GENETIC DIVERGENCE IN THE ASCIDIAN *PYURA PRAEPUTIALIS* (= *PYURA STOLONIFERA*) (HELLER, 1878) FROM MAINLAND AUSTRALIA AND TASMANIA

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(with one text-figure and one table)

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In Australia, the sea squirt *Pyura praeputialis* is found off the mainland and off the island of Tasmania. The extent of genetic divergence between insular and mainland continental samples was determined by performing a DNA analysis using PCR–RFLP on the internal transcribed spacer region of ribosomal DNA. The two mainland populations were polymorphic and showed similar type frequencies, but both were significantly different from the Tasmanian population. This was also monomorphic, showing a unique type, absent from the two continental populations. The genetic differentiation could be explained by the lack of genetic flow observed between both mainland and Tasmanian populations.

Key Words: sea squirt, genetic divergence, PCR-RFLP, ITS, Australia, Tasmania, *Pyura praeputialis*.

INTRODUCTION

The sea squirt *Pyura praeputialis* (=*Pyura stolonifera*) (Heller, 1878) is a large solitary ascidian species that occurs in dense beds on rocky intertidal reefs, from low intertidal habitats to a depth of 10–12 m along Australia's mainland and insular coasts (Edgar 1997). The species has an aggregated distribution along the rocky intertidal region (Dalby 1995) from Western Australia to Queensland and also in Tasmania (Kott 1985, Edgar 1997). These sea squirts have also been detected off the Chilean coast of South America (Castilla & Guiñez 2000, Castilla *et al.* 2002), most likely introduced there anthropogenically (Castilla *et al.* 2004).

Our study focuses on the disjunct distribution of the sea squirts *P. praeputialis* from the mainland Australian continent and Tasmania. All populations are believed to be conspecific. It is assumed that larval drift is sufficient to maintain continuous gene flow between the mainland and the island and, therefore, that Bass Strait is not a barrier for sea squirt dispersal; although some morphological differences have been reported between insular and continental specimens (Kott 1985).

Tasmania is separated from mainland Australia by the Bass Strait which is over 200 km wide. However, in the late Pliocene and Pleistocene (glacial maxima) the Bass Strait region was a dry land bridge (Knox 1980) that presumably blocked gene flow between eastern and western marine populations, also creating continuous habitats for sessile intertidal organisms currently occurring on mainland Australia and Tasmania. These geological events largely have determined the present composition of the terrestrial and marine flora and fauna, through vicariant events or dispersal driven by ocean currents (some examples are in Waters & Roy 2003, Waters *et al.* 2004, Waters 2008, York *et al.* 2008).

Any barrier, either oceanographic or habitat discontinuity, that interrupts gene flow is expected to result in increased genetic differentiation (Hedgecock 1986), and when sufficiently persistent, this can generate marine speciation processes (Barber *et al.* 2000). Therefore, the degree of population genetic differentiation within a species can be used to determine its level of gene flow and also could permit an estimate of the isolation time, once gene flow connectivity was interrupted, thereby, allowing a reconstruction of the biogeography of the species (Wong *et al.* 2004, Waples & Caggiotti 2006, Waters *et al.* 2007).

This paper reports the first results of work in progress on the degree of genetic differentiation in marine organisms found in Tasmanian and mainland Australian localities using PCR-RFLP analysis of the internal transcribed spacer (ITS) fragments of ribosomal DNA.

METHODS

Individuals were obtained at two sites off the Australian continent (Brisbane: 27°30"S; 153°01"E, and Barwon Heads: 38°35"S; 144°30"E) and from one site off Tasmania (Kingston: 42°58"S; 147°19"E) (fig. 1).

DNA was extracted from the siphon tissue of each individual using standard phenol/chloroform/isoamyl alcohol, and ethanol precipitation extraction methods (Doyle & Doyle 1987). A PCR-RFLP was carried out on the ribosomal DNA ITS fragment, which covered the ITS-1, 5.8S, and ITS-2 fragments. The ITS primers were designed based on the conserved regions of sequences extracted from GenBank of nine different tunicate species. The design of the forward primer used sequences of the 26S gene from the following species: *Herdmania momus* (Savigny, 1816) and *Styela plicata* Herdman, 1882 (Ascidiacea), and *Oikopleura* sp. and *Thalia democratica* (Forskal, 1775) (Thaliacea). The reverse primer was designed using sequences of the 18S gene from the following species belonging to the family Pyuridae (Ascidiacea): *Pyura vitatta* (Stimpson, 1852), *P. mirabilis* (Drasche, 1884), *Halocynthia roretzi* (Drasche, 1884), *Herdmania momus*, *H. curvata* Kott, 1952, and *H. mirabilis* (Drasche, 1884). The program Webprimer (http://

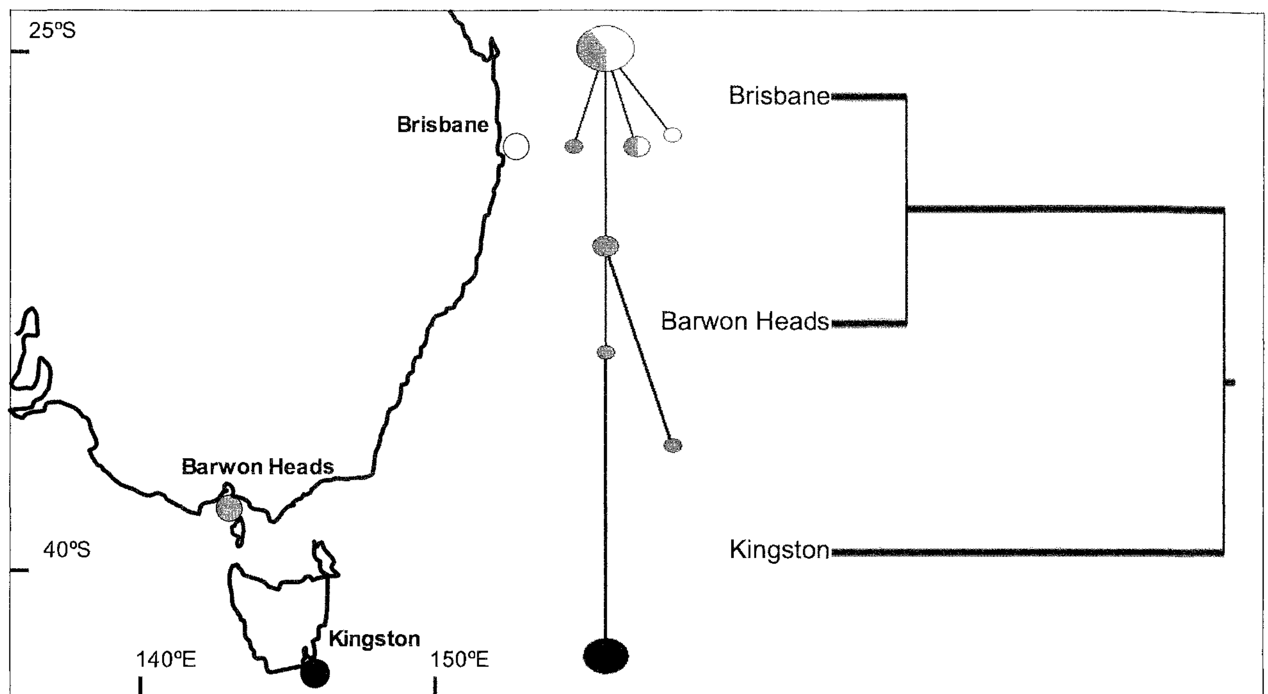

FIG. 1 — Map of the southern coast of Australia showing the analysed populations, in relation with the network analysis that groups the compound types based on their frequencies. Hierarchical clustering analysis (UPGMA) shows the relationships between the analysed populations based in Nei's genetic distance.

genome-www2.stanford.edu/cgi-bin/SGD/web-primer) was used to design both primers. The evaluation of each designed oligonucleotide was carried out using the program Net Primer software (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html).

PCR amplification

The ITS region was amplified by PCR using the designed primers in a mix made up of 5 µL 10X buffer PCR (Perkin-Elmer), dNTPs 10 mM, and 4 µL (10 µM) of each primer, $MgCl_2$ 50 mM, purified BSA, 5 Units/mL of Ampli Taq, and ddH_2O. The reactions were run in a thermocycler Eppendorf with an initial denaturation period of 1 minute at 95°C, followed by 40 cycles that included a denaturation period at 94°C for 1 minute, primer annealing at 58°C for 40 seconds, and extension at 72°C for 2 minutes; the cycles were followed with a final extension of 8 minutes at 72°C.

Digestion of the ITS fragment

Twelve restriction enzymes (*Alu*I, *Ava*II, *Bam*HI, *Dpn*II, *Hae*III, *Hha*I, *Hind*III, *Hinf*I, *Msp*I, *Nde*I, *Rsa*I, *Taq*I) were tested to determine which would reveal variability. Three of these (*Ava*II, *Hae*III, *Msp*I) were selected for the subsequent analyses. PCR products were digested using 10 µL amplified DNA, 1 µL of each enzyme, and 1.2 µL of buffer NEB2 for *Hae*III and *Msp*I and NEB 4 for *Ava*II. Digests were incubated overnight at 37°C, and then subjected to electrophoresis in a 3% TBE agarose gel.

Data analysis

PCR-RFLP is a dominant marker. Because of this, the variation of composite RFLP patterns of the ITS rDNA fragments generated by the enzymes was used to group into rDNA types. Genetic divergence between localities (island versus continent) was estimated with Fisher's Exact Test (Weir & Cockerham 1984) using the program GENEPOP 3.1. (Raymond & Rousset 1995), and a cluster analysis based on Nei's genetic distance values (Nei 1978) was applied to estimate the genetic relationships, using the program TFPGA (Miller 1997). Intraspecies gene genealogies were inferred, using the median-joining network approach (Bandelt *et al.* 1999) as implemented in NETWORK 4.1.08.

RESULTS

Digestion of the ITS fragment (1400 bp) of *P. praeputialis* samples with the enzymes *Ava*I, *Hae*III, and *Msp*I, produced 11 restriction fragments with an average of 3.7 fragments per enzyme. Eight restriction patterns or types were observed, the enzyme *Hae*III revealed the greatest diversity. Table 1 shows the frequencies, with the continental Brisbane and Barwon Heads localities presenting five types each; h5 was the most frequent type at both sites. A unique type was found at the Kingston site in Tasmania, which was absent from the continental sites.

Unsurprisingly, therefore, genetic differentiation was significant between the continental and island populations (Brisbane-Kingston $p<0.001$; Barwon Heads-Kingston $p<0.001$), with the pairwise comparisons including the Kingston site also showing the highest genetic distance values ($D = 0.788 \pm 0.09$). The two continental sites did not show significant genetic differentiation (Brisbane-Barwon Heads $p = 0.839$). The network analysis revealed a distribution in which a genetic type of major frequency was shared by both continental sites, with branches showing somewhat similar to "star shaped" diversity and another type unique to the Tasmania site (fig. 1). This differentiation pattern was observed in the cluster obtained from the hierarchic clustering analysis (fig. 1).

TABLE 1
Frequencies of the eight different types of restriction fragment pattern

Type	Brisbane (n=11)[1]	Barwon Heads (n=9)	Kingston (n=6)
1	0.000	0.000	1.000
2	0.273	0.111	0.000
3	0.091	0.000	0.000
4	0.455	0.556	0.000
5	0.000	0.111	0.000
6	0.091	0.000	0.000
7	0.000	0.111	0.000
8	0.091	0.111	0.000

[1] number of individuals at each locality

DISCUSSION

A highly significant genetic differentiation was found between island and continental *P. praeputialis* individuals, with a fixed type characterising the Tasmanian samples.

The fact that we did not detect genetic types shared among the island and the mainland samples, could be explained either because the coasts are not connected today with no genetic flow, or perhaps because there are low frequencies of shared types but these were not evident in the sample examined, with this number of loci analysed. Both continental sites showed similar types and both differed from the Tasmanian sample. As a result of the isolation between the populations, and/or the limitations in the exchange of genes, is expected that the populations will diverge by genetic drift or selection for alleles that adapt to each population local niche. If a lack of gene flow exists at a sufficiently low level, then otherwise isolated populations will diverge genetically, and they will become disjointed and evolve as separated evolutionary units. However, genetic divergence among subpopulations resulting from random genetic drift could be prevented by low levels of migration.

The genetic flow is provided by the dispersal capacity of planktonic larval phases and this is related to the larval life span; for *P. praeputialis*, the free-swimming larval period is only two hours (Clarke *et al.* 1999), implying a reduced dispersal capacity. However, dispersal is also determined by the oceanographic characteristics of the occupied habitat (e.g., water masses and currents in Bass Strait) that facilitate or restrict larval dispersal. At present, the coasts of Tasmania are bathed by the Eastern Australian Current and, from the west, the Leeuwin Current. This should facilitate dispersal between the mainland and island coasts, as confirmed by the fact that Tasmania and the continent share fauna from the same biogeographic marine regions (the Peronian and Maugean zones) (O'Hara & Poore 2000, Waters & Roy 2003, Dawson 2005).

On the other hand, the sea squirt distribution could be explained by past events such as vicariant processes, reflecting the dynamics of the changes in the union and separation of Tasmania from the mainland, with repeated openings and closings of Bass Strait, and large temperature differences in the Plio-Pleistocene (Knox 1980). These have favoured genetic differentiation, allopatric divergence and possibly speciation in some species present on both coasts (examples in Soh *et al.* 1998, Waters & Roy 2003, Waters *et al.* 2004, Colgan *et al.* 2005).

Alternatively, given the geological events discussed above, the extant population of *P. praeputialis* of Tasmania may have originated through past dispersal from continental *P. praeputialis* populations, with a series of geologic changes that occurred between the island and the continent facilitating the current breakdown in gene flow.

This scenario should be more fully evaluated in the future, by designing a population genetic analysis that includes taking more samples, covering more populations over a wider geographic range including eastern and western regions of southern Australia, and probably using molecular markers with greater variability.

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