

## Regional patterns of mtDNA diversity in *Styela plicata*, an invasive ascidian, from Australian and New Zealand marinas

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**Abstract.** The ascidian *Styela plicata* is abundant in harbours and marinas worldwide and has likely reached this distribution via human-mediated dispersal. Previous worldwide surveys based on mitochondrial cytochrome oxidase one (COI) sequences have described two divergent clades, showing overlapping distributions and geographically widespread haplotypes. These patterns are consistent with recent mixing among genetically differentiated groups arising from multiple introductions from historically distinct sources. In contrast, a study of Australian *S. plicata* using nuclear markers found that population differentiation along the eastern coast related to geographic distance and no evidence for admixture between previously isolated genetic groups. We re-examined the genetic patterns of Australian *S. plicata* populations using mtDNA (COI) to place their genetic patterns within a global context, and we examined New Zealand populations for the first time. We found that the haplotypic compositions of Australian and New Zealand populations are largely representative of other worldwide populations. The New Zealand populations, however, exhibited reduced diversity, being potentially indicative of a severely bottlenecked colonisation event. In contrast to results from nuclear markers, population differentiation of mtDNA among Australian *S. plicata* was unrelated to geographic distance. The discrepancy between markers is likely to be a consequence of non-equilibrium population genetic processes that typify non-indigenous species.

**Additional keywords:** cosmopolitan, human-mediated dispersal, invasive, mitochondrial DNA, non-indigenous species, phylogeography.

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### Introduction

Aquaculture and maritime transport are the main sources of introductions of non-indigenous species (NIS) in the marine environment (Ruiz *et al.* 1997; Carlton 1999; Wonham and Carlton 2005). NIS are particularly prominent in human-modified marine environments, such as marinas and harbours (Geller *et al.* 1993), occupying breakwaters, pontoons, pylons, mooring lines and boat hulls; such artificial structures might also assist in the invasion process by providing a local competitive advantage to NIS over native species that may not be well adapted to settling on foreign substrates (Bulleri and Airolidi 2005; Glasby *et al.* 2007; Tyrrell and Byers 2007). Retention of seawater by man-made structures may contribute to establishment of NIS (Dupont *et al.* 2009), because gametes and larvae may be concentrated and contained in harbours and marinas, rather than being swept away by ocean currents. Long-distance dispersal among marinas and harbours does not necessarily rely on chance movements of planktonic larvae; dispersal of only a few metres can potentially lead to extremely long-distance transport by the aforementioned human vectors (Minchin and Gollasch 2003). Thus, harbours and marinas can act as invasion hubs, allowing high reproductive output and rapid dispersal to

adjacent ‘node’ populations and to locations far outside an organism’s innate dispersal neighbourhood (Floerl *et al.* 2009).

Molecular markers and analyses are important tools for identifying native source populations of NIS, uncovering cryptic species and sometimes revealing post-establishment population dynamics among populations outside of their native range (Miura 2007; Dupont *et al.* 2009; Geller *et al.* 2010). A present area of research interest is whether NIS found in harbours and marinas generally result from multiple independent introductions from a geographically distant source (or sources) or from stepping-stone spread within a geographic region following single introductions (Geller *et al.* 2010; Rius *et al.* 2012). Similarly, the degree to which established populations are connected by ongoing gene flow is relevant to the management of NIS populations (Dlugosch and Parker 2008); if gene flow is low or non-existent, then NIS populations might be managed effectively in isolation, whereas if gene flow is high, then local eradication strategies may be ineffective.

Through human-assisted dispersal, many NIS attain cosmopolitan distributions. Ascidians are well known for their propensity to flourish in new environments, particularly in ports and marinas (Lambert 2007). Molecular surveys have been

employed in several studies of cosmopolitan ascidians to investigate routes and vectors of dispersal. For example, west- and east-coast populations of *Botryllus schlosseri* in North America were found to differ in mtDNA haplotype composition (Lejeune *et al.* 2011) and microsatellite allele frequencies (Stoner *et al.* 2002), consistent with separate introductions. Similarly, in Europe, at least two groups of invasive *Styela clava* are inferred from microsatellite markers, suggesting multiple introductions (Dupont *et al.* 2010, and other similar examples reviewed therein). Typically, at both global scales and within coastlines, genetic differentiation among non-indigenous ascidian populations is not correlated with geographic distance (Dupont *et al.* 2010; Goldstien *et al.* 2011; Pineda *et al.* 2011), being indicative of non-equilibrium population dynamics such as bottlenecks and long-distance colonisation.

The solitary ascidian *Styela plicata* (Lesueur, 1823) is a cosmopolitan species that is abundant in many human-altered environments (Lambert and Lambert 1998). Barros *et al.* (2009) provided a comprehensive review of global first observations and although preliminary results seem to indicate an origin in the North-west Pacific (individuals have been found on natural substrates in Japan), the true native origins of *S. plicata* remain unclear (Pineda *et al.* 2011). The earliest Australian record of *S. plicata* is from Sydney Harbour (New South Wales) in 1878 (Heller 1878; Barros *et al.* 2009). Subsequent records indicate that the species was present in Victoria by 1957, and Queensland by 1972; and records in New Zealand date back to 1948 (see Barros *et al.* 2009, for details on global historical occurrences).

MtDNA surveys using COI sequences have found no conclusive epicentre of genetic diversity to indicate a location or ocean basin of origin for *S. plicata* (Pineda *et al.* 2011). In fact, populations within ocean basins were found to be just as genetically distinct as populations among ocean basins (Pineda *et al.* 2011). *S. plicata* COI haplotypes fall into two well differentiated but sympatric clades (herein referred to as Group 1 and Group 2; Barros *et al.* 2009; Pineda *et al.* 2011). Sequences of the nuclear *ANT* gene from worldwide samples also revealed two distinct lineages but no concordance between COI and *ANT* patterns at the population or individual level (Pineda *et al.* 2011). Thus, global patterns indicate historic admixture among distinct genetic groups but provide no evidence for reproductive isolation among groups such as if each represented a cryptic sibling species.

The most intensive nuclear marker survey in *S. plicata* used nuclear amplified fragment length polymorphism (AFLP) markers and tested for cryptic population structure within the eastern coast of Australia (David *et al.* 2010). Populations 1000 km or more distant were more differentiated than were local populations ( $\Phi_{CT} = 0.11$ ,  $\Phi_{SC} = 0.03$ ); however, there was no evidence for strong differentiation among individuals either between or within groups, such as if two distinct lineages had been sampled (i.e. indicating the presence of Group 1 and Group 2). The detection of a regional population structure was interpreted as evidence for either (1) gene flow among populations being inversely proportional to geographic distance, or (2) regionally independent introductions followed by local spread (perhaps facilitated by boat movements among nearby marinas, David *et al.* 2010). This geographic differentiation for AFLP loci contrasts with the lack of geographic signal

evidenced from global surveys using COI sequences (Barros *et al.* 2009; Pineda *et al.* 2011). Here, we sample these same Australian populations for COI sequence variation to determine whether patterns within the Australian coastline match COI-sequence patterns from other locations. Specifically, we examine whether the two distinct lineages (Group 1 and Group 2) are widespread in Australia, and thus genetic differentiation would not relate to geographic distance, or if local gene flow homogenises geographically proximate populations but not distant populations as indicated by AFLP results. In addition, *S. plicata* from New Zealand is sequenced for COI for the first time, expanding global genetic coverage of this prominent cosmopolitan species.

## Materials and methods

### Sample collection and molecular methods

*Styela plicata* individuals were collected from floating pontoons and solid jetties from locations in Australia (including individuals from David *et al.* 2010) and New Zealand (Table 1, Fig. 1). Individual ascidians were dissected, and the gonads were preserved in ethanol (90%) or dimethylsulfoxide (DMSO). Genomic DNA was extracted following David *et al.* (2010). The mitochondrial cytochrome oxidase 1 (COI) gene was amplified using forward and reverse primers from Folmer *et al.* (1994), using Clontech titanium *Taq* polymerase under standard conditions (Clontech, CA, USA). PCR products were purified with an Exo-Sap purification protocol (New England Biolabs, Ipswich, MA, USA) and sequenced by capillary electrophoresis on an ABI 3730 (Macrogen, Korea). Trace file was edited using Codon Code Aligner v. 3.7.1 (www.codoncode.com). All sequences were manually aligned using SeAlv2.0 (Rambaut 1996) and trimmed to the same length (561 base pairs).

### Genealogical analyses

To verify that our observed haplotypes formed a monophyletic group, we compared them against the *S. plicata* COI sequences obtained from Barros *et al.* (2009) and Pineda *et al.* (2011), using *Styela motereyensis* and *S. gibbsii* as outgroups. A total sequence file was reduced to unique haplotypes and tree searches performed under both Bayesian and parsimony criteria. In MrBayes (ver. 3.12, Ronquist and Huelsenbeck 2003), we employed a general time-reversible model with a gamma distribution and allowing invariant sites. A Markov Chain Monte Carlo search of one million steps was used with a burn-in of 250 000 steps. Under the parsimony criterion, we used the heuristic search strategy in PAUP version 4.0b10 (Swofford 1998). Considering only the dataset that included our observed haplotypes, we constructed a haplotype network using TCS v1.18 (Clement *et al.* 2000), with a 90% connection limit.

### Genetic diversity and population structure

Patterns of genetic diversity and population structure were explored in two distinct manners. First, we used standard sequence-based estimators, whereby the number of nucleotide differences between haplotypes was taken into consideration. In this manner, we estimated the genetic diversity as the average number of pairwise differences between sequences (Tajima

**Table 1. Sampling locations and observed haplotypes**

Haplotype names correspond to those described by Barros *et al.* (2009) in a global dataset, followed by those of Pineda *et al.* (2011). Haplotype 20 (H\_20) is described only from Pineda *et al.* (2011). NSW, New South Wales; QLD, Queensland; VIC, Victoria

| Location           | Haplotype |        |       |       |       |      |       | N   |
|--------------------|-----------|--------|-------|-------|-------|------|-------|-----|
|                    | 2/H_10    | 3/H_19 | 5/H_1 | 7/H_1 | 8/H_2 | H_20 | 9/H_5 |     |
| Australia          |           |        |       |       |       |      |       |     |
| Newport, QLD       |           | 1      |       |       | 1     | 4    | 4     | 10  |
| Scarborough, QLD   | 1         |        |       |       | 6     |      | 3     | 10  |
| Manly, QLD         | 2         |        |       |       |       |      | 12    | 14  |
| Clontarf, NSW      | 1         | 1      | 1     | 3     | 1     |      | 3     | 10  |
| Cronulla, NSW      |           | 1      |       | 3     |       |      | 6     | 10  |
| Burraneer Bay, NSW |           |        |       | 2     |       |      | 8     | 10  |
| Williamstown, VIC  | 2         |        | 1     | 3     | 2     |      | 2     | 10  |
| St Kilda, VIC      |           |        |       | 3     |       |      | 7     | 10  |
| Yaringa, VIC       |           |        |       | 4     |       |      | 6     | 10  |
| New Zealand        |           |        |       |       |       |      |       |     |
| Auckland           |           |        |       |       |       |      | 9     | 9   |
| Whangarei          |           |        |       |       | 1     |      | 7     | 8   |
| Total              | 6         | 3      | 2     | 18    | 11    | 4    | 67    | 111 |

1989) and, similarly, used nucleotide differences to estimate population structure (AMOVA – see below). Also using full nucleotide sequences, we tested for deviations from neutral, equilibrium conditions using Tajima's *D* test (Tajima 1989), which compares  $\pi$  against the number of segregating sites, and Fu's  $F_s$ -statistic (Fu 1997), which compares the number of haplotypes against the number of segregating sites. Because the process of NIS introductions may include both bottlenecks and admixture of historically isolated genetic variants (e.g. from multiple geographic sources), we do not expect genetic diversity in non-indigenous populations to conform to patterns anticipated under migration-drift equilibria (Dupont *et al.* 2009). Tests of selection and measures of  $\pi$  were obtained using DNAsp v5.00.07 (Rozas *et al.* 2003).

Our second approach to examining genetic diversity treated each haplotype as an allele. In the NIS context, we expect native populations to contain the majority of standing genetic variation (that is, the greatest array of mtDNA haplotypes in this case) and non-indigenous populations to contain a subset of those genetic variants (haplotypes), perhaps with contributions from multiple sources. Under this scenario, a consideration of diversity at the nucleotide level may be misleading. Consider a hypothetical example, whereby the native (ancestral) population contained 10 haplotypes and two descendent populations were formed from this ancestral stock, but through the bottleneck of introduction, only two haplotypes were introduced into each descendent population. In the first descendent population, the two haplotypes differed at several base pairs, whereas in the second descendent population, the two haplotypes differed at a single base pair. A sequence-based estimator of genetic diversity (such as  $\pi$ ) would indicate greater diversity in the first than in the second population. In the context of non-indigenous introductions, however, the key aspect is that both daughter populations represent an equal subset of the diversity present in the ancestral population. Building on this logic, we coded each haplotype as a distinct allele and estimated the effective number of haplotypes

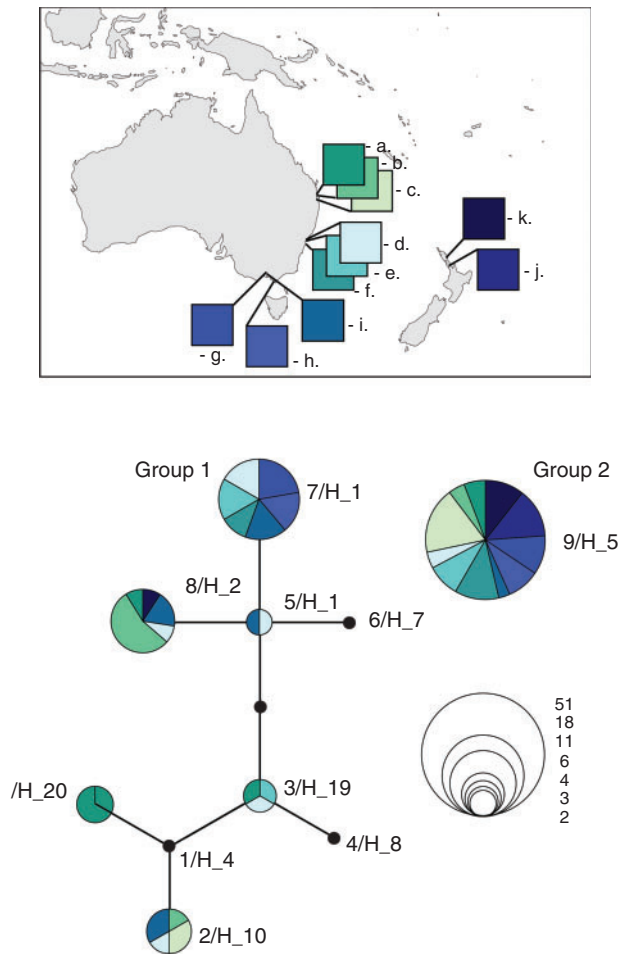
per population (a metric that takes into account the sampling size of each population) and the haplotype diversity per site (Nei 1987) by using GenoDive version 2.0b19 (Meirmans and Van Tienderen 2004).

We used both sequence-based and haplotype-based approaches to estimate population structure. Pairwise  $F_{ST}$ -values were estimated among all population pairs, using Meirmans' formulation of  $F_{ST}$  (Meirmans 2006), which is not biased by the relative amount of variation within each population. Among Australian populations, we tested for hierarchical population structure in the AMOVA framework (Excoffier *et al.* 1992), whereby populations were grouped by regions (corresponding to the states of Queensland, New South Wales and Victoria) in the same manner as David *et al.* (2010).  $F_{ST}$  and AMOVA calculations were conducted in GenoDive, with 4999 and 999 permutations, respectively. We also tested for isolation by distance among Australian populations, by using Mantel tests of geographic distance (km) and pairwise  $F_{ST}$ -values (both based on sequences and haplotypes; 999 permutations) in GenAlEx version 6.4 (Peakall and Smouse 2006).

## Results

### Genealogical analyses

Our newly obtained *S. plicata* sequences formed a well supported monophyletic group, sister to *S. clava* (all sequences are deposited in GenBank: Accession nos JX885713–JX885722). The monophyly of *S. plicata* (that is, the haplotypes described by Barros *et al.* (2009), Pineda *et al.* (2011) and the present study) was well supported both in Bayesian (100% posterior probability) and parsimony (100% consistency out of 13 equally parsimonious trees – not shown) searches. Our new sequences recovered the same haplotypes as identified by Barros *et al.* (2009) and Pineda *et al.* (2011). For convenience, we follow their notation (see Table 1, Fig. 1). One haplotype from Newport that was not reported by Barros *et al.* (2009) corresponded with



**Fig. 1.** Sampling locations and haplotype networks of Groups 1 and 2. Sampling locations are represented by letters (corresponding to Table 2) and shaded. Shading within the haplotype networks denotes where the haplotypes are found. The area of each haplotype is proportional to its observed frequency (see key, bottom right); unobserved haplotypes are filled black. Numbers adjacent to each haplotype indicate identity used in text, with the first number from Barros *et al.* (2009) and the second from Pineda *et al.* (2011).

H<sub>20</sub> from Pineda *et al.* (2011), being reported as a singleton from California. Within the *S. plicata* clade, there was reciprocal monophyly between Group 1 (Haplotypes 1–8, H<sub>20</sub>) and Group 2 (Haplotype 9). These two major clades were 2.5% divergent (14 changes from a length of 561 nucleotides along the connecting branch) and did not connect in the haplotype networks, even with a 90% connection threshold in TCS (Fig. 1). Australian populations comprised individuals with both Group 1- and Group 2-type haplotypes, whereas all but one New Zealand individual had Haplotype 9 (= Group 2).

#### Genetic diversity and population structure

All measures of diversity were quite variable among locations (Table 2). The statistics of Tajima's *D* and Fu's *F<sub>s</sub>* reflect the balance between the number of polymorphic (segregating) sites and  $\pi$  or the number of haplotypes, respectively. For both statistics, there was a preponderance of positive values, many of

them significant, being indicative of an excess of polymorphic sites such as resulting from admixture of historically differentiated lineages. These positive deviations from equilibrium expectations were extreme for some Australian sites (see Table 2), whereas the Tajima's *D* value for Whangarei, New Zealand, was negative, potentially reflecting an extreme bottleneck or selective sweep (Tajima's *D* could not be calculated for Auckland). The two New Zealand locations consistently had lower levels of genetic diversity than did the Australian locations. Within Australia, AMOVA and pairwise *F<sub>ST</sub>*-values revealed significant partitioning of variation by population and no structure between regions (for nucleotide diversity:  $\Phi_{CT} = -0.061$ ,  $P = 0.734$ ;  $\Phi_{SC} = 0.168$ ,  $P = 0.006$  and for haplotype diversity:  $\Phi_{CT} = -0.002$ ,  $P = 0.422$ ;  $\Phi_{SC} = 0.144$ ,  $P = 0.005$ ). There was also no evidence for isolation by distance among Australian populations, using either nucleotide-derived ( $r = -0.088$ ,  $P = 0.314$ ) or haplotype-derived ( $r = 0.157$ ,  $P = 0.249$ ) *F<sub>ST</sub>* metrics (see Fig. 2).

#### Discussion

In the present study, we expanded global investigations of *S. plicata* to include mtDNA sequences from nine Australian locations and two New Zealand locations. Within the eastern coast of Australia, we observed genetically distinct populations, with no distance-related patterns of genetic differentiation, being similar to patterns observed in *S. plicata* worldwide. In New Zealand, both populations had little genetic diversity, being suggestive of either a strong bottleneck or a post-introduction selective sweep.

Among our samples (like those of Barros *et al.* 2009 and Pineda *et al.* 2011), there was widespread sympatry between a set of mitochondrial sister clades (except for the Auckland population). Although these results might indicate two cryptic species, the lack of within-population differentiation for nuclear markers for Australian *S. plicata* (David *et al.* 2010), which includes high frequencies of both clades and individuals shared with our current study (Table 1; Fig. 1), does not support this conclusion. Similarly, Pineda *et al.* (2011) found that nuclear *ANT* loci did not form linkage associations with the two mtDNA groups, among global samples. Divergent haplotypes within populations, such as those observed for many *S. plicata* populations, are indicative of admixture from diverse source populations and consistent with multiple invasions originating from differentiated native sources (e.g. Roman 2006; Geller *et al.* 2010; Liggins *et al.* 2013). In the absence of mating experiments, however, conclusions regarding reproductive isolation, or lack thereof, remain speculative. For the remainder of the discussion, we assume that our populations comprise one interbreeding species.

Evidence for admixture is also reflected in the estimates of genetic diversity for Australian locations (Table 2; Tajima's *D* and Fu's *F<sub>s</sub>* statistics), because of an excess of nucleotide diversity and a deficit of unique haplotypes conditioned on the number of polymorphic sites. In natural populations, mtDNA loci tend to yield negative values for both statistics (Rand 2001; Wares 2010) perhaps as a result of selective pressures on mtDNA. Thus, our Australian samples showed highly atypical patterns of mtDNA diversity, relative to expectations for native



**Table 2. Genetic diversity of *Styela plicata* by location**

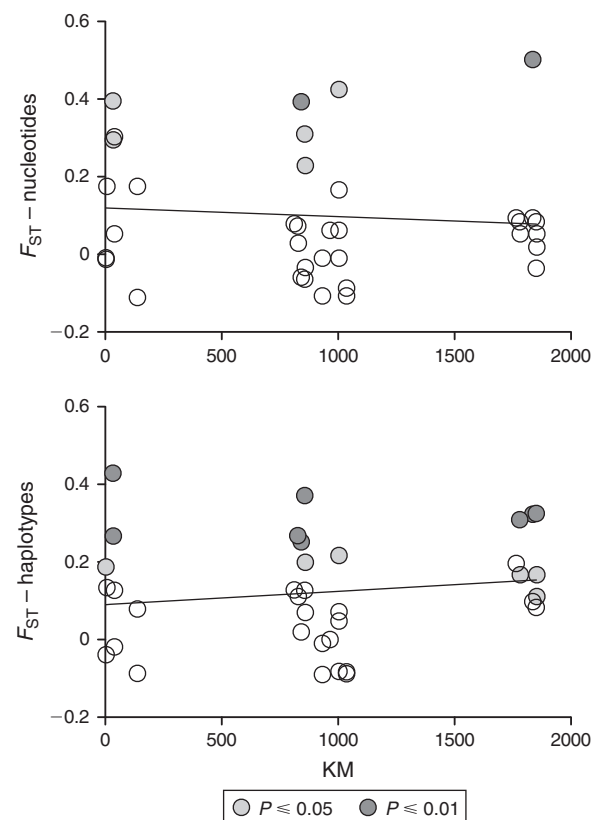
Underlined letters denote locations represented in Fig. 1. Gene diversity (by haplotype) follows Nei (1987). Gene diversity ( $\pi$ , by site, %) follows Tajima (1989). \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ ; \*\*\*values remain significant following Bonferroni correction. n.a., too few sequences to perform test. NSW, New South Wales; QLD, Queensland; VIC, Victoria

| Region      | Location               | No. of haplotypes | Effective haplotypes | Haplotype diversity | Gene diversity (by haplotype) | Gene diversity ( $\pi$ , by site, %) | Tajima's $D$ | Fu's $F_s$ |
|-------------|------------------------|-------------------|----------------------|---------------------|-------------------------------|--------------------------------------|--------------|------------|
| QLD         | <u>a.</u> Newport      | 4                 | 2.94                 | 0.2                 | 0.73                          | 2.01                                 | 2.44**       | 6.09*      |
|             | <u>b.</u> Scarborough  | 3                 | 2.17                 | 0.4                 | 0.6                           | 1.51                                 | 0.66         | 7.21**     |
|             | <u>c.</u> Manly        | 2                 | 1.32                 | 0.21                | 0.26                          | 0.99                                 | -0.68        | 9.69***    |
|             | Total                  | 5                 | 2.65                 | 0.64                | 0.64                          | 1.78                                 |              |            |
| NSW         | <u>d.</u> Clontarf     | 6                 | 4.55                 | 0.2                 | 0.87                          | 1.71                                 | 1.1          | 2.02       |
|             | <u>e.</u> Cronulla     | 3                 | 2.17                 | 0.6                 | 0.6                           | 1.77                                 | 1.9          | 8.02**     |
|             | <u>f.</u> Burraner Bay | 2                 | 1.47                 | 0.3                 | 0.36                          | 1.14                                 | 0.03         | 8.86**     |
|             | Total                  | 6                 | 2.5                  | 0.67                | 0.62                          | 1.7                                  |              |            |
| VIC         | <u>g.</u> Williamstown | 5                 | 4.55                 | 0.2                 | 0.87                          | 1.44                                 | 0.2          | 2.96       |
|             | <u>h.</u> St Kilda     | 2                 | 1.72                 | 0.5                 | 0.47                          | 1.71                                 | 2.38**       | 11.08***   |
|             | <u>i.</u> Yaringa      | 2                 | 1.92                 | 0.2                 | 0.53                          | 1.71                                 | 2.38**       | 11.08***   |
|             | Total                  | 5                 | 2.7                  | 0.66                | 0.65                          | 1.74                                 |              |            |
| New Zealand | <u>j.</u> Auckland     | 1                 | 1                    | 0.22                | 0                             | 0                                    | n.a.         | n.a.       |
|             | <u>k.</u> Whangarei    | 2                 | 1.28                 | 0.13                | 0.25                          | 0.71                                 | -1.81*       | 5.80*      |
|             | Total                  | 2                 | 1.13                 | 0.12                | 0.12                          | 0.34                                 |              |            |

populations. However, this pattern is what is expected under a scenario of admixture from multiple differentiated source populations.

Within Australia, we did not find evidence for regional structuring of mtDNA or evidence for isolation by distance (Fig. 2). Instead, we found that geographically proximate populations were just as likely to be statistically differentiated for mitochondrial COI as were more distant populations. These results are quite different from previous results on an overlapping set of individuals using nuclear markers (David *et al.* 2010), where neighbouring populations were more alike than were geographically distant populations. This contrast between markers is puzzling. It could be that population bottlenecks within marinas occur frequently and that mtDNA haplotype frequencies shift because of the bottleneck (more so than nuclear markers with larger effective population sizes); such a recently bottlenecked population may have significant  $F_{ST}$  values with many other populations, both nearby and distant (as seen in Fig. 2). However, it is also conceivable that our limited sampling strategy for mtDNA (<15 individuals per population) had less power to accurately detect differences in allelic frequencies than did the more extensive sampling for nuclear loci ( $\geq 39$ ).

Despite our small sample sizes, our study recovered much of the COI diversity described in previous studies of *S. plicata* (Barros *et al.* 2009; Pineda *et al.* 2011). For example, our *de novo* dataset included all seven of the haplotypes found to be shared across study locations in the global phylogeography of Pineda *et al.* (2011). Further, our dataset included three haplotypes previously identified as being private to a single location in their genetic survey (Pineda *et al.* 2011). Moreover, the genetic patterns we described (genetic differentiation independent of geographic distance) were corroborated by studies of *S. plicata* over greater geographical scales (Barros *et al.* 2009; Pineda *et al.* 2011) and using larger sample sizes (Pineda *et al.*



**Fig. 2.** The relationship between geographic distance (kilometres, KM) and genetic differentiation within Australia (Mantel test, after 999 permutations: nucleotide  $r = -0.088$ ,  $P = 0.314$ ; haplotype  $r = 0.157$ ,  $P = 0.249$ ). Individually significant pairwise values are filled with grey shading (4999 permutations, following Bonferroni correction); unshaded values are not statistically significant.

2011) For these reasons, it is unlikely that incongruence between our mtDNA findings and those of David *et al.* (2010) using nuclear markers is due to differences in sample size and statistical power. Different results between markers and studies are not unprecedented for NIS. For example, among European populations of introduced mitten crabs, isolation by distance was detected using microsatellite markers (Herborg *et al.* 2007), whereas mtDNA-based studies showed both population structure (Wang *et al.* 2009) and no population structure (Hänfling *et al.* 2002) in the same geographic region.

The extreme lack of diversity in the New Zealand may be indicative of a strong bottleneck, such as a single introduction of individuals, or very few introductions from divergent populations (in the case of Whangarei). Considering the level of global admixture and low haplotype diversity in New Zealand, a single introduction from a population containing both Haplotypes 8 and 9 (perhaps Australian populations in Clontarf, Williamstown, Newport or Scarborough) seems possible. The nearly complete fixation of Haplotype 9 is notable, because most previously surveyed locations from around the world have found Haplotype 9 in moderate frequencies and always in sympatry with Group 1 haplotypes (Barros *et al.* 2009; Pineda *et al.* 2011; although Pineda *et al.* (2011) also reported near fixation of Haplotype 9/H\_5 in Manly, Australia, and Port Elizabeth, South Africa, and high frequency in Misaki, Japan). Neither previous study included New Zealand populations, although we caution that with only two populations of eight and nine individuals sampled, respectively, this may overlook a significant portion of standing genetic diversity (as with much of our restricted sample set).

We also found high frequencies of Haplotype 9 in Manly and in the rest of Australia, this being the dominant haplotype in most populations (see Table 1). A similar pattern was found for the invasive ascidian *Didemnum vexillum*, where the native population was found to contain three separate COI clades, whereas only one clade was found outside the native range, potentially indicating variation in characteristics associated with invasion success, such as tolerance to extreme environmental conditions (e.g. temperature and/or salinity, Stefaniak *et al.* 2012). Alternatively, such haplotypic dominance could be a result of stochastic, repeated bottlenecks, as is common in sequential colonisations (natural and human-mediated). It will be interesting to see whether the extreme haplotype compositions of these locations (e.g. Auckland and Whangarei in New Zealand; Manly in Australia) persist over multiple generations or are eroded by new introductions of propagules carrying different mitotypes.

Our study has highlighted some of the complexities in using molecular markers to make inferences about the origins and present-day dynamics of NIS. As with global surveys, we found deeply divergent lineages that were broadly sympatric, and most *S. plicata* populations contained overlapping sets of haplotypes not conforming to neutral equilibrium expectations (Pineda *et al.* 2011). Thus, patterns within the Australian coastline mirrored patterns at the global scale. Although other genetic studies of contemporary ascidian invasions have found discernible patterns of both stepping-stone dispersal or multiple introductions (e.g. contrasting patterns for *Botrylloides schlosseri* on the eastern and western coastlines of the USA), it appears that

the genetic signature of *S. plicata*'s invasion history has been obscured by recurrent introductions from multiple sources that may themselves be admixed populations typical of a NIS (Dupont *et al.* 2009; Lejeune *et al.* 2011; Stefaniak *et al.* 2012). Long-distance movements appear to be sufficiently frequent so that haplotypes have broad geographic distributions, yet drift is sufficiently strong within populations to inflate population subdivision. Because geographically proximate populations are just as likely to be genetically differentiated as are distant populations (for mtDNA), we cannot exclude distant populations as being the sources of establishing colonists. Therefore, invasion scenarios of a single introduction followed by spread are unlikely for *S. plicata* in Australia and probably for several other cosmopolitan ascidians (Rius *et al.* 2008; Dupont *et al.* 2010; Goldstien *et al.* 2011). Greater sampling efforts of *S. plicata* and continued monitoring of New Zealand populations in particular may provide a valuable insight into the population dynamics of the marine invasion process.

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