic clumps on the Tasmanian coastline (Bennett & Pope, 1960). The presence of this species at the northern tip of New Zealand's North Island, where it can be found both on natural and artificial substrata (K. Walls, pers. observ.), suggests that it is only able to survive in the warmest parts of this island.

Additional cryptic species in the *Pyura stolonifera* complex?

The description of *P. doppelgangera* has contributed significantly towards resolving the taxonomy of a group of intertidal ascidians that is characterized by a complicated pattern of 'nested' cryptic diversity (Teske *et al*., 2011). Genetic data further indicate that there are several additional lower-level evolutionary lineages in the *P. stolonifera* species complex that require further scrutiny as they may represent distinct species. This is the case for the African species *P. herdmani* that occupies clearly defined biogeographical regions in temperate southern Africa, subtropical/tropical southern Africa, and northwestern Africa (Teske *et al*., 2011). Future studies might reveal cryptic biodiversity and/or patterns of sympatric hybridization with the sympatric species *P. stolonifera*.

CONCLUSIONS

Understanding the mechanisms that allow the establishment of range boundaries is important to identify rates of speciation and radiation, both in regions that remain relatively unexplored and in those that have been thoroughly surveyed. We have provided a detailed study of coastal temperate Australasia, the region of the world that harbours the highest diversity of members of the *Pyura stolonifera* species complex, and where the ascidian fauna of the temperate coasts has been extensively studied (Kott, 1952, 1985, 2006, 2009). In addition, we have formally described a cryptic species that can be found along the coast of mainland Australia, as well as in Tasmania and New Zealand. Taken together, the present study illustrates the usefulness of a combined morphogenetic approach in unravelling overlooked diversity in relatively well-studied regions. Future studies are required to reveal what evolutionary and ecological mechanisms have shaped the current distribution of the species within this species complex.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article on the publisher's web-site:

Appendix S1. The file 'PdoppelCOI_SuppInfo.mas' contains cytochrome c oxidase subunit I (COI) sequences of *Pyura doppelgangera* (GenBank numbers KC751899-KC751948).

Appendix S2. The file 'PdoppelANT_SuppInfo.fas' contains adenine nucleotide transporter (ANT) sequences of *Pyura doppelgangera* (GenBank numbers KC751949-KC752006).

Appendix S3. The file 'PpraeCOI_SuppInfo.mas' contains cytochrome c oxidase subunit I (COI) sequences of *Pyura praeputialis* (GenBank numbers KC751875-KC751898).

Appendix S4. The file 'PpraeANT_SuppInfo.fas' contains adenine nucleotide transporter (ANT) sequences of *Pyura praeputialis*.