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Research Article

Multiple introductions and regional spread shape the distribution of the cryptic ascidian *Didemnum perlucidum* in Australia: an important baseline for management under climate change

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Abstract

Anthropogenic agents of ocean change such as biological invasions, overfishing, habitat destruction, pollution and ocean acidification and warming are known to have a dramatic impact on marine ecosystems worldwide. They are also intrinsically connected. In Western Australia, a "hot-plate" settlement panel system aimed at investigating the effect of ocean warming on fouling communities led to the first report of a notorious worldwide invasive ascidian species *Didemnum perlucidum*. This species was subsequently recorded from numerous locations along the coast and included in the Western Australia Prevention List for Introduced Marine Pests. In the present study we used microsatellite markers to determine whether these populations are the result of single or multiple introductions to Australia and if anthropogenic vectors might have facilitated its spread. By including samples collected worldwide, we further aimed to evaluate broad-scale patterns of variation and ascertain whether regional differences could be used to determine the source of introductions of *D. perlucidum* to Australia. Our results report an extended geographic range for *D. perlucidum* in Australia and worldwide, in what is the most comprehensive genetic study of this species. Our data further supports the introduced status of *D. perlucidum* in Australia, an introduction that our results suggest having occurred most likely as a result of multiple events with subsequent admixture. The similarity between genotypes at locations in Western Australia suggests that domestic transport plays a crucial role in shaping these populations, most likely enhanced by climatic anomalies such as heat waves. The identification of the *D. perlucidum* native range and sourcing of international samples from potential sites of introduction to Australia, particularly south-east Asia, remains essential to understand the presence of this species in the country. Nevertheless, during this study we were able to increase our understanding of *D. perlucidum* populations in Western Australia. This work provides an important baseline for *D. perlucidum* management and protection of high value marine areas, in Australia and worldwide.

Key words: microsatellite markers, Tunicata, population genetics, invasive marine species, marine biosecurity, heat waves, high value marine areas

Introduction

Marine biological invasions have registered a dramatic increase in the last decades. This has led to its significance being well recognized alongside other anthropogenic impacts such as overfishing, habitat destruction, pollution and ocean acidification and warming. Such agents of ocean change are also intrinsically connected, the risk of invasions being directly related to trade, human developments and environmental changes at both global and local scales (Bax et al. 2003; Molnar et al. 2008; Sorte et al. 2010; Carlton 2011; Seebens et al. 2017). Only a small percentage of marine introduced species become invasive and they will likely fall within previously listed marine invasive species around the world (Williamson and Fitter 1996; Lockwood et al. 2013; Dias et al. 2017). They however often belong to highly cryptic and understudied groups that can establish and remain undetected and/or non-invasive for long periods until different factors or conditions come into play. The way species and communities that harbor invasive species might shift is particularly worrying under the current climate change scenario and has therefore been a growing subject of study (Canning-Clode et al. 2011; Côté and Green 2012; Mellin et al. 2016; Atkinson et al. 2020; Coleman et al. 2020; Jacox et al. 2020).

Ascidians comprise a highly diverse group of solitary and colonial species and are among the most commonly introduced species groups worldwide (Lambert 2002; Shenkar and Swalla 2011; Pagad et al. 2015). The colonial ascidian *Didemnum perlucidum* (Monniot, 1983) has been widely recorded as an introduced or cryptogenic species at tropical and temperate locations associated with anthropogenically impacted sites and artificial structures such as harbours and aquaculture operations (Godwin and Lambert 2000; Kremer et al. 2011; Dias et al. 2016a). This species was first detected in Western Australia (WA) during the autumn of 2010 from a "hot-plate" settlement panel system aimed at investigating the effect of ocean warming on the recruitment, growth and interactions of fouling communities in their natural environment in the Swan River, Perth (Smale et al. 2011; Smale and Childs 2012). The notably increased coverage of *D. perlucidum* on the heated plates (compared to controls) in the Swan River in April 2010 and detection on settlement panels in February 2011 at Hillarys Marina raised concerns on the potential introduced status, distribution and ecological and economic impacts of this species in WA (Smale and Childs 2012).

Such concerns were justified between 2012 and 2015 as *D. perlucidum* was recorded from numerous locations along the WA coast, from Esperance in the south to Cygnet Bay in the north, and the Northern Territory (NT). Most records were obtained from artificial substrates in harbours and marinas, monitored as part of the WA Government Department of Fisheries (now Department of Primary Industries and Regional Development – DPIRD) introduced marine pest monitoring program, and

fouling of shellfish aquaculture farms in WA and the NT (Bridgwood et al. 2014; Dias et al. 2016a). Although *D. perlucidum* was reported overgrowing seagrass meadows in the Swan River, it was not present in any other natural habitats (such as reefs) that were regularly observed at sites close to *D. perlucidum* infected areas such as Hillarys marina (Simpson et al. 2016a, b; Dias et al. 2016a). Given the demonstrated invasive characteristics (Smale and Childs 2012; Bridgwood et al. 2014; Muñoz et al. 2015) and absence of previous records in Australia (Kott 2001, 2005; McDonald et al. 2005; Shenkar and Swalla 2011), *D. perlucidum* was rapidly flagged as an introduced marine pest in WA (Bridgwood et al. 2014). Two possible explanations were put forward by Bridgwood et al. (2014) to explain the detection and expansion of *D. perlucidum* in WA. The first was that the species was long introduced and already established but remained undetected due to low abundance or simply, due to the high biodiversity of the group, been taxonomically overlooked. The second possibility is that the species has been recently introduced into WA by either multiple inoculation events or a single event with rapid secondary dispersal. In this latter case, due to the short life span of didemnid larvae, the authors suggested that anthropogenically assisted dispersal was likely given the high number of disjunct locations at which *D. perlucidum* has been detected in WA.

Didemnum perlucidum is currently widely established in WA and although it is highly unlikely that the species could be eradicated, implementing control measures aimed at excluding these and other introduced species from high-value environmental areas (e.g. marine parks) has been deemed a priority. Such measures could include developing and instigating vessel management protocols to prevent translocation of *D. perlucidum* from known infected locations, both within Australia and from countries where the species is known to be prevalent (Bridgwood et al. 2014; Dias et al. 2016a). The use of genetic information has previously proven to be of great value in resolving the status, origin, distribution and dispersal of species (Holland 2000; Cristescu 2015). Resolution of this question is generally based on the principle that genetic diversity of a species is often relatively high in its native range due to an accumulation of mutations within natural populations over a long timeframe. When species are first introduced into a new location, only a small subset of that genetic diversity is initially transported and becomes established, which creates a founder effect (Dlugosch and Parker 2008) that is reflected in a reduced genetic diversity within the new expanding population. This is particularly true for recently introduced populations, as subsequent long-term multiple introduction events from different sources are known to increase genetic diversity and "blur the genetic signal" that would allow tracing of the origin of introduced populations (Sakai et al. 2001; Dlugosch and Parker 2008). In a comprehensive global genetic study of *D. perlucidum* to date, Dias et al. (2016a) reported on a striking lack of cytochrome *c* oxidase

subunit I gene (COI) diversity worldwide, the one haplotype detected in Australia being the predominant haplotype worldwide. This finding supported previous suggestions that *D. perlucidum* was recently introduced to Australia.

Fast evolving mitochondrial markers such as the COI are particularly useful in species identification and population genetics of metazoan species over wider spatial scales (Estoup and Guillemaud 2010). While COI can be more sensitive to genetic drift (random fluctuations in allele frequency leading to the fixation and loss of founder alleles), abundant and hypervariable nuclear microsatellite markers tend to give higher population structure resolution over finer spatial scales (Holland 2000; Darling et al. 2008). In the present study, we build on the study by Dias et al. (2016a) and, using purposely developed microsatellite markers (Dias et al. 2016b), set to investigate the invasion history of populations of *D. perlucidum* reported throughout WA and the NT in recent years. In particular, we aimed to determine whether these populations are the result of single or multiple introductions and if anthropogenic vectors might have facilitated its spread. By including samples collected worldwide, we further aimed to evaluate broad-scale patterns of variation and ascertain whether regional differences could be used to determine the source of introductions of *D. perlucidum* to Australia.

Materials and methods

Sampling, DNA extraction and D. perlucidum identification

The present study includes DNA samples of *D. perlucidum* used in the study by Dias et al. (2016a). These samples were from Australia, Hawaii (USA), Brazil and the Gulf of Mexico (Mexico), with each location represented by a minimum of 12 individuals. In the present study, a single sample collected from the Northwestern Hawaiian Islands is included for the first time. Additional tissue samples are also included from WA and the NT (total of 19 locations), collected between 2012 and 2015 as part of monitoring and research surveys conducted by the Aquatic Biosecurity team at DPIRD (see map Figure 1 and Table S1 for list of samples and details). Tissue samples were collected as per Dias et al. (2016a) from individual (discontinuous) colonies of white colonial ascidians suspected of being *D. perlucidum*, by hand while snorkelling or scuba diving, or from easily accessible artificial structures such as buoys, ropes and docks. All tissue samples were preserved in 70–100% ethanol and transported to the Western Australian Fisheries and Marine Research Laboratories (DPIRD) for processing.

DNA was extracted from a ~ 5 mg sample of ascidian tissue (with tunic) using a Fisher Biotec Favorgen FavorPrep Tissue Genomic DNA Extraction Mini Kit (Fisher Biotec, Australia, Wembley, WA), following the manufacturer's instructions. Samples were screened using the real-time PCR

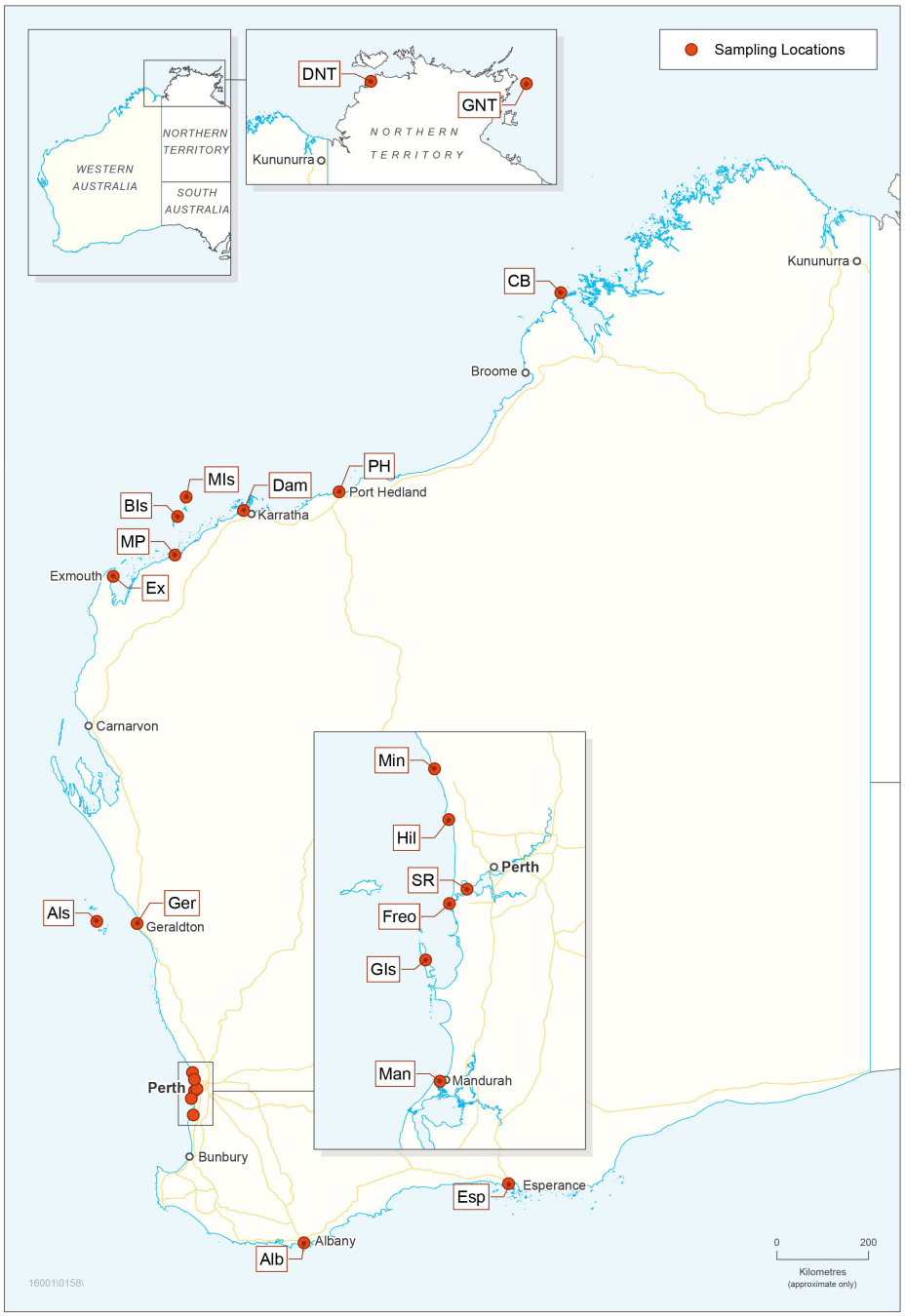

Figure 1. Sampling locations for *Didemnum perlucidum* in Australia (Western Australia and Northern Territory). Abbreviations are as per Table S1.

assay developed by Simpson et al. (2016a) for rapid identification of *D. perlucidum*. This species-specific assay allowed for molecular verification of hundreds of *D. perlucidum* samples during DNA amplification, saving time and costs associated with downstream processing and sequencing. Confirmed *D. perlucidum* DNA extracts were then quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific), diluted to ~ 10 ng/µL and stored at −20 °C until required. The taxonomic identification of negative (non-*D. perlucidum*) samples was determined by sequencing the mtDNA COI gene region as per Dias et al. (2016a).

Microsatellite genotyping and analysis

Genotypes were determined at 14 microsatellite loci (GenBank KT694049–KT694054, KT694056, KT694057, KT694059, KT694060, KT694062–KT694065) for each sample using primers and polymerase chain reaction (PCR) conditions described by Dias et al. (2016b). PCR products (2.5 µL) were analysed on an ABI 3730 Genetic Analyser with GeneScan 500 (LIZ) internal size standard. Genotypes were scored using GENEMARKER (SoftGenetics) software. For each microsatellite marker locus at each sampling location with more than 12 samples, we tested for the presence of null alleles (failure to amplify expected alleles), large allele dropouts and scoring errors, using the MICROCHECKER software package (van Oosterhout et al. 2004). Linkage disequilibrium between microsatellite loci was assessed by testing the significance of association between genotypes using the online version of GENEPOP 4.2 (Raymond and Rousset 1995; Rousset 2008). A sequential Bonferroni correction was applied to the tests for linkage disequilibrium (Rice 1989). We also used the web version of GENEPOP 4.2 to test for departure from Hardy-Weinberg Equilibrium (HWE) in each population and locus (1000 dememorization steps, 100 batches and 1000 iterations per batch).

Genetic diversity at each locus and sampling location was quantified by calculating allelic richness (A_R, a measure of the number of alleles independent of sample size) and gene diversity (H, expected heterozygosity). Analyses for a deficit or excess of heterozygotes (deviations from random mating) within each site were conducted using randomization tests, with results characterized by the inbreeding coefficient (F_{IS}) statistic. Significantly positive F_{IS} values indicate a deficit of heterozygotes relative to a random mating model, while negative results indicate an excess of heterozygotes. Estimates of genetic variation and F_{IS} were calculated using FSTAT v.2.9.3 (Goudet 2001). Differences in genetic diversity (A_R, H) and F_{IS} among locations were tested using Friedman's ANOVA in R (R Core Team 2019).

We also tested for recent reductions in effective population size by testing for excess in expected heterozygosity (Wilcoxon signed rank test) and by assessing allele frequency distributions with the software package BOTTLENECK (Piry et al. 1999). Following a bottleneck expected heterozygosity should be higher than the equilibrium heterozygosity predicted in a stable population (because the number of alleles decreases faster than expected after a bottleneck, Maruyama and Fuerst 1985) and the allele frequency distributions should be shifted away from a typical L-shaped distribution (because rare alleles are lost after a bottleneck, Luikart et al. 1998). Simulations in POWSIM v 4.0 (Ryman and Palm, 2006) were used to evaluate the power of the microsatellite data to detect genetic differentiation at five levels of divergence. 1 000 simulations each were run with a Ne of 10 000 and various generations of genetic drift (t) to yield FST

values of 0.001, 0.0025, 0.005, and 0.01, with default program values for the iteration/permutation factors required for the Fisher exact test of significance (Ryman and Palm, 2006).

Population structure was assessed by calculating the Weir and Cockerham (1984) estimator of F_{ST} and Nei's (1987) genetic distance using FSTATv.2.9.3 (Goudet 2001). We also carried out an individual-based Bayesian clustering method for assessing population structure in STRUCTURE v.2.3.4 (Pritchard et al. 2000; Falush et al. 2003). This method identifies genetically distinct clusters (K) based on allele frequencies across loci. All analyses were based on an ancestry model that assumed admixture and correlated allele frequencies, with the locations of samples incorporated as prior information for the model. Ten independent runs were performed for each value of K (1–20) with a burn-in of 10 000 followed by 100 000 Markov Chain Monte Carlo (MCMC) iterations. STRUCTURE HARVESTER (http://taylor0.biology.ucla.edu/struct_harvest) was used to determine the most likely number of clusters by comparing the likelihood of the data (Ln K) for different values of K [ln P(X | K)] and by examining the standardized second-order rate change of ln P(X | K), ΔK (Evanno et al. 2005; Janes et al. 2017). Although STRUCTURE is the most widely used Bayesian clustering method, it is recommended that it should be used as an *ad hoc* guide. The accuracy of STRUCTURE assignments is known to depend on several factors, including the number of samples, sample size and admixture (Pritchard et al. 2000; Evanno et al. 2005). Uneven sampling has been particularly pointed to lead to wrong inferences on hierarchical structure and downward-biased estimates of the true number of subpopulations (Puechmaille 2016). To avoid wrong inferences due to sampling unevenness, we subsampled 12 individuals (number corresponding to the smallest sample) from each population and ran STRUCTURE analysis on both the full and shorter dataset.

STRUCTURE is recognised to perform well in identifying groups of individuals corresponding to the uppermost hierarchical level. However, in order to find potentially hidden within-group structure, subsequent analyses of subsets created by the best individual assignment provided by the program, is recommended (Evanno et al. 2005; Puechmaille 2016; Janes et al. 2017). To investigate potential substructure within the uppermost hierarchical clusters identified, we conducted separated STRUCTURE analysis at different spatial scales, namely on populations from Australia only. Different population structure analysis methods are based on different models and assumptions, and the use of more than one program is often advocated to compare results (Evanno et al. 2005; Latch et al. 2006; Puechmaille 2016). For this reason, we also performed discriminant analysis of principal components (DAPC) using the *adegenet* package in R (Jombart 2008) to explore structure between all samples, and potential additional substructure within populations sampled from Australia only.

Results

Sampling, DNA extraction and D. perlucidum *identification*

In this study, we included the collection of a total of 567 samples of white colonial ascidians (putative *D. perlucidum*) at 23 sites within commercial harbours, recreational marinas and marine protected areas in Australia and abroad (Figure 1, Table S1). From the 567 samples collected, 537 (95%) were identified as *D. perlucidum* (Dias et al. 2016a, this study). DNA barcoding identification at the species level was only possible for three (GenBank accession numbers MN215879–81) of the remaining 30 ascidians sampled at nine of the sites, whose sequence had a high identity (~ 99%) and query cover (100%) to other molecularly identified putative *Lissoclinium fragile* deposited in the NCBI GenBank database, including KJ725150.1 (Abdul et al. 2016) and MN586609.1 (Lopez-Guzman et al. 2020). These samples, however, need taxonomic (morphological identification) verification. The sample collected from the Northwestern Hawaiian Islands was identified as *D. perlucidum* using real-time PCR, with subsequent sequencing revealing it had COI haplotype 1 (Dias et al. 2016a).

Microsatellite genotyping and analysis

Microsatellite genotyping was performed for 536 samples of *D. perlucidum* collected at 19 locations (*D. perlucidum* samples represented by a minimum of 12 individuals). The number of alleles per locus was typically high, but varied between loci, ranging from one to twelve. Although 17 of the 19 populations presented null alleles at one to four microsatellite loci, no loci showed null alleles consistently across all populations. There was no evidence of linkage disequilibrium between pairs of loci across populations, therefore, all the loci were considered to provide independent information and were included in the analysis. We found no significant differences in allelic richness, gene diversity and F_{IS} between sampling locations. All populations were in Hardy-Weinberg equilibrium. Most populations showed evidence of reductions in effective population size with significant heterozygosity excesses (one-tailed tests, $P < 0.00263$) and/or shifted allele distributions. The exception is the sample collected at the Abrolhos Islands ($P = 0.03381$, Table S3).

There was evidence of population genetic structure across all world populations (pairwise F_{ST} ranged 0.048 to 0.225) and between locations within Australia (pairwise F_{ST} ranged −0.005 to 0.207) (Table S2). These results are complemented by STRUCTURE and DAPC analysis, which revealed two overarching distinct genetic clusters (K = 2) formed one, by the samples obtained from populations abroad and the other, by the samples obtained from populations in Australia (Figures 2A, 3A). STRUCTURE analysis on both the full and shorter (12 individuals only) dataset yielded

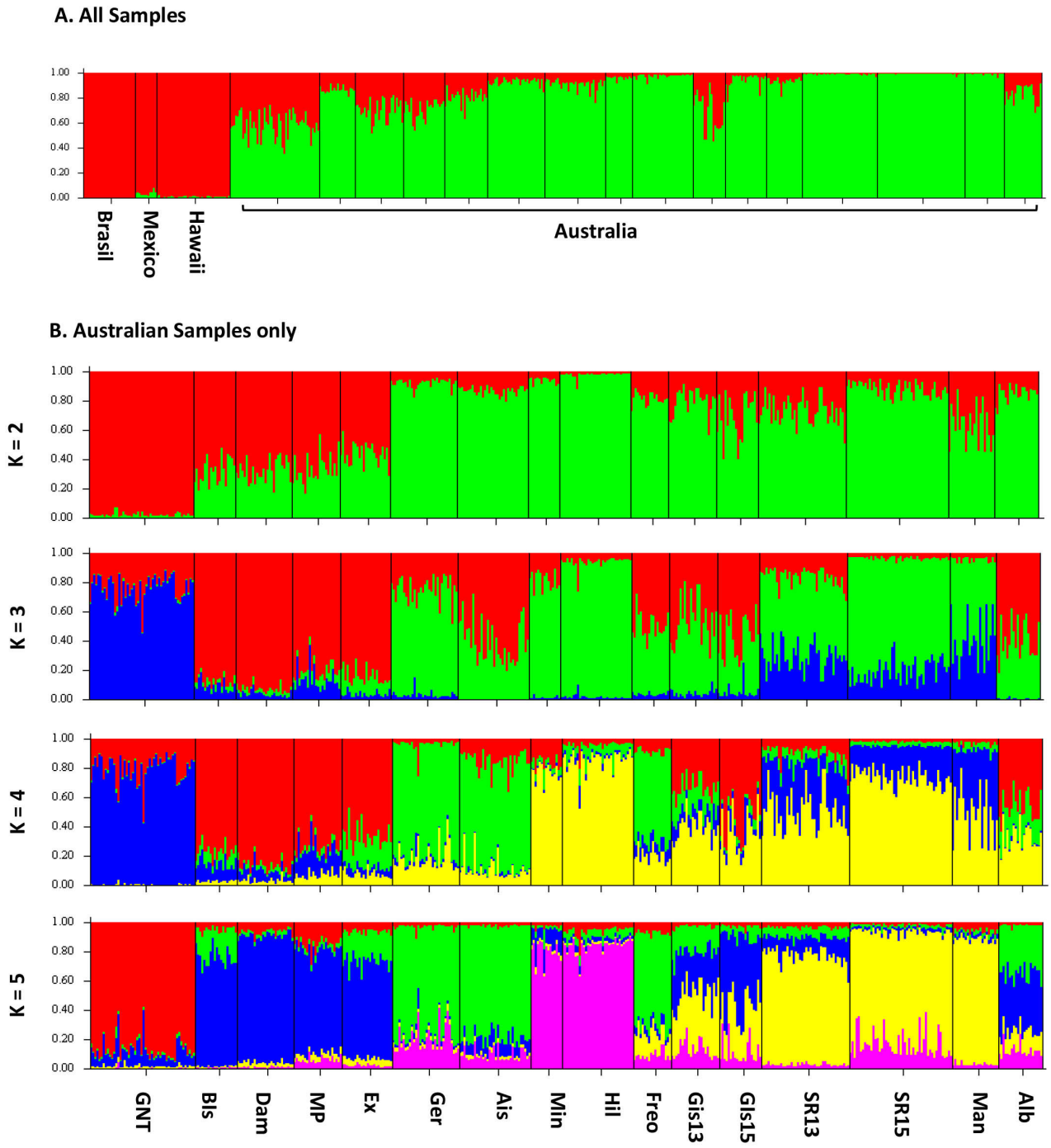

Figure 2. Genetic clustering of *Didemnum perlucidum* samples. Results obtained with the Bayesian clustering of individual genotypes implemented in STRUCTURE are given for (A) all samples K = 2 genetic clusters and (B) Australian samples K = 2 to K = 5. Each vertical line represents an individual. Box width is proportional to sample size. Genetic clusters are represented by different colours. Abbreviations refer to sampling locations as per Table S1.

similar results, so we opted to present the more informative graphical display from STRUCTURE analysis, for the full dataset (Figure 2). STRUCTURE and DAPC analysis of samples from Australia suggests two further genetic clusters (K = 2) within Australia represented by populations in the NT and WA (Figures 2B, 3B).

Previous studies had reported that STRUCTURE performed well in identifying subpopulations even when they were weakly differentiated

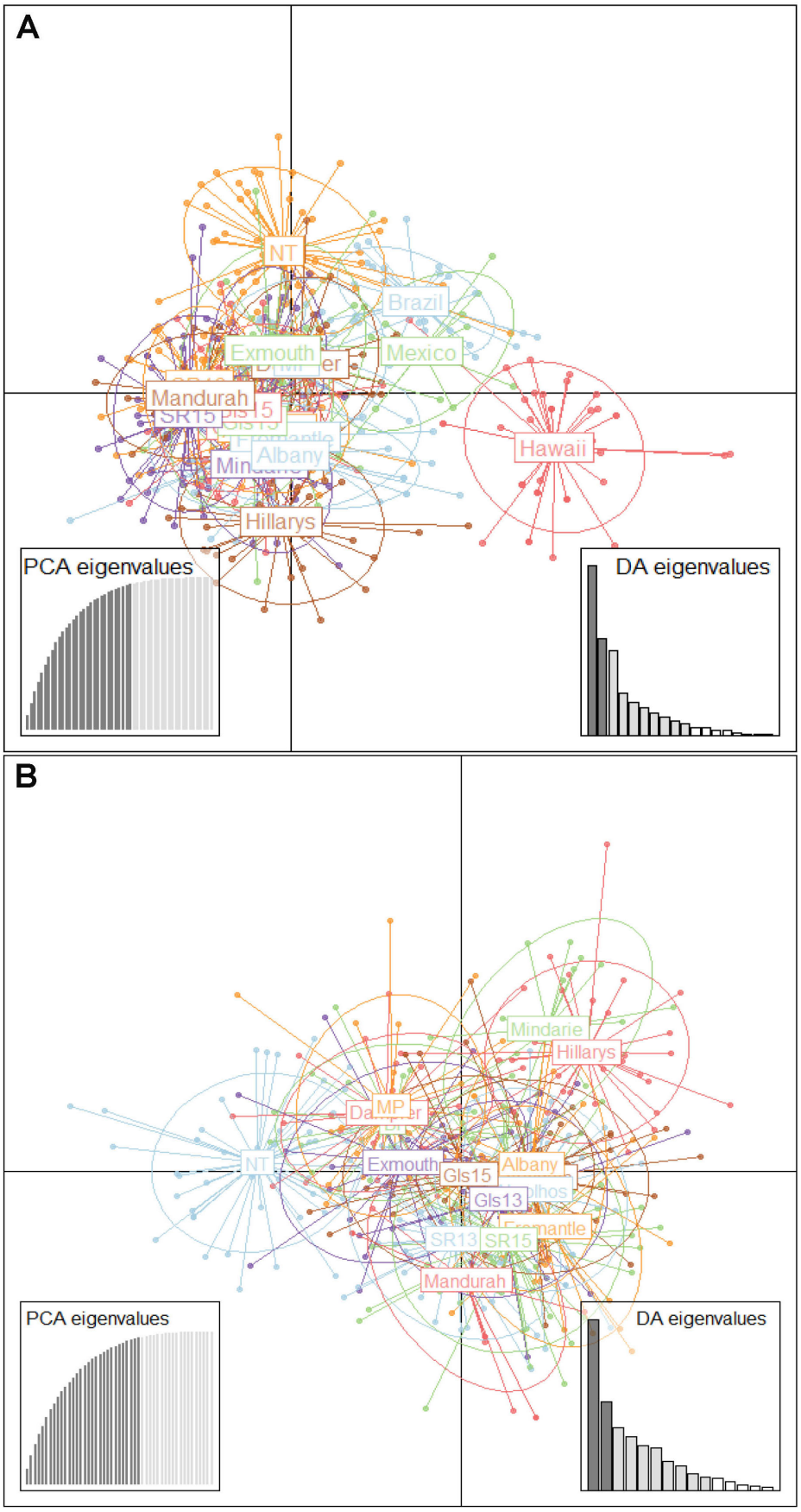

Figure 3. Discriminant analysis of principal components (DAPC) of samples of *Didemnum perlucidum* obtained from: (A) all populations and (B) populations in Australia. Abbreviations refer to sampling locations as per Table S1.

(F_{ST} = 0.002–0.03), which can be observed from close inspection of its graphic output (Latch et al. 2006; Puechmaille 2016). Genetic substructure can indeed be observed from close inspection of the STRUCTURE Australian clusters (Figure 2B). Genetic substructure is particularly evident between the samples collected from a pearl farm in the NT (GNT) and all remaining samples and is supported by F_{ST} values (Table S2). F_{ST} values also support the cluster of samples from populations in Exmouth, Mangrove Passage, Dampier and Barrow Island, which are not significantly different from the samples collected at Garden Island and the Swan River. Shallower genetic substructure can be observed between populations of Geraldton/Abrolhos, Mindarie/Hillarys, Swan River/Mandurah and all others within the west and southwest coast.

Discussion

One or multiple introductions to Australia?

Our results show the extensive presence of *D. perlucidum* in Australia to be the result of multiple introductions with subsequent spread. Population substructure was identified corresponding to two main genetic clusters in Australia, consisting of samples from the NT and WA. This suggests the introduction of *D. perlucidum* to have resulted from at least two separate events. Staff that provided the samples from the pearl farm in Gove, NT, reported observing white colonial ascidians fouling the aquaculture structures for many years. Gove has been recently noted as an area of transit for sailing vessels travelling between Indonesia and the east coast of Australia and New Zealand (Huhn et al. 2020).

Further, significant genetic divergences found between the populations sampled from the north coast of WA and the west/southwest coast of WA suggest these populations could have been the result of at least two further separate introductions with subsequent mixing. The similarity between the samples collected in Exmouth, Mangrove Passage, Dampier and Barrow Island, and the similarity between these four locations and the populations sampled at locations around the Perth metropolitan area such as Garden Island and the Swan River suggests that this could have been a source of introduction to north WA, subsequently spread by local vessel traffic. The higher admixture observed at the main commercial ports sampled in the west/southwest coast, in relation to the recreational marinas, suggests the first could be the main point of introductions from overseas. The genetic clustering of samples from recreational marinas that are geographically closer, such as Mindarie and Hillarys further suggests recreational vessels play a role in the subsequent local spread of *D. perlucidum*.

Bridgwood and McDonald (2014) identified the greatest inoculation risk to the north coast bioregion of WA to be from vessels that travelled within state waters, while the greatest inoculation risk to the WA west and

southwest coast bioregion was from vessels travelling from international last ports of call (LPOCs). This is in line with the observations above. Although the population divergences found within Australia are relatively small (e.g. than between the samples from abroad and Australia), we believe it provides a realistic and useful picture of the genetic and demographic processes affecting *D. perlucidum* introduction, transport and consequent admixture in the region. Janes et al. (2017) advocates the focus on evidence for genetic subdivision, rather than on interpreting genetic clusters as isolated, panmictic "populations". Considering the problems with obtaining an optimal K reported in similar studies, the quality of population substructure evidence is important, as an absence of evidence for subdivision is not necessarily evidence of an absence of subdivision (Janes et al. 2017).

Worldwide picture and potential sources

The relatively low genetic diversity and genetic bottlenecks found at sampling locations support previous suggestions by Dias et al. (2016a) that the populations sampled in Brazil, Mexico, Hawaii and at locations in Australia are all most likely introduced. *Didemnum perlucidum* was first described from the Caribbean island of Guadeloupe (Monniot 1983) and subsequently from other tropical locations across the Atlantic (Goodbody 2000; Rocha et al. 2010) and Indo-Pacific oceans (Monniot and Monniot 1996, 1997; Monniot et al. 1985; Lambert 2002, 2003). Molecular studies of *D. perlucidum* populations at these locations are deemed essential to determine its native range, which is currently considered to be unknown (Dias et al. 2016a; Lambert 2002; Monniot and Monniot 1997).

The main pattern arising from the genetic clustering analysis separated Brazil, Mexico and Hawaii from locations sampled within Australia suggesting none of them were the primary source of introduction of *D. perlucidum* to Australia. A comprehensive inoculation, infection and establishment likelihood analysis, of introduced marine pests (IMP) being translocated by commercial vessels to the main WA ports and bioregions, was produced by Bridgwood and McDonald (2014). Among the factors taken in consideration were the number, type and flag of vessels visiting from a source, the existence of a viable source of IMPs at the LPOC and the environmental compatibility of that species (salinity and temperature tolerance) to WA ports and bioregions. That study identified Singapore, Indonesia, China and Japan as the LPOC posing the higher likelihood of introduction of marine pests to WA ports. Molecular studies of *D. perlucidum* populations from south-east Asia locations are therefore particularly urgent to determine potential sources of high risk and prioritise management actions relating to vessels entering WA.

The similarity found between the samples from Fremantle Harbour and Mexico suggest a potential North American population source, but this

could also be an artefact resulting from the small size and/or admixture of these populations. International commercial vessels often operate in many countries before arriving at a LPOC. Therefore, given the multiple introductions and admixture scenario observed, we cannot rule out that an introduction from North America could have contributed to the *D. perlucidum* population currently present at Fremantle Harbour.

A complex anthropogenic scenario

Extensive morphology-based taxonomic research has been devoted to ascidians in Australia, but such work has historically been conducted on specimens collected from natural areas and typically preserved in formalin solutions that are not conducive to DNA amplification (Kott 2001, 2005; McDonald et al. 2005; Shenkar and Swalla 2011). Surveys involving the identification of fouling communities from vessels and harbours in Australia are more recent and generally conducted for marine biosecurity reasons, targeting a list of species of concern. Although marine biosecurity inspectors are aware of the need to observe and report any ascidians demonstrating invasive behaviour, there were no reports of *D. perlucidum* documented before 2012 when it was flagged as a species of concern and included in the WA Prevention List for Introduced Marine Pests (Department of Fisheries 2016; Dias et al. 2017).

In the present study, the collection of multiple species of "*D. perlucidum* look-a-like" white colonial ascidians from nine of the 23 sites sampled highlights the cryptic diversity of this group at anthropogenically impacted sites such as ports, harbours and marinas. Although DNA barcodes were successfully amplified from all samples, only three of the 30 non-*D. perlucidum* samples sequences had a match in Genbank to species level. Unfortunately, these molecular sequences are not linked to a taxonomically vouchered sequence that can verify the molecular identification. The still underrepresented identification effort of marine invertebrate speciose groups such as ascidians and the need to generate DNA barcodes associated to voucher specimens, that could assist identification, has been highlighted as a priority in research (Shenkar and Swalla 2011; Dias et al. 2017). Indeed, the fact that Dias et al. (2017) generated a voucher and associated DNA barcode for *D. perlucidum* has greatly assisted in the subsequent identification of samples of this species.

In this study we also report the presence of *D. perlucidum* at three new sites, in addition to the 15 sites previously reported (Smale et al. 2011; Smale and Childs 2012; Bridgwood et al. 2014; Dias et al. 2016a). New locations in WA include Mandurah and Mangrove Passage. The DNA barcoding of an ascidian sample collected at the Midway Atoll in the remote Northwestern Hawaiian Islands, and identified as *D. perlucidum*, also represents an important new record for the USA. The fact that the sequence obtained from the sample belongs to the most common *D. perlucidum* COI

haplotype worldwide prevents the identification of the potential source population for this new location. Given the cryptic and taxonomic diversity of ascidians and lack of historical comprehensive baseline studies in harbours, the undetected or overlooked presence of *D. perlucidum* at locations in Australia and throughout the world cannot be discarded.

Irrespective of whether *D. perlucidum* was long present in low abundance or has been recently introduced in Australia, current conditions have allowed colonies to form particularly extensive mats up to 900 cm^2 in area during summer, occupying over 90% of available space (Bridgwood et al. 2014; Muñoz et al. 2015). Western Australian coastal waters experienced a series of short-term warming events and anomalies in 2008, in November 2010–April 2011 during the La Niña marine heatwave and again in 2011–2012, extending the duration of the heatwave event to 24 months. These events were followed by many atypical ecological events being recorded, including the southward migration of tropical marine species and incursions of non-native species (Pearce and Feng 2007, 2013; Pearce et al. 2011; McDonald 2012; Feng et al. 2013; Caputi et al. 2014; Hewitt et al. 2018). The potential for oceanic warming to cause an increase in biofouling rates on artificial surfaces has been previously suggested, particularly in temperate regions (Poloczanska and Butler 2010). *Didemnum perlucidum* was observed to grow in summer and retract in winter (or wet season) in WA (Dias et al. 2016a; Muñoz et al. 2015). This supports the initial observation by Smale et al. 2011 (when observing *D. perlucidum* on their hot-plate experiment simulating oceanic warming) that temperature influences the species recruitment and growth, and therefore, is likely to play a crucial role in explaining the detection and distribution of *D. perlucidum* in WA.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Country, location and number of *Didemum perlucidum*, other colonial ascidian species and total of samples obtained per site.
Table S2. Measure of genetic differentiation based on microsatellite loci pairwise comparisons.
Table S3. BOTTLENECK results for the 19 sampling locations included in the population genetics study.

This material is available as part of online article from:
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