Conservation Genetics of Antarctic Heart Urchins (*Abatus* spp.)

by

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Thesis submitted in fulfilment of the requirements for the degree of Master of Science in Biological Sciences

University of Tasmania

January 2015

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Acknowledgements

I would like to thank my main supervisors, Dr. Karen Miller and Dr. Chris Burridge. It has been a great experience to work with you, not only in Academic terms (you are inspiring scientists) but also simply because you are very nice people and that makes a big difference. Thank you for your continuous support! Dr. Catherine King was not a supervisor "on paper" but has been a very active member of this team, thanks for your valuable input, and for working so efficiently when I needed to get things done. Also thanks to Dr. Barbara Wienecke for your input in this work and also for showing me the Antarctic world with such a passion, you are an inspiring woman.

Thanks to the Australian Antarctic Division for the unforgettable opportunity to spend 6 months in "the world's largest freezer" and to all the amazing expeditioners with whom I shared the experience, too many to name them all. I would like to make special mention of Helen Achurch: fieldwork with you in Antarctica was a fantastic experience, and you've become a friend with a very special place in my heart.

Thanks to the University of Tasmania for scholarships to work on this project and to the Biological Sciences Department for their support and for making the transition there so easy. Special thanks to Adam Smolenski for help in the lab.

I'd like to acknowledge Catherine King for the urchin samples, Leslie Watson for bringing the DDU urchins (and everyone involved in collecting samples), and Ashley Miskelly for the morphologic identification of specimens and for sharing his expertise and answering my many queries by email.

Thanks to all the beautiful friends that I was so lucky to meet in Hobart…so many to thank for those precious fun times! Also thanks to my fantastic lab and office mates.

Thanks to Alyce Hanckock for the picture of the cover!

To all my family and friends in Argentina, especially my brother Santiago and my father Daniel, you are immensely missed but even from a distance I get your support and inspiration. And to my in-laws in Canada (Marilyn, Curtis, Jason and Annik) you've truly become part of my family and my heart.

Last but certainly not least, to Eric. It's easy for me to thank you for your help with the figures, with R and for your numerous comments and English corrections, but I have no words to thank you for ALL the rest, you are my home and I love you.

Abstract

Antarctic marine biodiversity is largely contained in the continental shelf zone and is characterized by high levels of endemism and a high proportion of invertebrate species with brooding life histories. Benthic marine species in Antarctica are considered highly vulnerable to the predicted warming environment, and therefore there is an urgent need for research to aid their effective management and preserve biodiversity. Our knowledge of the processes affecting population genetic diversity and structure in the Antarctic benthos remains very limited, particularly in East Antarctica. The irregular heart urchins (genus: *Abatus*, Spatangoidea: Schizasteridae) are endemic to the sub-Antarctic and Antarctic regions, and their brooding life history is expected to restrict their dispersal capabilities, leading to isolated and potentially inbred populations that may be susceptible to the effects of environmental change. The broad aim of this thesis is to characterize the genetic diversity and structure of heart urchin populations at different spatial scales (hundreds of meters to thousands of kilometers) in order to elucidate past and contemporary processes affecting heart urchin populations in East Antarctica.

Two mitochondrial markers (16S and COI) were used to examine large-scale phylogeographic relationships in *Abatus ingens* and *Abatus nimrodi*. Samples were collected near three Antarctic stations separated by over 1000 km: Davis, Casey, and Dumont D'Urville. In addition, three other *Abatus* species: *A. shackletoni*, *A. philippii*, and *A. cavernosus*, and the closely related *Amphipneustes lorioli*, were included in the analysis in order to clarify taxonomic relationships within the genus. Large-scale genetic patterns were indicative of populations that survived in shelf refugia and experienced pronounced bottlenecks during Quaternary glaciations.

Genetic diversity was higher at Davis than in Casey and Dumont D'Urville for *A. ingens* and *A. nimrodi*. Populations of *A. ingens* from the three study areas were genetically distinct, while populations of *A. nimrodi* from Casey, and Dumont D'Urville shared a single haplotype and were differentiated from Davis individuals. Phylogenetic inferences suggest that the species classified as *A. nimrodi* is more closely related to *Amphipneustes lorioli* than other *Abatus* spp. and requires taxonomic revision.

Microsatellite DNA markers were developed from *A. ingens* to study the fine-scale (ca. 5km) patterns of population genetic diversity and structuring in the area surrounding Davis Station. The markers successfully cross-amplified in *A. shackletoni* and *A. philippii* allowing for a multispecies comparison. In order to test for potential impacts by anthropogenic pollutants, samples were taken at five sites at varying distances from the outfall of wastewater discharge from the station. There was no evidence of an effect of pollution from Davis Station on genetic variation, and patterns of population genetic differentiation varied among the species. Gene flow occurs across distances of at least 5km, which is not consistent with the hypothesis of limited dispersal associated with the brooding life history, and suggests life history is not a good predictor of fine-scale population structure in these species. There was genetic evidence of a long-term population decline in all three species, but the estimated timing at the decline precedes any likely impact due to anthropogenic activities. Furthermore, two genetic clusters inferred in *A. ingens* and *A. shackletoni* suggest secondary contact after population differentiation in glacial refugia. The declining effective population size inferred for these three benthic species highlights their fragility and the need for conservation concern**.**

Taken together, the results from this study provide a significant insight into the processes that have shaped patterns of genetic diversity and structure of Antarctic heart urchin populations in East Antarctica. This research has implications for the management and preservation of Antarctic marine biodiversity. A clear influence from the cyclic glaciations of the Pleistocene on the present day population genetic differentiation patterns of the Antarctic heart urchin species studied is evidenced at both large and fine spatial-scales. The brooding development of these species does not appear to restrict their dispersal capabilities at fine spatial-scales, and population connectivity may be promoted by physical factors such as rafting on floating sea ice. The vulnerability of *Abatus* populations in East Antarctica was suggested by their reduced effective population sizes and population isolation at a large scale, which can contribute to a higher risk of local extinction.

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Chapter 1

General Introduction

1.1 *The Antarctic continental shelf and its biodiversity*

The continental shelf in Antarctica is unique in a number of ways, including its oceanography, cryosphere, and biological properties (Griffiths 2010). The shelf is unusually deep, over 1000 m in some locations, and has an average depth of 450 m. Although narrow in some areas, it has an average width of ca. 125 km (Griffiths 2010). Physical, biological, and environmental parameters are highly seasonal, e.g. light levels, nutrients, primary production (Constable et al. 2003), and most notably sea-ice cover. Each winter, the area covered by sea ice doubles the size of the continental surface of Antarctica (Gloersen 1992). Sea ice can have major influences on the shelf biota (Barnes & Conlan 2007). For example, sea-ice scouring, pressure ridges, and icebergs can physically change the seafloor topography, alter sediments characteristics, and modify current flows (Barnes & Conlan 2007). Additionally, anchor-ice which forms on the seafloor and occurs at depths up to 30 m, can detach benthic organisms when it becomes buoyant and releases from the seafloor (Dayton et al. 1969). This high frequency of disturbance, in combination with low recolonization rates inherent to Antarctic organisms, results in a high degree of patchiness in both the diversity and abundance of benthic communities (Barnes & Conlan 2007).

The Antarctic marine benthos is considered one of the most isolated ecosystems on earth, as it is surrounded by deep sea basins and the Antarctic Circumpolar Current (ACC), which formed ca. 25 Mya (Clarke et al. 2005). Associated with these long term isolating influences, the Antarctic shelf harbours a unique fauna with high levels of endemism, e.g. over 50% for several Bryozoan and Molluscan groups (Griffiths

2010). Significant international efforts have been made to document Antarctic marine species diversity, such as the Census of Antarctic Marine Life and the Scientific Committee on Antarctic Research Marine Biodiversity Information Network. As a result, we know that diversity levels in the Antarctic shelf are actually comparable to that of temperate regions (Griffiths 2010). Moreover, while some groups are poorly represented, e.g. fishes, the species richness of other taxa is higher in Antarctic waters than anywhere else, e.g. pycnogonids (Griffiths et al. 2011). In addition, genetic studies have revealed species complexes with no diagnostic morphological features (cryptic species), suggesting Antarctic benthic biodiversity is underestimated (Rogers 2007).

The recent development of comprehensive databases and the incipient accumulation of molecular evidence have provided the groundwork to understand broad diversity patterns and geographic distribution (biogeography) in the Antarctic benthos (Rogers 2007, Griffiths et al. 2009, Pierrat et al. 2013). At a large spatial scale and higher taxonomic level there is strong evidence that the Antarctic benthos has long been structured by the presence of the Antarctic Polar Front (APF) acting as a barrier to latitudinal dispersal, as well as the eastward ACC and westward Antarctic Coastal currents promoting dispersal along the shelf (Griffiths et al. 2009, Pierrat et al. 2013). Comparative studies using the most up to date databases of the classes Echinoidea, Bivalvia, Gastropoda, Cyclostomata and Cheilostomata each support a single biogeographic province for the Antarctic benthos, with no biogeographic splits across the entire continental shelf (Griffiths et al. 2009, Pierrat et al. 2013). The importance of the cyclic glaciations during the Pleistocene is evident from the genetic structure and differentiation persisting in many benthic species. However, such differentiation patterns rarely coincide across taxa, suggesting that no single mechanism shapes

large-scale spatial structuring of marine diversity in Antarctica (Convey et al. 2014). Much work is still needed to understand the mechanisms influencing the distribution of biodiversity at smaller spatial scales.

1.2 *Threats to Antarctic marine biodiversity*

The unique marine ecosystems of Antarctica, characterised by endemic species, are affected by human activities including fisheries, localized pollution, introduced species and climate change (Aronson et al. 2011). Antarctic benthic species in particular are considered highly vulnerable to the predicted warming environment (Peck, 2005; Barnes & Peck, 2008; Barnes & Souster, 2011). Peck (2005) proposed that physiological (stenothermic), life history (long development and life span) and dispersal (high isolation) characteristics of Antarctic benthic species could constrain their capacity to respond to a changing environment. Although the impact of climate change on the biology of species is difficult to predict, a link between the warming climate and low survival of bryozoans has been observed in the Antarctic Peninsula (Barnes & Souster, 2011). Polar organisms with calcified shells or skeletons, e.g. sea urchins (Echinodea), are considered particularly vulnerable to the ocean acidification (OA) associated with increased levels of CO_2, because mineral forms of calcium carbonate have lower saturation states at high latitudes (Aronson et al. 2011). Specifically, brooding irregular sea urchins from the Family: Schizasteridae are predicted to be one of the most impacted because calcification of the offspring occurs at depths above the calcite saturation horizon (Sewell & Hofmann 2011). However, recent evidence suggests that the regular Antarctic sea urchins (broadcaster spawners of the Family: Echinidae) might be more resilient to OA than initially thought (Collard et al. 2014).

Wastewaters are commonly discharged into coastal areas adjacent to Antarctic stations and the impacts of these complex mixes of contaminants on Antarctic biota are poorly understood (Aronson et al. 2011). Impacts on community composition and reduced benthic biodiversity was shown to be associated with contamination from two stations in East Antarctica −McMurdo and Casey− where ostracods, cumaceans and echinoderms were particularly sensitive (Stark et al. 2014). In addition, elevated levels of toxic contaminants from wastewater at Davis Station (metals, polybrominated diphenyl ethers, nutrients and faecal sterols) were found to be accumulating within 2 km of the station's outfall (Stark et al. In Press). Moreover, more severe histopathologies were observed in Antarctic rock-cod collected in close proximity (within 800 m) of the Davis station outfall compared to fish sampled from further away (Corbett et al. 2014). Notably, although the impacts on benthic communities associated with pollution are generally localized to within a few hundred meters of the stations (Aronson et al. 2011), most of the 84 research stations in Antarctica (COMNAP, 2014) are located in ice-free coastal areas that represent a rare (0.01% of the continental area) and very important habitat for the benthic ecosystem (Stark et al. 2014). Therefore anthropogenic pollution from research stations can be considered a serious threat to the Antarctic benthos and it is important that the full impacts of these activities in Antarctica are understood.

1.3 *Population genetic structure in the Antarctic benthos*

With increasing pressure on Antarctic ecosystems from humans, associated with station activities, tourism and fishing, there is an urgent need for research to aid the effective management and preservation of the marine biodiversity of Antarctica. For example, estimates of genetic diversity and population connectivity are essential to inform the design of conservation initiatives such as Marine Protected Areas (MPAs)

(Palumbi 2004). This is because the efficiency of MPAs depends on the right placement and optimal size to both protect diversity within, and ensure dispersal between, the reserve and its surroundings (Palumbi 2004). The high relevance of preserving genetic diversity for the conservation of biodiversity has long been recognised (Lande, 1998), not only for its role in the long-term persistence of species (Frankham, 2009), but also to enhance ecosystem resilience to extreme climatic events (Reusch et al. 2005).

The application of different molecular markers in genetic studies can provide essential information for management initiatives at different levels depending on the markers' mutation rates (Feral, 2002). Fast evolving markers such as microsatellites can detect variation at the intra-species level, e.g. population connectivity. Slower evolving coding regions, such as the mitochondrial ribosomal sub-unit 16S or the cytochrome oxidase sub-unit 1 (CO1), can determine deeper phylogeographic and phylogenetic relationships (among populations and species, respectively). Our knowledge of the distribution of genetic diversity of Antarctic benthic species is mostly derived from large-scale studies implementing phylogenetic approaches using mitochondrial markers, but little is known about the processes acting at the population level which require the development of species-specific markers with higher mutation rates (Held & Leese, 2007).

The geographic structuring of genetic variation at the intraspecific level is the result of evolutionary processes that can act in opposition, i.e. with diverging or homogenising effects (Stalkin, 1987). Novel genetic variation is generated through mutation, and populations can diverge in allele frequency through independent genetic drift or natural selection. On the contrary, gene flow tends to homogenise populations but it can also promote evolution by spreading gene variants throughout a

species' range (Stalking, 1987). At the same time, levels of gene flow among populations depend on the dispersal capacities of the species as well as on the presence of barriers in the landscape or, in the case of marine populations, in the 'seascape' (Manel et al. 2003).

Brooding marine species are expected to have restricted dispersal capabilities (and consequently higher levels of population structure) relative to species with pelagic larvae (Bradbury et al. 2008). However, the relationship between life history and population structure is not always straightforward. For example, the dispersal capacity of Scleractinian corals in the tropics is not easily predicted based on their life history (Miller & Ayre 2008). Relatively few genetic studies have tested these predictions in Antarctic marine invertebrates (Hoffman et al. 2010, Hoffman et al. 2011, Baird et al. 2012, Hoffman et al. 2013), especially when compared to the vast literature available for lower latitudes (Bradbury et al. 2008). However, of the few published studies that have examined fine-scale population structure in Antarctica using fast evolving markers (such as microsatellites and AFLP) results have been consistent with the expectations based on life history i.e. in the brooding gastropod *Margarella antarctica* (Hoffman et al. 2010, Hoffman et al. 2013) and the amphipod *Orchomenella franklini* (Baird et al. 2012). On the other hand, strong population structure has been found in the broadcast spawning limpet *Nacella concinna* (Hoffman et al. 2011) as inferred by AFLP markers, so it is unlikely that broad generalisations about life history and population genetic structure will be supported in the Antarctic. In addition, large-scale studies using less variable mitochondrial markers have increasingly reported genetic homogeneity at long distances (<1000 km) in several brooding Antarctic species (Hunter & Halanych 2008, Wilson et al. 2009, Baird et al. 2011).

In addition to reflecting life-history characteristics and revealing ecological patterns, population genetic structure can retain the signature of historical processes, such as climate variability (Allcock & Strugnell 2012). Given the long-term isolation of the Antarctic marine environment, the extant benthic fauna appears to have survived Quaternary glacial cycles *in situ*, and the molecular signatures of survival in different refugia have been characterized and summarized in a review of the available literature by Allcock & Strugnell (2012). For brooding species, these authors describe a pattern of reduced genetic diversity and fragmented haplotype networks indicative of isolated populations that survived in glacial refugia on the shelf and underwent pronounced bottlenecks. On the other hand, a pattern of less dramatic reduction in haplotype diversity and a 'parochial' haplotype network with most haplotypes restricted to a single geographic location, is associated with populations of brooding eurybathic species that are proposed to have survived in deep-sea refugia. As most population genetic studies on Antarctic benthic species have been conducted in the Antarctic Peninsula, further genetic studies of benthic brooders would help clarify whether the patterns characterised by Allcock & Strugnell (2012) can be extrapolated to other regions in Antarctica including East Antarctica.

1.4 *Antarctic heart urchins*

There is a high proportion of brooding species among Antarctic coastal invertebrates, many of which are echinoderms (Poulin et al. 2002). The irregular heart urchins (genus: *Abatus*, Spatangoida: Schizasteridae, Fig.1) comprise 11 known species endemic to the subantarctic and Antarctic regions (David et al. 2005). They are deposit feeders and live buried or partially buried in fine silt or mud (Poulin & Feral 1995). All species have direct development with their offspring protected within the female's brood pouches until they are released to the environment as juveniles. The

reproductive biology of *A. cordatus* is fairly well studied (Schatt & Feral, 1991, 1996; Gil et al. 2009), in which case development protection takes about 9 months (Schatt & Feral, 1991). This species exhibits annual brooding cycles, while *A. nimrodi*, *A.ingens*, and *A. shackletoni* exhibit pluriannual brooding cycles (Pearse & McClintock, 1990; Chenuil et al. 2004; Galley et al. 2005).

Fig. 1 Adult specimens of *Abatus* spp. Photo by A. Miskelly.

1.5 *Aims of the study*

The broad aim of this study is to characterize the genetic diversity and structure of Antarctic heart urchin populations at different spatial scales (hundreds of meters to thousands of kilometres) in order to elucidate past and contemporary processes affecting heart urchins population genetic diversity and structure in East Antarctica.

Specific goals include:

First, the aim is to examine large-scale intraspecific patterns of genetic diversity and structuring of two *Abatus* species: *A. ingens* and *A. nimrodi* collected near three Antarctic stations, each separated by over 1000 km within East-Antarctica: Davis (68°35′S 77°58′E), Casey (66°16′S 110°31′E), and Dumont D'Urville (66°39′S 140°0′E) (Fig. 2). To do this I use sequences of two mitochondrial markers (COI and 16S). Additionally, I include samples of three other *Abatus* species: *A. shackletoni*, *A. philippii*, and *A. cavernosus*, as well as the closely related *Amphipneustes lorioli*, in order to examine the phylogenetic relationships of species from these genera, and clarify some taxonomic uncertainties among these species that could confound the interpretation of evolutionary processes in this group.

Fig. 2 Map showing the location of the sampling areas at three research stations in East Antarctica.

The second aim is to compare the demographic history and patterns of population genetic diversity and structuring at a fine spatial scale (<5 km) of three *Abatus* species: *A. ingens*, *A. shackletoni* and *A. philippii* in the area surrounding Davis Station. In addition, I test for potential impacts of the Davis Station wastewater discharge on the genetic diversity, population structuring, and effective population size of these three species. In order to do this a set of microsatellite markers specific to *A. ingens* was developed, that successfully cross-amplified in *A. shackletoni* and *A. philippii*, thereby allowing for a multispecies approach. Urchins were collected from five sites located at different distances from the station's wastewater outfall. Three of the sites are considered heavily impacted and two control sites are removed from the predominant longshore current that transports the wastewater plume in a south easterly direction along the coast (Stark et al. In Press).

The two specific aims described above are developed in Chapters 2 and 3 of this thesis, respectively. These chapters are presented as manuscripts that are currently in preparation for submission to scientific journals.

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Chapter 2

How did glaciations affect benthic biodiversity in East Antarctica? Genetic insights from the heart urchins of the genus *Abatus* (Spatangoidea: Schizasteridae)

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Authors' contributions to this manuscript:

Cecilia Carrea: contributed to the idea, was responsible for the data collection (laboratory work), analysis and interpretation, and wrote the manuscript (≥ 80% work).

Jake van Oosterom: collected samples and sequence data for the Davis and Casey sampling sites.

Christopher Burridge: assisted with data analysis and interpretation and provided editorial revisions to the manuscript.

Catherine King: assisted with the idea formulation, provided editorial revisions to the manuscript and obtained funds to develop the project.

Karen Miller: assisted with the idea formulation, data analysis and interpretation, editorial revisions to the manuscript. She also obtained the funds to develop the project.

The undersigned agree with the above stated proportion of work undertaken for this manuscript that is currently in preparation to be submitted for publication

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Abstract

Remarkably, much of the Antarctic benthic biota appears to have survived the massive disturbance caused by Quaternary glaciations *in situ*, and contemporary populations carry genetic signatures of how and where these species survived. However, genetic diversity and differentiation patterns at the intraspecific level are poorly understood in benthic species of East-Antarctica. We examine large-scale (over ca. 1000 km) phylogeographic relationships and intraspecific patterns of genetic diversity using 656 bp of mitochondrial DNA (315 bp of COI and 341 bp of 16S), in two brooding species of Antarctic heart urchins (*A. ingens* and *A. nimrodi*) from three different regions of East-Antarctica: Vestfold Hills, Windmill Islands and Durmont D'Urville. Three additional *Abatus* species (*A. shackletoni, A. philippii* and *A. cavernosus*) and the closely related *Amphipneustes lorioli* were included to assess the evolutionary relationships of species from these genera. The genetic diversity patterns observed in *A. ingens* and *A. nimrodi* are consistent with patterns observed in other Antarctic brooding species that survived as small populations in shelf refugia undergoing severe bottlenecks during Quaternary glaciations. Relative high genetic diversity in the Vestfold Hills region for both species suggests long persistence and the potential for glacial refugia to be located in this area. In *A. nimrodi* the high levels of divergence between Vestfold Hills and the combined Windmill Islands and Dumont D'Urville populations suggest that those areas were probably recolonized from different refugia and that cryptic speciation is possible. Finally, our results suggest that *A. nimrodi* is more closely related to *Amphipneustes lorioli* than to the rest of the *Abatus* species, providing a framework to further investigate the taxonomic relationships among these species as well as the species composition of these genera.

Introduction

The evolution of Antarctic marine biodiversity has been influenced by a combination of oceanographic, climatic, geological and biological processes (Rogers 2007). In particular, the current distribution of populations in the shallow Antarctic benthos has been profoundly shaped by the variation in habitat availability associated with Quaternary glacial cycles (Allcock & Strugnell 2012). Notably, at glacial maxima, a grounded ice-sheet covered the continental shelf around Antarctica (Anderson et al. 2002) exposing the benthos to a massive reduction in habitat (Thatje et al. 2005). To what extent the marine benthos survived in refugia on the continental shelf, the slope, or the deep sea during the last glacial maximum (LGM), and whether present day shallow-water populations were colonised from these refugia remains largely unknown (Clarke & Crame 2010). In addition, recolonization of shallow habitats from peri-Antarctic refugia has been proposed as an alternative biogeographic model for benthic marine invertebrates, e.g. from subantarctic islands and the Western Antarctic Peninsula (Gonzalez-Wevar et al. 2013).

The nature of refugia and the duration over which species were confined to them during glacial cycles may be highly variable among species, and subsequently may have had differing effects on present day genetic diversity (Stewart et al. 2010). Evidence supporting the origin of contemporary Antarctic shelf benthos in shelf, deep sea and subantarctic refugia has been found for different species across the Antarctic (Table 1). In a recent review of molecular studies, Allcock & Strugnell (2012) characterized the genetic signature found in populations of Antarctic species proposed to have originated from shelf vs. deep refugia. Species originating in shelf refugia show evidence of pronounced bottlenecks, e.g. low diversity and one dominant

haplotype over large areas, while deep-sea refugia implied a less drastic decrease in population size and therefore resulted in more genetically diverse contemporary populations. Not surprisingly, genetic patterns on contemporary populations also reflect the species' life history and its associated dispersal capabilities, i.e. direct development (brooders, low dispersal) vs. indirect development (broadcast spawners with dispersive larval stages).

There is an unusually high proportion of brooding species among Antarctic marine invertebrates, and particularly in echinoids (Poulin et al. 2002). Although initially interpreted as an adaptation to cold environments (Thorson 1936), hypotheses involving macroevolutionary processes, such as selection against pelagic development and vicariant mechanisms enhancing speciation of brooders, are currently more widely accepted (Poulin et al. 2002). Glacial refugia in the continental shelf may have held fragmented populations of brooders, promoting their speciation (Clarke & Crame 1992), and the Antarctic Circumpolar Current (ACC) may have infrequently facilitated dispersal of brooding species to new locations, where they would diverge from their ancestral population under limited gene flow (Pearse et al. 2009). Within the echinoids restricted to Antarctica, all schizasterids (Spatangoidea: Schizasteridae) have non-pelagic direct development, with offspring brooded in the female's aboral pouches (Poulin & Feral 1994). The mechanisms by which schizasterids, also known as heart urchins, radiated and attained their current distributions remain unclear, although evidence from *Abatus cordatus* in the Kerguelen Islands suggests genetic differentiation due to limited gene flow occurs in this species (Poulin & Feral 1994, Ledoux et al. 2012).

Intraspecific genetic diversity and structure is difficult to predict for Antarctic marine invertebrates (Baird et al. 2011). Despite the perceived low dispersal capacities of

benthic brooders, genetic homogeneity at large scales has been reported in a variety of taxa including an amphipod (Baird et al. 2011), a brittle star (Hunter & Halanych 2008), a nudibranch (Wilson et al. 2009) and a pycnogonid (Krabbe et al. 2009). On the other hand, fragmented populations surviving in refugia likely diverged under different environmental conditions (Clarke & Crame 1992), and their recolonization of the shelf can explain the high prevalence of cryptic species (without morphological diagnostic features but genetically distinct) that have been found in the Antarctic benthos, often in sympatry (Janosik & Halanych, 2010). Molecular studies of Antarctic organisms are therefore essential to elucidate the diversity of lineages, their distribution and evolutionary relationships, and provide an independent tool to test taxonomic and biogeographic hypotheses such as how they came to presently occupy the Antarctic shelf (Janosik & Halanych 2010). However, genetic studies analysing intraspecific diversity and differentiation focusing on East-Antarctica are relatively scarce, especially when compared to West-Antarctica and particularly the Antarctic Peninsula (Table 1).

Some taxonomic uncertainties exist for the Antarctic heart-urchins, regarding species composition of *Abatus*, the status of *Pseudabatus*, and the relationships among the species from those genera and *Amphipneustes* (Kroh & Mooi 2014), and this could confound the interpretation of evolutionary processes in this group. The taxonomy of the Spatangoid urchins relies largely on the morphological classification established by Mortensen (1951), David et al. (2005) includes 11 species in the genus *Abatus* and considers *Pseudabatus* as a subgenus. On the other hand, the Echinoid directory (Smith & Kroh 2014) elevates *Pseudabatus* to the genus level, comprising four species (*P. nimrodi, P. beatriceae, P. shackletoni* and *P. ingens*). Although progress has been made on the phylogeny of Spatangoid urchins at a higher taxonomic level

(Stockley et al. 2005, Kroh & Smith 2010), the phylogenetic relationships at the species-level remain to be resolved for *Abatus*, *Pseudabatus*, and *Amphipneustes* (Kroh & Mooi 2014).

We use two mitochondrial markers, the cytochrome oxidase sub-unit 1 (COI) and the large ribosomal sub-unit (16S), to examine the intraspecific patterns of genetic diversity and structuring of two brooding species of Antarctic heart urchins (*A. ingens* and *A. nimrodi*) from three different regions of East Antarctica, in order to elucidate their biogeographical history. In addition, we include samples of three other *Abatus* species, *sensu* Mortensen (1951), and the closely related *Amphipneustes lorioli*, in order to examine the phylogenetic relationships among mitochondrial lineages and thus contribute to the understanding of the evolutionary relationships of species from thesegenera.

Table 1: Review of published studies showing support for different survival strategies during glaciations in different benthic species and highlighting the scarcity of studies focusing on East-Antarctica

Benthic species	Development	Study area*	Molecular	Origin inferred by Genetic	Reference
Chorismus antarcticus	Indirect	CP (E-A, 1 site)	COI, 16S, 28S	Peri-Antarctic/Shelf Refugia	(Raupach et al. 2010)
Nematocarcinus lanceopes	Indirect	CP (E-A, 1 site)	COI, 16S, 28S	Deep sea	(Raupach et al. 2010)
Epimeria spp	Direct	CP (E-A, 1 site)	COI	Shelf Refugia	(Loerz et al. 2009)
Macroscapha spp	Direct	CP (E-A, 1 site)	COI, ITS	Peri-Antarctic/Shelf Refugia	(Brandao et al. 2010)
Nymphon australe	Direct	CP (E-A, 1 site)	COI, 16S	Deep sea	(Arango et al. 2011)
Pareledone turqueti	Direct	CP (E-A, 2 sites)	COI, microsats	Shelf Refugia	(Strugnell et al. 2012)
Doris kerguelenensis	Direct	CP (E-A, 2 sites)	COI, 16S	Shelf Refugia	(Wilson et al. 2009)
Eusirus spp	Direct	CP (E-A, 3 sites)	COI, CytB, ITS2	Deep Sea/Shelf Refugia	(Baird et al. 2011)
Pareledone spp	Direct	CP (E-A, 3 sites)	COI	Deep sea/Shelf Refugia	(Allcock et al. 2011)
Nacella concinna	Indirect	W-A	COI	Peri-Antarctic	(González-Wevar et al. 2011,
Abatus agassizii	Direct	W-A	COI	Peri-Antarctic	(Diaz et al. 2012)
Parborlasia corrugatus	Indirect	W-A	COI, 16S	Deep sea/Shelf Refugia	(Thornhill et al. 2008)
Sterechinus spp	Indirect	W-A	COI	Peri-Antarctic	(Díaz et al. 2011)
Promachocrinus kerguelensis	Indirect	W-A	COI, CytB	Shelf Refugia	(Wilson et al. 2007)
Colossendeis megalonyx	Direct	W-A	COI	Shelf Rfugia	(Krabbe et al. 2009)
Odontaster spp.	Indirect	W-A	COI, 16S	Deep sea	(Janosik et al. 2010)
Astrotoma agassizii	Direct	W-A	COI, 16S	Shelf Refugia	(Hunter & Halanych 2008)

Materials and Methods

Study area and data collection

A total of 126 Spatangoid specimens was included in the analysis. Individuals were identified morphologically using an adapted version of Mortensen (1951) and following David et al. (2005). Twenty-seven *Abatus* spp. individuals were collected in the area offshore the French station Dumont D'Urville 66°40'S 140° 01'E (hereafter DDU), in Adélie Land, Antarctica during the 2012/2013 austral summer. Of those, 11 were identified as *Abatus nimrodi,* 16 as *Abatus ingens* and three as *Amhispneustes lorioli*. All samples were collected by trawling from depths of 40-110 m and were preserved whole in 96% ethanol. In addition, mitochondrial DNA sequences from 48 specimens of *Abatus nimrodi* (48 of CO1 and 43 of 16S) and 48 specimens of *Abatus ingens* (48 of CO1 and 48 of 16S) were obtained from van Oosterom (2013). Those sequences correspond to individuals collected from within 1 km of the shoreline in the Windmill Islands (hereafter WI) area surrounding the Australian research base Casey Station 66°16'S 110°31'E in Wilks Land during the 2008/2009 austral summer, and the Vestfold Hills (hereafter VH) area surrounding the Australian Davis Station 68°34'S 77°58' E in Princess Elizabeth Land, during the 2009/2010 austral summer. Samples were collected by SCUBA divers, snorkelers or by dip nets from depths of 1-25m at each location within approximately 1 km of each other (Fig.1). Mitochondrial DNA sequences from specimens of three other *Abatus* species were also obtained from van Oosterom (2013) to incorporate into the genetic analysis: three *A. shackletoni,* one *A. philippii* and one *A. cavernosus* from Davis Station (VH). Gonad tissue was dissected from all samples and preserved in 96% ethanol for genetic analysis.

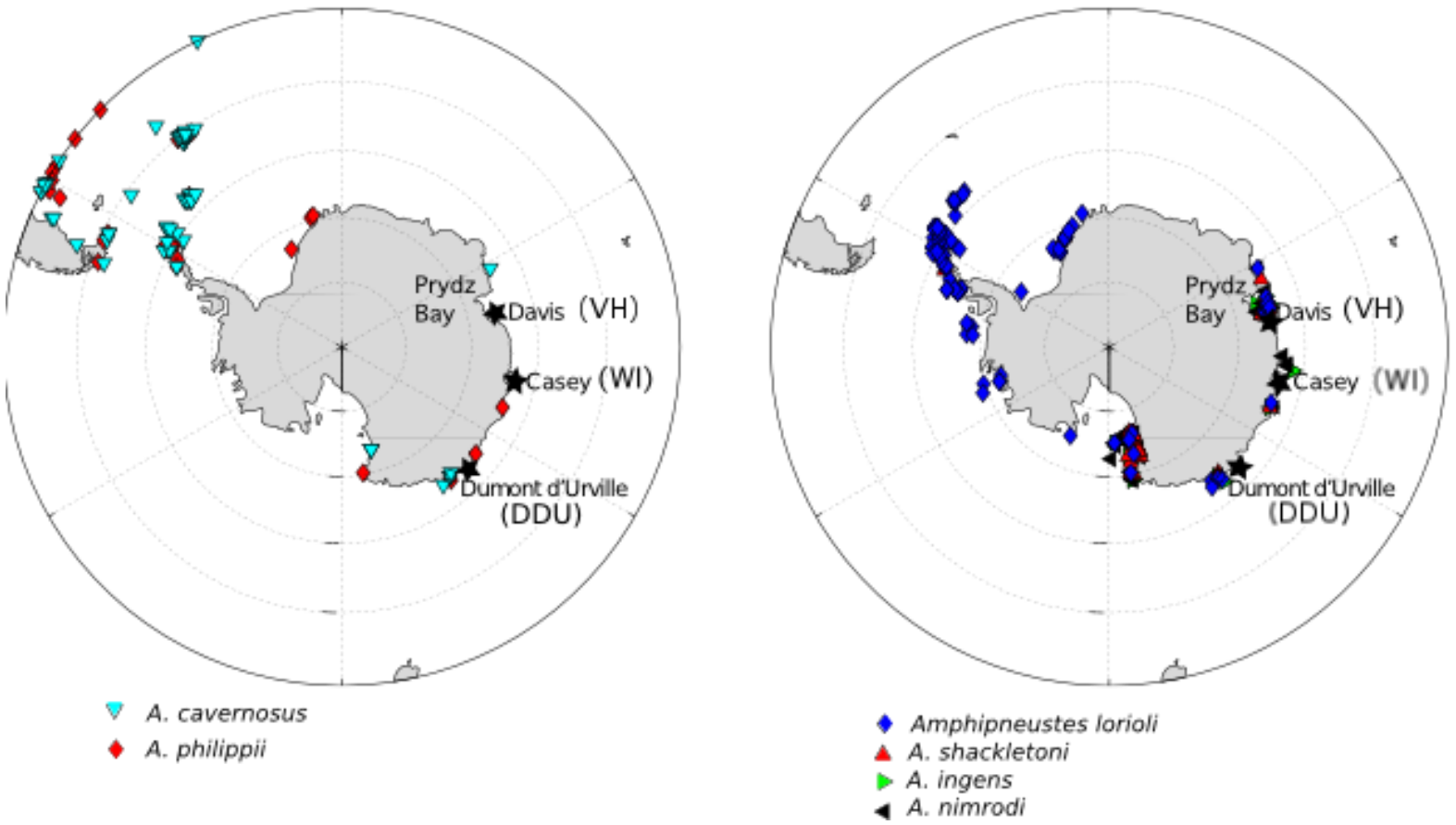

Figure 1. Map showing sampling areas (indicated by black stars) in East Antarctica and occurrence data compiled from the Antarctic Echinoid Database (Pierrat et al. 2012) for the six Antarctic heart-urchins species included in our analysis. Left: Species with subantarctic and Antarctic distributions. Right: Species confined to the Antarctic continent.

Molecular laboratory methods

Genomic DNA was extracted from the gonad samples using Qiagen DNeasy kits according to the manufacturer's instructions. Cytochrome oxidase sub-unit 1 and the large ribosomal sub-unit 16S were PCR amplified using primers developed by van Oosterom (2013): for C01 (F: CCTCGTAATGATCTTCTTCATGG and R: GGTCTGTGTTAAAAGCATTGTTATTGC), under 30 cycles of thermal cycling: 94°C 30s, 51.5°C 45s, 72°C 1 min. Universal primers were used for 16S (Palumbi 1996). Fragments were sequenced in both directions at the Australian Genome Research Facility (Melbourne). The accession numbers for additional sequences obtained from Genbank are shown in Table 1 of the Appendix for this chapter (Appendix C2). However, the Genebank sequences for *Amphipneustes lorioli* (AJ639905 and AJ639804) were later excluded from the analysis as their position in the tree topologies was inconsistent with sequences obtained in this study for *A. lorioli* from DDU for both genes. The tree was rooted using *Brisaster latifrons*, *Paleopneustes cristatus* and *Spatangus raschi* following the phylogeny proposed by (Stockley et al. 2005).

Molecular data analysis

Sequences were aligned using the ClustalW algorithm as implemented in MEGA 6.0 (Tamura et al. 2011). Each gene sequence was truncated to a consistent length for comparison: 315 bp of CO1 (78% inter primer region) and 341 bp of 16S (58% inter primer region). Genetic diversity indices such as the number of haplotypes, haplotype diversity, nucleotide diversity (mean number of pairwise differences) and number of polymorphic (segregating) sites were calculated using ARLEQUIN 3.5 (Excoffier & Lischer 2010). Sequences from the two mitochondrial markers were concatenated

using Sequence Matrix (Vaidya et al. 2011). In order to test for genetic structure among populations of the three East-Antarctic regions an hierarchical analysis of molecular variance (AMOVA) and pairwise F_{ST} were calculated based on haplotype frequencies among geographic localities using ARLEQUIN 3.5; significance tests were performed using 10,000 permutations.

In order to investigate the demographic dynamics of *A. ingens* and *A. nimrodi* populations in East Antarctica, we analysed the distribution of pairwise nucleotide differences among individuals (i.e. mismatch distribution) in combination with neutrality tests. We compared the observed mismatch distribution to the distribution expected under the sudden population expansion model (Rogers & Harpending 1992) in each of the three study areas. This model assumes that population growth and decline events leave characteristic patterns: expanding populations exhibit L-shaped distributions, while populations in equilibrium or declining can show bi modal or multimodal distributions patterns (Rogers & Harpending 1992). We used the sum of squared deviations (SSD) to test goodness of fit to the model as implemented in ARLEQUIN 3.5. Statistical significance was tested using 1000 permutations. The raggedness index (r) and its significance were also calculated using 1000 permutations in ARLEQUIN 3.5 for each population. High and significant r-values are indicative of bottlenecked populations. In addition, to assess whether each population is at a neutral mutation-drift equilibrium we used DNAsp 5.10 (Librado & Rozas 2009) to compare tests statistics with different sensitivities to changes in population size (Fu 1997): Tajima's D (Tajima 1989), Fu's F_S (Fu 1997), D* and F* (Fu & Li 1993). Both D and F_S statistics are expected to be negative when a population has undergone a recent expansion or is under purifying selection, or

positive when the population has undergone a bottleneck or is under overdominance selection. The D* and F* statistics are especially sensitive to background selection.

Bayesian inference of phylogeographic and phylogenetic relationships among populations and species, respectively, were performed with MrBayes 3.2.1 (Ronquist & Huelsenbeck 2003). Initially the sequences from CO1 (truncated to 315 bp) and 16S (truncated to 341 bp) were analysed separately, but since the results were in agreement, concatenated data (656 bp) were used in order to increase statistical power. Partitioned analysis was performed by treating each gene under a different substitution model as determined by Modeltest 3.7 (Posada & Crandall 1998). The partition corresponding to CO1 was treated under the GTR model (Tavare 1986) and the 16S partition was treated under the HKY model (Hasegawa et al. 1985). A proportion of nucleotide sites were considered invariant, with a gamma shaped distribution of rates across sites. The analysis was performed using the MrBayes default prior probability distributions for the model parameters, and duplicate MCMC searches were conducted with four chains of 10,000,000 generations and a tree sampling frequency of 500 generations in each case. Three of the chains were heated according to 'Temp = 0.1'. The first 25% of the samples from the cold chain were discarded as 'burn-in'. Tracer 1.5 (Rambaut et al. 2014) was used to establish if asymptotes were attained for the values of lnL and substitution model parameters in the trace plots in order to ensure stationarity and adequate mixing of the chains. Convergence was also determined when the average standard deviation of split frequencies was lower than 0.005.

Alternative phylogenetic hypotheses were tested with the Shimodaira-Hasegawa (SH) and the Shimodaira Approximately Unbiased (AU) tests implemented in PAUP 4.0 (Swofford 2003), using 1,000 bootstrap replicates. The fit of the best Maximum

Likelihood (ML) trees (unconstrained), where *Abatus* resulted paraphyletic in agreement with the topology obtained by Bayesian inference as described above, was compared to the likelihood of the best tree with *Abatus* spp constrained to be monophyletic. The ML analysis was performed using the heuristic search algorithm with 10 replicates of random sequence addition, and the substitution model used for the COI and 16S concatenated sequences was the GTR model (Tavare 1986) as determined by Modeltest 3.7 (Posada and Crandall 1998).

Mean pairwise sequence divergence among genetic lineages suggested by the Bayesian analysis was calculated using MEGA 6.0 under the Kimura 2-Parameter model. Divergences were calculated only for COI and under this model to make them comparable with the ranges of genetic variation calculated for this marker for spatangoids by van Oosterom (2013) (Fig. A1, Appendix C2) and Ward et al. (2008); the ranges vary between 0-2% within species, between 1-5% among species and 5-20% among genera. In addition, haplotype genealogy was examined by a statistical parsimony haplotype network with 95% connection limits constructed using TCS 1.21 (Clement et al. 2000).

Results

Genetic diversity and demography of A. ingens and A. nimrodi in East Antarctica:

All *Abatus nimrodi* individuals from the Windmill Islands and Dumont D'Urville shared a single identical haplotype (null haplotype and nucleotide diversities) both in COI and 16S; in the Vestfold Hills, *A. nimrodi* populations were more diverse, with ($h = 0.17$ and $\pi = 5 \times 10^{-4}$ for 16S (Table 2). In addition, a unique 16S haplotype was

found in VH for this species. In contrast, *A. ingens* exhibited haplotype and nucleotide diversities ranging from h=0 (WI) to h=0.67 (VH) and $\pi = 0$ (WI) to $\pi = 0.004$ (VH) for CO1, and from h = 0 (DDU) to h = 0.66 (VH) and $\pi = 0$ (DDU) to $\pi = 0.004$ (VH) for 16S (Table 2). In *A. ingens*, the highest number of unique haplotypes (5) was found in VH both for COI and 16S (Table 2).

Given the lack of variation in WI and DDU in the case of *A. nimrodi*, the mismatch distribution was constructed only for the VH population of this species. The mismatch distributions for *A. nimrodi* from VH and *A. ingens* from WI is L-shaped, while *A. ingens* from VH and DDU showed bimodal patterns (Fig. 2). However, the sudden expansion model was rejected only in the case of *A. ingens* from DDU (SSD= 0.06 $p < 0.05$), indicating that this population is either in equilibrium or declining. The null hypothesis of neutrality was not rejected in any population.

Intra-specific population structure

Genetic differentiation exists among populations both of *A. ingens* and *A. nimrodi* from the different geographic locations (AMOVA, Table 2). For *A. nimrodi*, there is strong evidence of genetic differentiation between VH and the other two East-Antarctic regions, WI and DDU, with high and significant pairwise F_{ST} values ranging from 0.8 to 0.9 (Table 3). However, no differentiation was detected between WI and DDU (Table 3). *Abatus nimrodi* individuals from DDU and WI form a separate mitochondrial lineage from VH individuals (Posterior probability =1, Fig. 3) and diverge by a 2.5 % (Table 4). Moreover, *A. nimrodi* haplotypes from the two regions fall into unconnected networks at a 95% level (Fig. 4).

For *A. ingens*, all pairwise F_{ST} values are significant, indicating genetic structure exists among the three East Antarctic regions (Table 3). However, posterior

probabilities for mitochondrial lineages corresponding to geographic locations are generally weak for this species (Fig. 3). Sequence divergence between *Abatus ingens* lineages from VH, WI and DDU range from 0.2-0.8%, which is within expected levels of intraspecific CO1 divergence in spatangoids (Fig A1, Appendix C2).

Phylogenetic relationships among Abatus species

Both the topology recovered from the partitioned Bayesian analysis (Fig. 3) and the ML analysis, show the genus *Abatus* as paraphyletic. *Amphipneustes lorioli* is contained within the *A. nimrodi* clade, and the rest of the *Abatus* species form a monophyletic group. Notably, within the *Abatus* species clade, the species that have an Antarctic and subantarctic distribution (*A. philippi* and *A. cavernosus*, Fig. 1), form a different group (PP=1, Fig 3) from species with a distribution that is restricted to the Antarctic continent (*A. shackletoni* and *A. ingens*, Fig 1). However, the null hypothesis of no statistical difference between the best ML topology (*Abatus* paraphyletic) and a tree with *Abatus* constrained to be monophyletic could not be rejected (SH p= 0.06 and AU p= 0.05).

Table 2: Summary statistics, AMOVA results and molecular diversity indices per gene region and per geographic location. Statistical significance is indicated as * = p-<0.05, or ns= non-significant. N: Sample size, K: Number of Haplotypes, H: Haplotype Diversity, PS: Number of Polymorphic Sites, PIS: Parsimony Informative sites, TS: transitions, TV: transversions, **π**: Nucleotide diversity, Nucleotide composition (%C, %T, %A, %G).

Species	Gene region	F_{ST}	Geographic Location	N	K	H	PS	PIS	TS	TV	π	%C	%T	%A	%G
Abatus nimrodi	CO1	1.0*	WI	24	1	0	0	0	0	0	0	26.9	28.9	27.9	16.2
			VH	24	1	0	0	0	0	0	0	26.9	28.9	27.6	16.6
			DDU	11	1	0	0	0	0	0	0	26.9	28.9	27.9	16.2
			Total	59	2	0.49	8	8	4	4	0.012				
	16S	0.99*	WI	21	1	0	0	0	0	0	0	17.6	35.5	26.7	20.2
			VH	22	2	0.17	1	0	1	0	0.0005	17.6	35.5	26.4	20.5
			DDU	7	1	0	0	0	0	0	0	17.6	35.5	26.7	20.2
			Total	50	3	0.54	8	8	4	4	0.01				
Abatus ingens	CO1	0.61*	WI	23	1	0	0	0	0	0	0	26.9	28.9	26.4	17.8
			VH	25	5	0.67	5	3	4	1	0.004	27.4	28.6	27.2	16.8
			DDU	16	2	0.13	1	0	0	1	0.0004	26.9	28.9	26.4	17.8
			Total	64	7	0.60	9	6	4	2	0.007				
	16S	0.69*	WI	23	2	0.08	1	0	1	0	0.0003	17.0	35.8	26.4	20.8
			VH	25	5	0.66	7	6	7	0	0.004	16.9	35.9	26.7	20.5
			DDU	10	1	0	0	0	0	0	0	17.0	35.8	26.7	20.5
			Total	58	7	0.73	8	6	8	0	0.004				

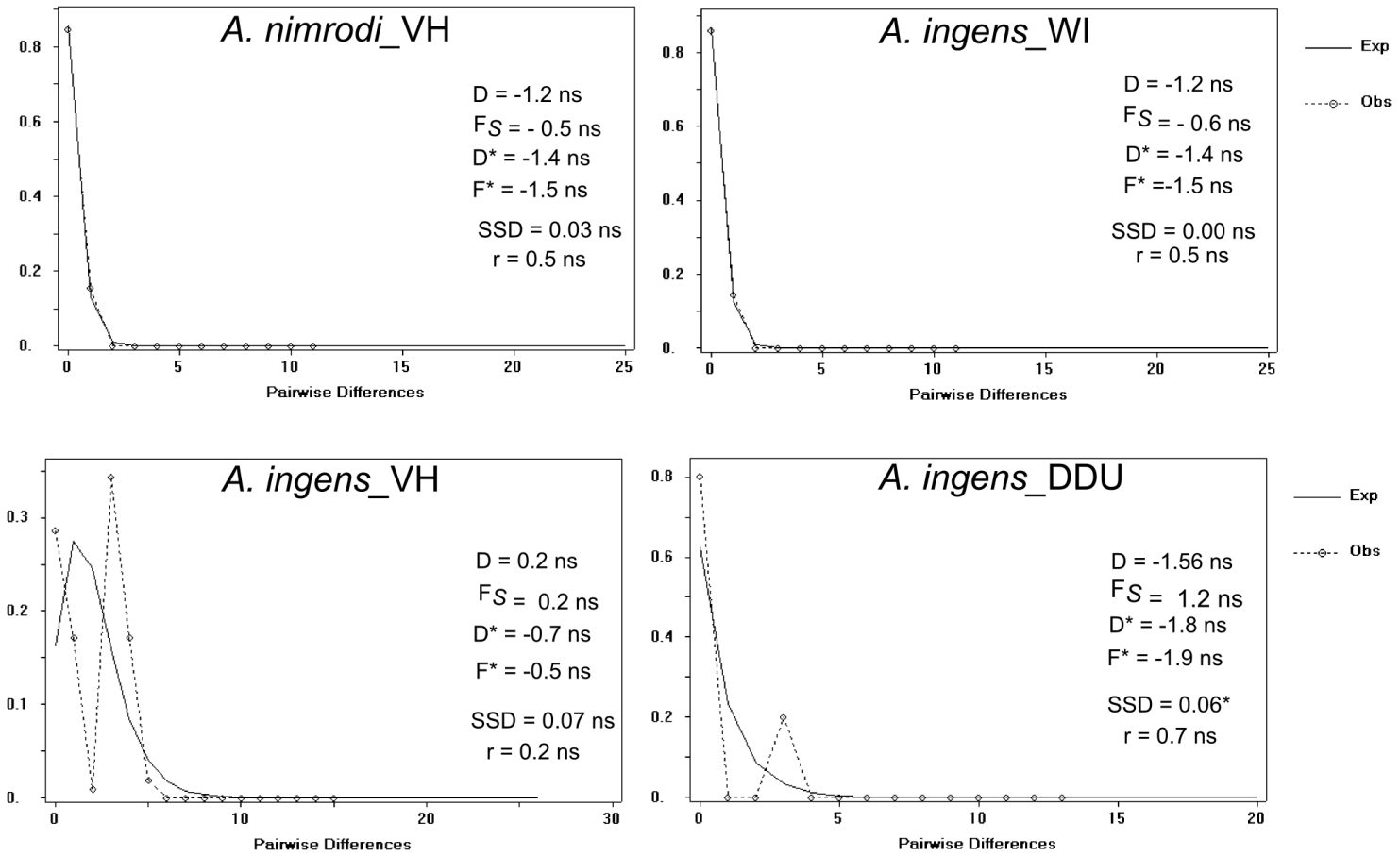

Fig.2. Mismatch distributions and Neutrality tests for *A. nimrodi* and *A. ingens* populations in East Antartica.

Table 3. Pairwise F_{ST} among the three Antarctic locations (VH=Vestfold Hills, Davis station, WI= Windmill Islands, Casey station and DDU= Dumont D'Urville station) based on concatenated sequence data. Values in upper diagonal correspond to *Abatus nimrodi* and values in lower diagonal correspond to *Abatus ingens.* Statistical significance to a 95% level is indicated with * or ns= non-significant.

Location	DDU	WI	VH
DDU	-	0	0.89*
WI	0.83*	-	0.90*
VH	0.49*	0.56*	-

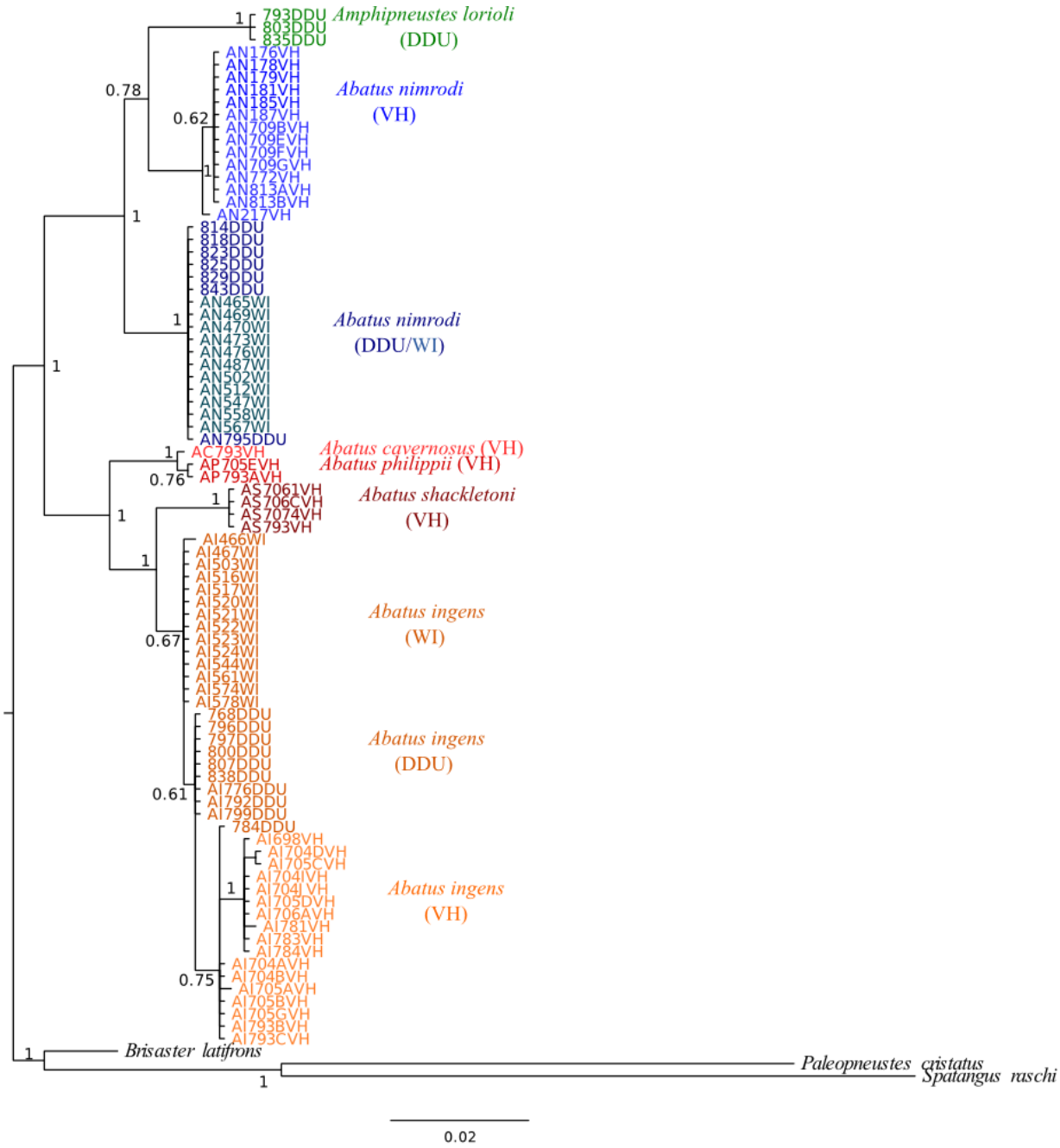

Figure 3. Bayesian tree topology obtained for COI and 16S concatenated data for five morphological *Abatus* spp. and *Amphipneustes lorioli* from East Antarctica. Node labels correspond to posterior probabilities.

Table 4. Estimates of the mean and standard errors (SE) percentage of evolutionary sequence divergence among the genetic lineages of *Abatus* spp. inferred by Bayesian analysis.

Species	**AL**		***A. nimrodi***						***A.ingens***				**AS**		**AP**		**AC**		**BL**	
Location	DDU	(SE)	VH	(SE)	WI_ DDU	(SE)	VH	(SE)	WI	(SE)	DDU	(SE)	VH	(SE)	VH	(SE)	VH	(SE)	BL	(SE)
***A. lorioli* (AL) DDU**	-	-	3.3	(0.6)	3.7	(0.7)	6.2	(1.0)	5.8	(0.9)	5.9	(0.9)	6.7	(1.0)	6.5	(1.0)	6.5	(1.0)	6.3	(0.9)
***A.nimrodi* VH**			-	-	2.5	(0.5)	5.3	(0.9)	5.1	(0.9)	5.3	(0.9)	5.1	(0.9)	5.6	(0.9)	5.6	(0.9)	5.6	(0.9)
***A. nimrodi* WI DDU**					-	-	4.9	(0.9)	4.9	(0.9)	5.1	(0.9)	5.1	(0.9)	5.3	(0.9)	5.3	(0.9)	5.6	(0.9)
***A. ingens* VH**							-	-	0.8	(0.0)	0.6	(0.0)	2.3	(0.5)	2.6	(0.6)	2.6	(0.6)	5.4	(0.8)
***A ingens* WI**									-	-	0.2	(0.0)	1.8	(0.5)	2.6	(0.6)	2.6	(0.6)	5.4	(0.8)
***A ingens* DDU**											-	-	2	(0.5)	2.8	(0.6)	2.8	(0.6)	5.6	(0.9)
***A.shackletoni* (AS)**													-	-	3.4	(0.7)	3.4	(0.7)	5.4	(0.8)
***A. philippii* (AP)**															-	-	0.1	(0.0)	4.9	(0.8)
***A.cavernosus* (AC)**																	-	-	4.9	(0.8)
***Brisaster latifrons* (BL)**																			-	-

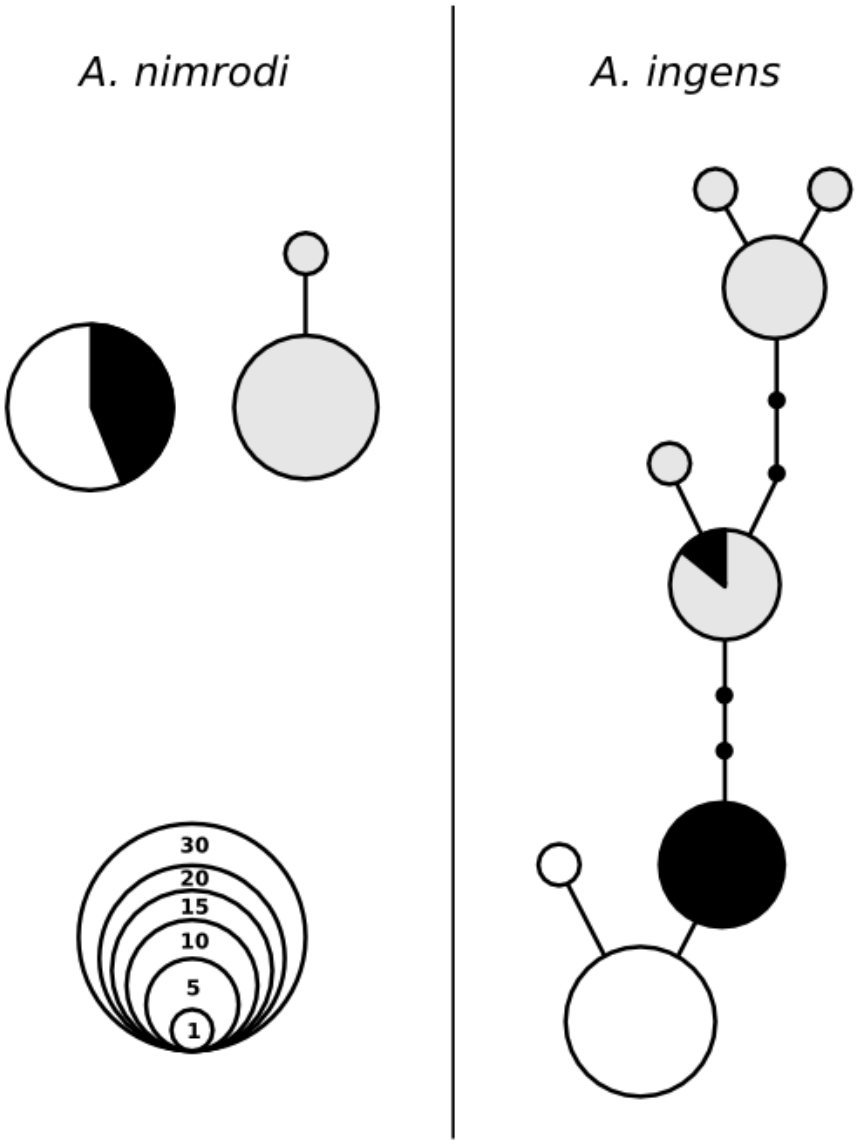

Figure 3. Haplotype networks (COI and 16S concatenated data) for *A. ingens* and *A. nimrodi*. Hollow nodes represent unsampled haplotypes. Different colours correspond to sampling sites: Black: DDU, White: WI and Grey: VH.

Discussion

Effects of historical glaciations on Abatus populations

While the idea of glacial refugia located on the Antarctic shelf has existed for more than 30 years (Dayton & Oliver 1977), evidence of the existence of refugia from molecular studies has started to accumulate only recently (Fraser et al. 2012). The genetic diversity patterns observed in *A. ingens* and *A. nimrodi* are consistent with patterns observed in other Antarctic brooding species that survived as small populations in shelf refugia and underwent severe bottlenecks during Quaternary glaciations (Allcock & Strugnell 2012). Low genetic diversity is expected in formerly glaciated areas with a small number of haplotypes dominating large areas (Maggs 2008), e.g. *A. nimrodi* individuals collected at WI and DDU share a single haplotype despite being separated by over 1000 km. In contrast, both *A. nimrodi* and *A. ingens* exhibit relatively higher genetic diversity including a higher number of unique haplotypes in VH, suggesting long persistence in glacial refugia in that area. Moreover, it has been proposed that during the LGM, coastal drainage systems maintained areas free of grounded ice on the shelf of Prydz Bay, where VH is located (Domack et al. 1998, O'Brien et al. 1999).

Populations of benthic marine species can be expected to have expanded from refugia since the present day interglacial climate was established in the Antarctic and habitat became available for recolonization (Provan & Bennett). The *A. nimrodi* population from VH and the *A. ingens* population from WI exhibited unimodal mismatch distributions, as expected under a population expansion model. The hypothesis of

population expansion could not be rejected for *A. ingens* in VH, however this should be considered cautiously since the genetic signature of a population expansion can be difficult to distinguish from the signature of a population decline (Rogers & Harpending 1992), and this population shows a bimodal mismatch distribution combined with positive D and F_S indices characteristic of a declining population. The hypothesis of expansion was rejected for *A. ingens* in DDU, suggesting this population is either at equilibrium or experiencing a decline. The expansion and retreat of ice during the LGM has not been symmetric around Antarctica (Anderson et al. 2002). The oldest radiocarbon ages of glacial marine deposits on the shelf, depicting estimated minimum ages of ice retreat, vary among our study areas (Anderson et al. 2002): the retreat appears to have been most recent in DDU (9 Ka BP), intermediate in WI (13 Ka BP) and occurred first in VH (20 Ka BP). Therefore, it is possible that *A. ingens* recolonized the DDU area more recently than VH or WI, resulting in less time for the evolution of genetic diversity in this more recent population.

Intra-specific population structure

Genetic structure was detected among the three East-Antarctic regions studied for *A. ingens*, consistent with its brooding mode of development, while only the VH population of *A. nimrodi* could be distinguished from WI and DDU, which were identical. The lack of genetic structure observed for *A. nimrodi* (Table 3, Fig. 3) between the WI and DDU regions (separated by over 1,000 km) is not expected for a brooding species, where geographic distance alone could promote isolation and genetic differentiation. This result most likely reflects common ancestry and recolonization of the two areas (WI-DDU) from the same stock in combination with

low mutation rates. Although less likely, long distance dispersal could be facilitated by either the Antarctic Circumpolar Current (ACC) in the Eastward direction or the Antarctic Coastal Current in the opposite direction. Large-scale genetic homogeneity has been reported in several Antarctic benthic brooders (Hunter & Halanych 2008, Allcock et al. 2011, Baird et al. 2011) and strong evidence exists for a long-term influence of the ACC on the distribution of the Southern Ocean benthos (Griffith et al. 2009). As a mechanism for long distance dispersal in brooders, passive rafting of juveniles or adults on floating substrata has been proposed (Baird et al. 2011). Dayton et al. (1969) directly observed the removal of benthic organisms by anchor ice (which forms on the seafloor but often becomes detached and floats) in the Ross seacoast in East-Antarctica, including entrapped motile organisms such as the regular sea urchin *Sterechinus sp.* However, until *A. nimrodi* populations from WI and DDU are surveyed using markers that exhibit polymorphism it is not possible to refute a combination of genetic isolation and low mutation rates.

Phylogenetic relationships among Abatus species

The phylogenetic analysis of *Abatus* spp. suggests that the species composition of the genus should be revised, particularly regarding the classification of *A. nimrodi* in the genus *Abatus*. Our results show that *A. nimrodi* more closely related to *Amphipneustes lorioli* than to the rest of the *Abatus* species, based on mitochondrial DNA data. Support for an alternative topology with *Abatus* species constrained to be monophyletic could not be rejected, however, the p-values obtained were small (SH p=0.06 and AU p=0.05) and these nonparametric are often conservative with reduced power when compared with parametric tests (Goldman et al. 2000). Moreover, the support for the *Abatus nimrodi- Amphipneustes lorioli* clade was high (p=1, Fig. 3)

and the mean pairwise genetic divergences between *A. nimrodi* and *A. lorioli* (3.3% and 3.7% for VH and WI-DDU *A. nimrodi* lineages respectively) suggest that these species may be considered congeneric. Similarly, the level of sequence divergence between *A. nimrodi* and the other *Abatus* species (*A. shackletoni* 5.1%, *A. ingens* 4.9-5.3%, *A. philippii* 5.3% and *A. cavernosus* 5.6%) are within the range of variation observed for different genera within Spatangoidea (5-20%, Fig. A1, Appendix C2). Future investigation including morphological examination is required. Interestingly, *Abatus* species with distributions including the Antarctic and subaSub-Antarctic regions (*A. cavernosus* and *A. philippii*) formed a distinct lineage within the *Abatus* species that are restricted to the Antarctic continent, and there was very low divergence between these species (in the intraspecific range for known divergences within the spatangoid group).

The genetic divergence between the *A. nimrodi* VH lineage and the WI-DDU lineage was 2.5 % for COI, which is slightly above the variation range (0-2%) observed during intraspecific comparisons for this gene within the Spatangoidea (Fig. 1, A C2), and could indicate cryptic speciation. Furthermore, the high F_{ST} values (> 0.89) between WI-DDU and VH populations are indicative of reproductively isolated species. Haplotypes of the two lineages fall into individual haplotype networks (Figure 4) obtained by statistical parsimony analysis with 95% connection limits, which has been widely assumed to represent cryptic speciation (Chen et al. 2010). However, the networks connect under 90% limits (results not shown) and the fact that the two lineages are not sympatric suggests that the deep divergence between them could also be explained by long-term isolation, e.g. in different glacial refugia. Future examination of morphological variability and potential for reproductive isolation between these lineages should help elucidate whether the observed mitochondrial

DNA pattern represents on-going allopatric speciation or intraspecific geographic structuring.

Acknowledgements

The authors thank the Davis Station summer marine teams of 2009/10 and 2012/13 for assistance in field collections and laboratory processing of samples, particularly Jonathan Stark, Ian Aitkenhead and Patricia Corbett. This study was supported by an Australian Antarctic Science Grants awarded to K. Miller (Project 3051) and C. King (Project 4100). Samples were collected in Terre Adélie (dumont d'Urville station) thanks to the "REVOLTA" campaigns of the French Polar Institute (Institut Paul-Emile Victor, program 1124 directed by Guillaume Lecointre).

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Chapter 3

Population structure and long-term decline in three species of heart urchins (*Abatus* spp.) near-shore in the Vestfold Hills region, East Antarctica.

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Authors' contributions to this manuscript:

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Christopher Burridge: assisted with data analysis and interpretation and provided editorial revisions to the manuscript.

Catherine King: assisted with the idea formulation, provided editorial revisions to the manuscript and obtained funds to develop the project.

Karen Miller: assisted with the idea formulation, data analysis and interpretation, editorial revisions to the manuscript. She also obtained the funds to develop the project.

The undersigned agree with the above stated proportion of work undertaken for this manuscript that is currently in preparation to be submitted for publication

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Abstract

Understanding the patterns of population genetic structure in benthic species is essential to preserve marine diversity in Antarctica. Genetic structuring of populations can show signals of historic processes (such as refugial populations in ice-free areas during Pleistocene glaciations), or microevolutionary processes (e.g. gene flow, selection, drift). In addition, anthropogenic factors such as potential impacts of pollutants in wastewaters discharged to coastal waters adjacent to Antarctic stations can produce changes in the magnitude and distribution of genetic diversity (e.g. by selection of tolerant genotypes or diversity loss associated with increased mortality). We used seven microsatellite markers to examine genetic variation in populations of three sympatric Antarctic irregular sea urchin species from the order Spatangoida (*Abatus ingens*, *A. shackletoni* and *A. philippii*), all with brooding life history strategies. Heart urchins were collected from sites at varying distances from the outfall of wastewater discharge from Davis Station. We found genetic evidence of a long-term population decline in all three species, but the estimated timing at the decline precedes any anthropogenic activities. Two genetic clusters inferred in *A. ingens* and *A. shackletoni* suggest secondary contact after population differentiation in glacial refugia. Life history is not a good predictor of fine-scale population structure in these species, with gene flow possible at distances of 5 km. There was no evidence for an effect of pollution from Davis Station on genetic variation, however, potential limitations to detect such an impact are discussed. The reduced effective population size observed for these Antarctic benthic species highlights their fragility and the need for conservation concern**.**

Introduction

The recognition of the vulnerability of Antarctic benthic biodiversity to global warming has raised the urgency of improving our understanding of the biological processes critical for effective ecosystem management and conservation (Barnes & Peck 2008, Barnes & Souster 2011). The study of genetic diversity and population structure is essential to inform conservation initiatives, e.g. to design appropriate boundaries for marine reserves that both protect diversity within, and ensure dispersal across those boundaries (Palumbi 2003). Most of our knowledge of genetic diversity in the Antarctic benthos is from large spatial scale (i.e., ca. 1000 km) phylogeographic studies using mitochondrial DNA markers. Intraspecific genetic structure at finer scales is less understood, particularly in Eastern Antarctica (but see Baird et al. 2012). Patterns of genetic population structuring can help elucidate how species survived historical climatic events in Antarctica, such as glaciations (Allcock & Strugnell 2012), as well as provide an insight into contemporary factors that may affect population divergence. Possible factors include microevolutionary processes (e.g. selection, drift) and the species' capability for dispersal and gene flow (Thatje 2012). Comparative multispecies approaches are particularly well suited to help elucidate the main processes shaping population genetic differentiation in the Antarctic benthos, given potential idiosyncrasies of individual species responses (Baird et al. 2011).

The advance of grounded ice sheets across the Antarctic shelf has been advocated as a process that largely eradicated benthic communities during the Last Glacial Maximum (Thatje et al. 2005). Different hypotheses have been proposed to explain how the benthic fauna survived glacial periods: (i) in the deep sea (Thatje et al. 2005, Thatje et al. 2008), (ii) in ice free refugia on the Antarctic shelf (Rogers 2007), and (iii) in subantarctic islands (Gonzalez-Wevar et al. 2013). In a recent review of the

genetic patterns observed for Antarctic benthic species, Allcock & Strugnell (2012) show that populations of low dispersive species considered to have recolonized from deep sea refugia have higher haplotype diversity than those considered to have survived in shelf refuges, since the latter would have experienced massive reductions in their effective sizes. Isolation in multiple glacial refuges in combination with subsequent limited dispersal has resulted in deep genetic population divergences involving cryptic speciation in numerous taxa (Allcock & Strugnell 2012).

The Antarctic benthic invertebrate community comprises a high proportion of brooding species (Dell 1972). To our knowledge, only a few studies have used fast evolving markers to infer fine-scale population genetic structure in Antarctic brooding species (Baird et al. 2012, Hoffman et al. 2013). These studies report patterns consistent with the expectation of direct development hindering connectivity and promoting population differentiation. Hoffman et al. (2013) found genetic population structure in the gastropod *Margarella antarctica* at a scale of less than 10 km in the Antarctic Peninsula. Baird et al. (2012) found limited gene flow across distances of 30 km in the amphipod *Orchomenella franklini* around Casey and Davis stations in East Antarctica. In addition, the subantarctic heart urchin *Abatus cordatus* (endemic to the Kerguelen Islands) shows evidence of population structure on scales of a few kilometres, as inferred by two allozymes (Poulin & Feral, 1994) and by three microsatellite and two introns markers (Ledoux et al. 2012). Together, these studies suggest that similar low dispersal capabilities for other Antarctic brooding *Abatus* species may be expected.

Effective population size is a critical parameter for conservation purposes as it can help predict the effects of inbreeding and neutral genetic diversity loss due to genetic drift in natural populations (Frankham, 1995). Molecular studies using fast-evolving

markers can help to detect recent changes in a population's effective size (Beaumont 1999). For example, numerous studies have detected population declines induced in the last decades or centuries as a result of anthropogenic environmental changes (Keller et al. 2005, Goossens et al. 2006, Olivieri et al. 2008). In Antarctica, it has been impractical to directly measure population sizes of benthic marine invertebrates, and therefore genetic data represents a valuable tool in this context to test for effective population size changes. Recently, differences in community composition and reduced benthic biodiversity were associated with environmental contamination by pollutants discharged to coastal waters adjacent to two stations in East Antarctica (Stark et al. 2014). Ostracods, cumaceans and echinoderms were particularly sensitive to the contamination (Stark et al. 2014). Changes in population effective size, as well as in the magnitude and distribution of genetic diversity of those benthic species can be expected from either selection or stochastic diversity loss (genetic drift) associated with increased mortality in areas impacted by pollution. However, such expectations have rarely been tested, but Baird et al. (2012) found reduced genetic diversity in an amphipod associated with localized pollution impacts at Davis and Casey stations in East Antarctica.

Here we compared fine-scale genetic structure of three sympatric species of brooding Antarctic heart urchins (*A. ingens, A. shackletoni* and *A. philippii*) around Davis Station. The largest distance between any two sampling sites was 5 km and sites were exposed to varying degrees to the wastewaters from the station. Populations are expected to have expanded since the LGM, approximately 15 kyr ago (Huybrechts 2002), but a recent local population decline may have resulted from the impact of anthropogenic pollutants. Furthermore, patterns of fine scale genetic structure are expected to reflect low dispersal abilities and be similar among species based on

sympatry and the shared presence of a brooding life history. A set of microsatellite DNA markers were developed that cross-amplified in the three species, thereby reducing potential bias during interspecific comparisons. Our main aims are: first, to compare the demographic history and patterns of population genetic diversity and structuring of three brooding heart urchin species, in order to improve our understanding of the processes affecting population differentiation in the Antarctic benthos. Secondly, to test for potential impacts of pollution from the Davis Station wastewater discharge on the genetic diversity, population structuring, and effective population size of these species.

Materials and methods

Study species and area

The genus *Abatus* (Schizasteridae, Spatangoida) comprises 11 known species endemic to the subantarctic and Antarctic, five of which occur around the coastline of Davis station (A. Miskelly, pers. comm.). All species have direct development (lacking dispersive larval stages) and their offspring are protected within the female's brood pouches until they are released to the environment as juveniles (Poulin & Feral 1994). Individuals of three species of *Abatus* were collected in the nearshore area surrounding Davis Station (68°35'S, 77°58'E) located on Prydz Bay, Vestfold Hills in Princess Elizabeth Land, East Antarctica (Fig. 1, Table 1): *A. ingens* (N= 112), *A. shackletoni* (N= 93) and *A. philippii* (N= 44).

A recent evaluation of the dispersion and dilution of wastewater at Davis Station found that elevated levels of toxic contaminants (metals, polybrominated diphenyl ethers, nutrients and faecal sterols) are accumulating within 2 km of the station's outfall (Stark et al. In Press). Moreover, histological alteration was observed in

Antarctic rock-cod collected in close proximity to the station's outfall, with more severe pathologies in fish from sites within 800 m of the outfall compared to fish sampled from further away (Corbett et al. 2014). Samples were collected from five sites located at different distances from the station's wastewater outfall. Three of the sites are considered heavily impacted: the OF, OF1, and OF2 are within 25 m from the outfall pipe. The two control sites are considered to have negligible impact from the wastewater discharge; OF3 is located approximately 2.4 km from the outfall pipe to the North-East, and OF4 approximately 2.6 km to the South-West (Fig 1). Moreover, both OF3 and OF4 sites are removed from the predominant longshore flow that transports the wastewater plume in a south easterly direction along the coast (Stark et al. In Press). Samples from OF1 and OF2 were collected at 4.5-7.0 m deep and 10-12 m respectively during the 2009/2010 season, and samples from OF, OF3 and OF4 were taken at depths <4 m, during the 2012/2013 season.

Microsatellite development and genotyping

Microsatellite markers were developed from the genome of three *Abatus ingens* individuals. An enriched library was made in July 2010 by Ecogenics GmbH (Zurich, Switzerland) from size-selected genomic DNA ligated into SNX forward/SNX reverselinker (Hamilton et al. 1999) and enriched by magnetic bead selection with biotin-labelled $(CT)_{13}$, $(GT)_{13}$, $(AAC)_{10}$ and $(AAG)_{10}$ oligonucleotide repeats (Gautschi et al. 2000a, Gautschi et al. 2000b). Of 528 recombinant colonies screened, 315 gave a positive signal after hybridization. Plasmids from 152 positive clones were sequenced and primers were designed for 32 microsatellite inserts, of which all were tested for polymorphism in 15 *A. ingens* individuals. Seven loci were suitable for population-level analyses (Table 1). Additionally, cross-species amplification was successful in *A. shackletoni* and *A. philippii* (Table A1).

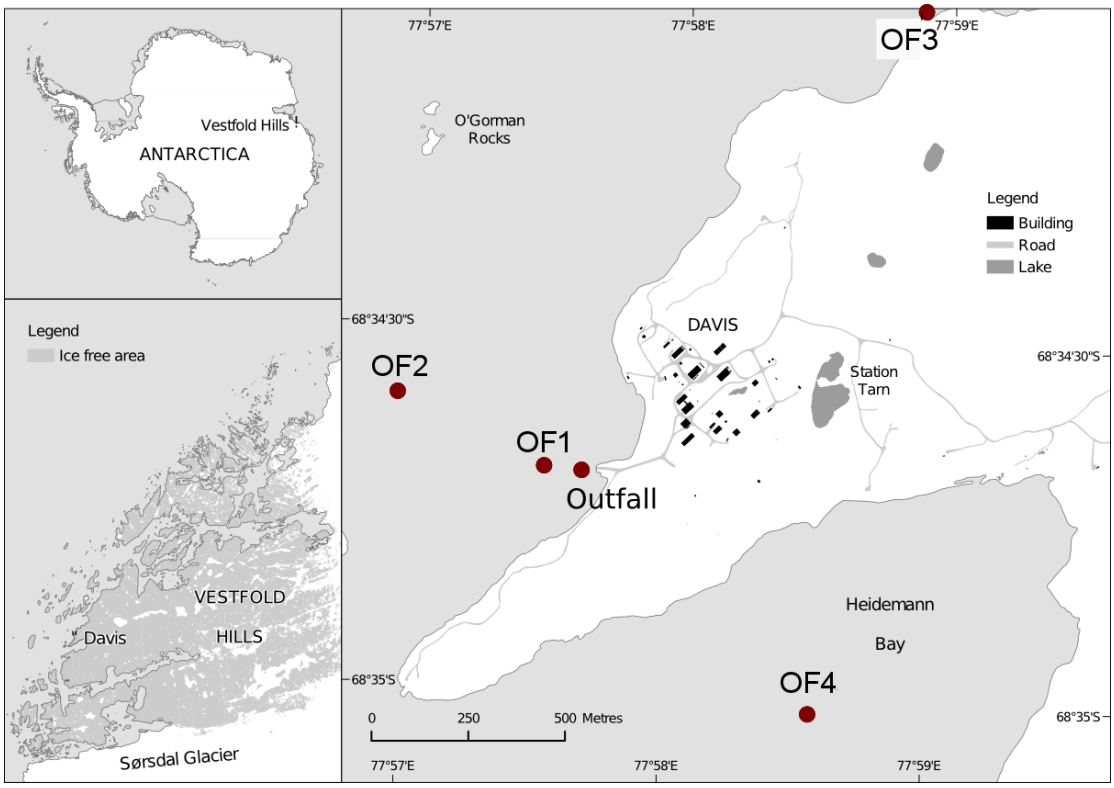

Fig. 1 Map showing the five sampling sites around Davis station, East Antarctica. The Outfall (OF), OF1 and OF2 sites are considered highly impacted by the station's wastewater discharge; OF3 and OF4 are reference non-impacted sites.

Genomic DNA was extracted from approximately 20 mg of gonad tissue from each individual, using the DNeasy Blood and Tissue Kit (QIAGEN) following the manufacturer's instructions. Three multi-locus PCR panels were designed (Table 1). Multiplex PCRs were performed using 12.5 µL reactions containing Multiplex PCR Mastermix (QIAGEN), Primer mix (final concentration varied for each primer, see details in Appendix C3), and 10−100 ng of template DNA. Fragments were separated on an automated sequencer (CEQ 8000, Beckman Coulter) and fragment size was scored using the CEQ 8000 Genetic Analysis System software version 8.0.

Genotypes were examined with MICROCHECKER (Van Oosterhout et al. 2004) to detect potential genotyping errors resulting from null alleles, large allele drop-out or stuttering. Datasets were formatted using Convert (Glaubitz 2004) and PGDSpider 2.0 (Lischer & Excoffier 2012) for use in other software packages. The null hypothesis of genotype frequencies under Hardy-Weinberg (H-W) equilibrium and linkage disequilibrium (LD) among loci were tested by exact tests (Guo & Thompson 1992) and G-tests (Weir 1996) respectively, using Genepop 4.0 (Raymond & Rousset 1995). A sequential Bonferroni correction *sensu* Rice (1989) was applied to significance levels for tests of HWE and LD. Departures from H-W expectations were further examined by calculation of the Weir & Cockerham's (1984) fixation index, F_{IS}, to determine the excess ($F_{IS} < 0$) or deficit ($F_{IS} > 0$) of heterozygotes.

Table 1. Characterization of seven microsatellite loci developed for *Abatus ingens.*

Multiplex	PCR	Locus	Primer Sequence	Dye	Repeat
1	[30s at 95 °C, 90s at 62 °C, 60s at 72 °C] x34	Ab_07	F: CTCGAGACTCCATCTTCTAGTCC R: CACAGATACGTCTTACCACTTCG	D4	$(TCT)_{15}…(TCA)_8$
		Ab_15	F: GCCATTAAGTTCCCCAAAGAG R: CGGTTTGCTTGTCATTTTTAGC	D2	$(GTT)_{22}$
2	[30s at 95 °C, 90s at 62 °C, 60s at 72 °C] x34	Ab_16	F: CCCCTCGCATCATCTGTAAG R: GAAGAACAACAACAAGAACTTGAATAG	D4	$(CAT)_{10}$
		Ab_17	F: GAGCAAACCACCCGTTG R: ACTGTGCGTCAAATCAAACG	D3	(GAA)8
		Ab_31	F: GAGTGTGGTGCTGTGAGGTG R: TACAATGTGCCTCCCGTCTC	D2	(GCT)6
3	[30s at 95 °C, 90s at 60 °C, 60s at 72 °C] x34	Ab_18	F: CATTTATCTGTCATCCTTTCACG R: TGGAGGAAAAATAAACGAGGAG	D4	(TCT)8
		Ab_29	F: GGCCGGGAGGATACTTTTAC R: GGGTCAAGGTAAGGAGACAG	D3	(ACA)6

In order to identify loci under the influence of selection a Bayesian approach was implemented using BayeScan 2.1 (Foll & Gaggiotti 2008). This method uses differences in allele frequencies between subpopulations and a common migrant gene pool, which is measured by a specific F_{ST} coefficient that is decomposed into a population-specific component and a locus-specific component. Selection is inferred when the locus-specific component is necessary to explain the observed pattern of diversity. The method has two main advantages: it considers a realistic ecological scenario for our study system, and it can handle very small sample sizes with no risk of bias as it incorporates the uncertainty of allele frequencies due to small sample size. Each species data set was tested separately, using the default parameters for the Monte Carlo Markov Chain (total number of iterations = 10^5), except the number of pilot runs that was doubled to 40.

Genetic diversity among species and populations

Mean observed and expected heterozygosities, number of different and private alleles, and percentage of polymorphism over all loci for each sampling location were calculated using GenAlEx 6.5 (Peakall & Smouse 2012). In order to compensate for differences in sample size when comparing genetic diversity among geographic sites, Allelic Richness and Private Allelic Richness (unique alleles in a population) were calculated using the rarefaction method implemented in HP-RARE (Kalinowski 2005). Three group comparisons were carried out by permutation tests in FSTAT 2.9.3 (Goudet 1995) using 10,000 permutations: (i) expected heterozygosity (He) was compared among species (ii) allelic richness (A_R) was compared among sites within species, and (iii) A_R was compared between impacted and non-impacted sites within species.

Genetic differentiation

Cryptic speciation is common in marine invertebrates (Knowlton 1993), and particularly so within the Antarctic benthos (Baird et al. 2011). Therefore, we performed a Bayesian hierarchical approach implemented in STRUCTURE (Pritchard et al. 2000) in order to examine genetic clustering among all individuals. Subsequently, data for each species was analysed separately to examine intra-specific groupings. Locus 29 was not included in the analysis since it was monomorphic in two of the species (Table A1). To estimate the number of genetic groups within each species a LOCPRIOR model, with sampling location as prior information, was set to assist clustering (Hubisz et al. 2009). In each case, five iterations for each K (K from 1 to 7) were run under the Admixture model with correlated allele frequencies. A total of 450,000 Markov Chain Monte Carlo replicates was run for each analysis, with a burn-in length of 150,000. Consistent parameter values were obtained from independent runs indicating an appropriate run length for parameter accuracy. The most likely K was determined by the highest posterior probability method (Pritchard et al. 2000) and the Evanno et al. (2005) method as implemented in STRUCTURE HARVESTER 0.6 (Earl & vonHoldt 2012).

Genetic differentiation among sampling sites within each species was tested using Analysis of Molecular Variance AMOVA (Excoffier et al. 1992) based on F_{ST} (Meirmans 2006), implemented by GenAlEx. A second AMOVA was conducted to test for differentiation among sites grouped according to impact by pollution (three impacted sites vs. two control sites). Tests of significance were based on 10,000 permutations. In addition, diversity partitioning measure G_{ST} (Nei 1973), and differentiation statistics G'_{ST} (Hedrick 2005) and D_{EST} (Jost 2008), were calculated for each pair of sampling locations to explore the spatial patterns of differentiation, using

the R package DiveRsity 1.9 (Keenan et al. 2013). Finally, to analyse trends observed in the data, confidence intervals at the 95% level were calculated for each statistical measure and visualisation plots were obtained with DiveRsity. Significance for the pairwise D_{EST} statistics were calculated with GenAlex based on 10,000 permutations.

Demographic parameters

We used a Bayesian coalescent-based approach to estimate posterior probabilities for demographic parameters as implemented in Msvar 1.3 (Beaumont 1999a, Storz & Beaumont 2002). The method's demographic model has four estimated parameters: current effective population size (N_0), ancestral effective population size (N_1), time since the demographic change occurred (*Ta*), and mutation rate (μ) of microsatellite loci (assumed to evolve under a stepwise mutation model). It assumes a panmictic population of size N_0 that has changed (expanded or declined) exponentially since *Ta* years ago. Therefore, for each species we used a subsample corresponding to a single genetic cluster inferred by STRUCTURE (with Q > 0.9) and pooled from sampling sites presenting non-significant pairwise differentiation measures. We included 43 *A. ingens* individuals (pooled from OF and OF1), 37 *A. shackletoni* individuals (pooled from OF and OF1) and 27 *A. philippii* individuals (pooled from OF and OF3).

Three Msvar runs were performed for each of the three species; run lengths were of $1x10^9$ iterations with a thinning interval of 20,000 steps. Different starting values of the model parameters and random seeds were set for each independent run. In order not to favour neither an hypothesis of population decline nor a population expansion, the same prior means for the ancestral and the current population sizes were chosen (N0=N1=1,000). The mean prior time for a population size change was 18,000 years, which is approximately the time since the last glacial maximum. High variances were

chosen for prior distributions in order to allow for high uncertainty in the prior mean values chosen and therefore make them "uninformative". Finally, we assumed a generation time of 20 years (average parental age in the population) based on the fact that Antarctic species are expected to have longer life spans than temperate echinoids (that can live 10−20 years), with the Antarctic species possibly reaching a maximum age of 50 years (Brey 1991).

Convergence among the three MCMC runs was assessed visually and by using the Gelman & Rubin's (1992) diagnostic implemented in the package CODA 0.16-1 (Plummer et al. 2006) in R. The first 10% of the total number of steps were discarded (burn-in). The posterior distributions of the model parameters were estimated by combining the three convergent runs to improve precision. Median values and 95% Highest Posterior Density (HPD) intervals for each parameter were estimated with CODA. In order to detect changes in population size, and the magnitude of such change, we calculated the mean of the ratio between current and past population size (r) as the $\log_{10}(r) = \log_{10}(N_0/N_1)$ following Chikhi et al. (2010). In order to evaluate the strength of evidence for a population decline versus a population expansion we performed a Bayes Factors (BF) analysis in R for each species. Bayes Factors were calculated as the ratio between the number of states in the chain with negative values of r (population decline) over the number of states in the chain with positive values of r (population expansion). Bayes factors equal to 1 indicate similar evidence for the two scenarios, while BF higher than 1 favour the population decline hypothesis, and values higher than 7 are considered significant (Storz & Beaumont 2002).

Results

Genotyping error and neutrality tests

Allele frequencies by locus and sampling site for the three species are shown in Fig. A1 (Appendix C3). Two out of 105 LD tests were significant for each of *A. ingens* and *A. shackletoni* (none in the case of *A. philippii*), but no test remained significant after sequential Bonferroni correction. Two out of 35 H-W exacts tests remained significant after the sequential Bonferroni for *A. ingens*, six out of 35 for *A. shackletoni* and one out of 21 in the case of *A. philippii* (Table A2, Appendix C3). Departures from H-W were all heterozygote deficits (positive F_{IS} values) but they did not involve a specific locus in multiple sampling sites, except for the case of Ab_18 in *A. shackletoni* (Table A2, Appendix C3). Therefore, subsequent analyses were performed both including and excluding this locus, but no effects on the results were detected. The same approach was taken with two outlier loci (locus Ab_16 and Ab_17) identified with BayeScan, for *A. shackletoni*. Negative alphas obtained for both outlier loci are indicative of balancing selection. No outlier loci were detected for *A. ingens* or *A. philippii*.

Genetic diversity

Expected heterozygosities ranged from 0.38 to 0.49 in *A. ingens*, from 0.53 to 0.56 in *A. shackletoni*, and from 0.43 to 0.46 in *A. philippii* (Table 2). Allelic richness and private allele diversity were not significantly different among sites in any of the three species, or within species with respect to the distance to the station outfall (Table 2). However, comparisons among species showed that *A. shackletoni* is genetically more diverse than *A. ingens* and *A. philippii* ($P < 0.01$). There was no difference in genetic diversity between *A. ingens* and *A. philippii*.

Table 2. Genetic diversity indexes and sampling size per *Abatus* species and per sample location at Davis Station, East Antarctica.

Species	Sampling Location	N	Mean ± SE H_O	Mean ± SE H_E	Allelic Richness	Mean ± SE No. of Alleles	% of polymorphic loci	No. of Private Alleles	Private Allelic Richness
A. ingens	**OF**	22	0.34±0.1	0.38±0.1	2.90	3.29± 0.8	71.4%	0	0.00
	OF1	36	0.49±0.1	0.45±0.1	3.39	4.57±1.3	85.7%	5	0.42
	OF2	16	0.41±0.1	0.38±0.1	2.33	2.86±0.6	71.4%	0	0.00
	OF3	17	0.43±0.1	0.42±0.1	3.09	3.57±0.9	71.4%	1	0.33
	OF4	21	0.51±0.1	0.49±0.1	3.45	4.71±1.0	85.7%	3	0.42
A. shackletoni	**OF**	17	0.40±0.1	0.53±0.1	3.10	4.0±0.9	85.7%	1	0.09
	OF1	32	0.56±0.1	0.56±0.1	3.62	5.7±1.1	100%	7	0.49
	OF2	7	0.61±0.1	0.54±0.1	3.13	3.6±0.29	100%	2	0.47
	OF3	19	0.49±0.1	0.53±0.1	2.96	4.14±1.1	85.7%	2	0.23
	OF4	18	0.48±0.1	0.55±0.1	3.15	3.8±0.9	85.7%	1	0.22
A. philippii	**OF**	12	0.39±0.1	0.43±0.1	2.49	2.7±0.4	85.7%	1	0.15
	OF3	15	0.36±0.1	0.46±0.1	2.54	2.7±0.5	85.7%	2	0.23
	OF4	17	0.38±0.1	0.43±0.1	2.61	2.8±0.4	85.7%	4	0.37

Genetic differentiation

When data from all individuals were included in the STRUCTURE analysis, three genetic groups were inferred corresponding to the three *Abatus* species sampled (Fig 2). Notably, four individuals showed admixed genotypes between *A.ingens* and *A. philippii*, suggesting hybridization (Fig. 2). Potential hybrid individuals were removed from further analyses. Patterns of spatial population structure differed among species. Two genetic clusters were inferred with STRUCTURE for both *A. ingens* and *A. shackletoni* (Fig. 2). Individuals of *A. ingens* belonging to both genetic groups were spread across sampling sites, and many were admixed in the same site (Fig. 2). *Abatus shackletoni* individuals from OF1 and OF2 correspond to one of inferred genetic groups, most individuals from OF3 and OF4 correspond to the other inferred group (despite being separated by approximately 5 km), and individuals from the OF either correspond to one of the groups or are admixed (Fig. 2). Only a single genetic group was inferred for *A. philippii* with STRUCTURE (results not shown).

The AMOVA shows significant genetic differentiation among sampling sites in *A. shackletoni* (F_{ST}= 0.04, P< 0.01) and *A. philippii* (F_{ST}= 0.045, P< 0.01), while the null hypothesis of no genetic differentiation among sites could not be rejected in *A. ingens* (F_{ST}= 0.007, P= 0.12). A significant proportion of the genetic variation was explained by pollution impact in *A. shackletoni* (AMOVA, F_{CT}= 0.06, P< 0.01), but not for *A. ingens* or *A. philippii* (Table 3). Confidence intervals for the diversity partitioning measures, $G_{ST,}$ and differentiation statistics, G'_{ST} and $D_{EST,}$ mostly overlap (Table A3, Appendix C3) and all estimates were significantly correlated: $R_{GST- G'ST}$= 0.99 (P<0.01), $R_{GST- DEST}$= 0.87 (P<0.01), and $R_{DEST- G'ST}$= 0.87 (P<0.01). We therefore focused on D_{EST} for pairwise comparisons among sites (Fig. 3). The most differentiated sites for *A. ingens* are OF2 relative to OF4 (P < 0.01). In the case of *A.*

shackletoni, the most differentiated sites were OF1 relative to OF4 ($P < 0.01$) and OF1 relative to OF3 ($P < 0.01$). Finally, for *A. philippii*, individuals from OF were differentiated from OF4 ($P < 0.01$), but no other pair of sites were differentiated. Despite being separated by the greatest distances in our sample scheme, no significant differences were observed between non-impacted sites for any of the three species.

Demographic history

Bayesian estimation of current and ancestral effective population sizes shows a population decline in all three *Abatus* species in the Vestfold Hills (Table 4, Fig. 4). Varying the starting values and parameter priors did not alter the results. The three species had median ancestral effective population sizes of approximately 30,000 to 80,000 individuals (Table 4), which decreased by over 90% to their current effective sizes of less than 1,000 individuals. Bayes Factors analysis (BF) consistently shows that evidence for a scenario of population decline is significantly stronger than a scenario of population expansion (Table 4). Median values for the posterior distributions of *Ta* ranged between approximately 12 kyr for *A. ingens*, 31.5 kyr for *A shackleton*i and 47.6 kyr for *A. philippii*. The 95% HPD in each case largely overlap among species (Table 4, Fig. 4).

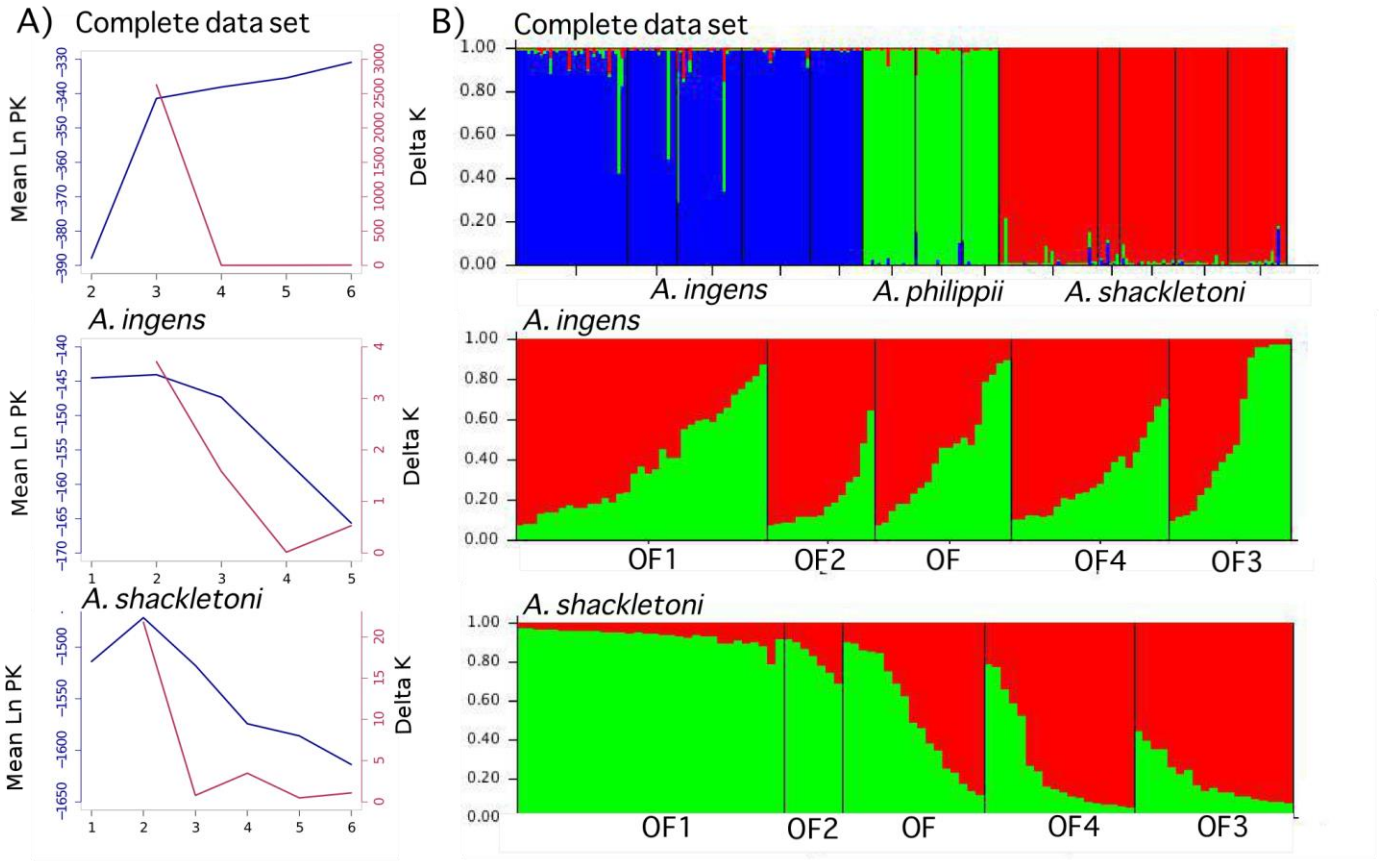

Fig. 2. Panel A Most likely K inferred by posterior probability method (blue) and Evanno et al. (2005) method (red). **Panel B**: Q membership coefficient bar plots, showing genetic clusters obtained with STRUCTURE. Black vertical lines separate sampling sites (information in Materials and Methods section)

Table 3. Hierarchical Analysis of Molecular Variance (AMOVA) for the three *Abatus* species.

Source of Variation	**F-stat**	***A. ingens***			***A. shackletoni***			***A. philippii***		
		% Var	F-value	P-value	% Var	F-value	P-value	% Var	F-value	P-value
Among individuals	F_{IT}	99%	-0.001	0.6	96%	0.06	**0.00**	95%	0.054	**0.03**
Among sites within groups	F_{SC}	1%	0.007	0.1	1%	0.002	0.41	3%	0.032	**0.003**
Between groups (impacted vs. non-impacted) sites	F_{CT}	0%	-0.001	0.5	3%	0.06	**0.00**	2%	0.023	0.1

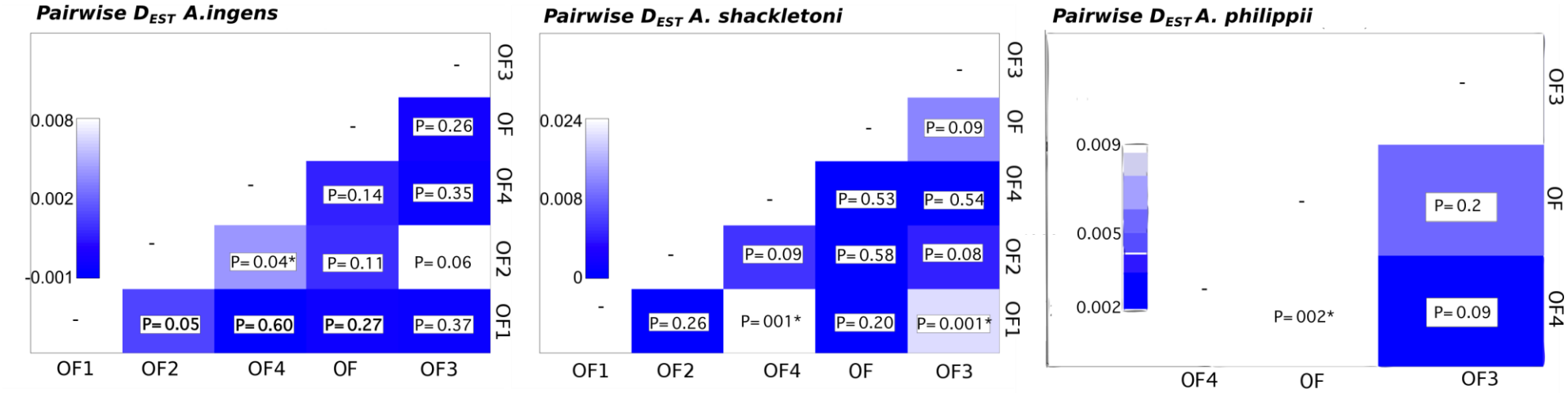

Fig 3. Visualization of pairwise differentiation between sample sites for the three species, as measured by D_{EST}-values. P-values were obtained using 10000 permutations in GenAlEx.

Table 4. Median values and 95% HPD intervals for the demographic parameter estimates obtained with Msvar: N_0 (Current population effective size), N_1 (Ancestral population effective size), and Ta (Time since change in effective size started). Negative values for the log of the ratio between N_0 / N_1 indicate population declines of over 90% in the three *Abatus* species. Bayes Factors (BF) were significant in the three species.

Species	N_0	**L95%**	**U95%**	N_1	**L95**	**U95**	***Ta***	**L95**	**U95**	**$\log_{10}$(r)**	**BF**
A. ingens	147.6	1.5	2105.5	33646.9	2577.8	490332.6	12385.6	115.1	269650.7	-2.5	3175
A. shackletoni	691.1	50.5	6831.6	14986.9	1460.4	166961.2	31573.5	962.9	746879.8	-1.4	40
A. philippii	208.2	12.7	2112.1	78151.4	5982.4	950459.1	47692.6	2310.6	618094.7	-2.6	Inf. *

*(no positive values of **$\log_{10}$(r)**)

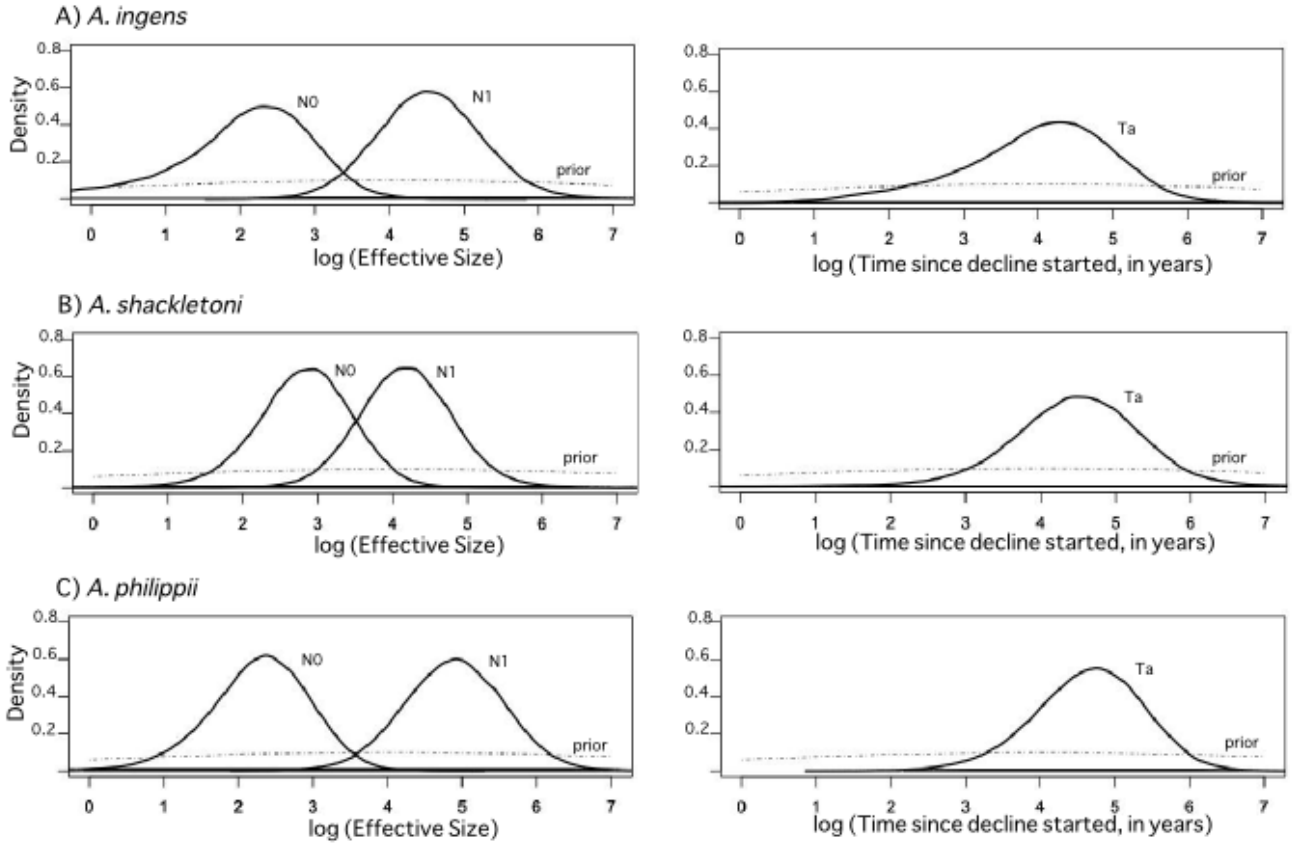

Fig. 4 Posterior probabilities for demographic parameters (given in $\log_{10}$ scale) inferred with MsVar: N_0 (Current effective population size), N_1 (Ancestral effective population size), and Ta (Time since change in effective size) for A) *Abatus ingens*, B) *A. shackletoni* and C) *A. philippii.*

Discussion

Our study provides genetic evidence for a remarkable long-term population decline of over 90% for three species of heart urchins in Vestfold Hills, Eastern Antarctica. In all cases, the estimated time since the start of the decline pre-dates any possible anthropogenic impact and is therefore more compatible with the effects of long-term natural climate variability. Antarctic ice core data indicate that the last 800 kyr have been characterized by eight glacial cycles with a strong 100-kyr periodicity (Laurent et al. 2004). In addition, two genetic clusters were inferred both for *A. ingens* and *A. shackletoni* that suggest long-term persistence and secondary contact after the two populations survived in different refugia, where the populations would have diverged. It has been proposed that ice-free areas existed on the shelf in Prydz Bay during the LGM (Domack et al. 1998, O'Brien et al. 1999), and these could have provided habitat for benthic organisms. Refugial populations and post-recolonization secondary population admixture has been identified in the Ross Sea (East Antarctica) in Adelie penguins (Ritchie et al. 2004), and has been documented as a genetic legacy to glacial cycles in different species from both the Southern (Zemlak et al. 2008) and Northern (Hewitt 2000) Hemispheres. Additionally, the low genetic diversity and low effective population size observed for the three species could be associated with a relatively recent recolonization of the area.

Radiocarbon dating of glacial marine deposits indicates that the ice retreat in Prydz Bay occurred ca. 20 Kya (Anderson et al. 2002) and we can expect benthic populations to have expanded (i.e., $N_0 > N_1$) since this deglaciation occurred. In fact, genetic evidence of a recent (ca. 10,000 years BP) demographic expansion has been found in the congeneric *A. agassizii* in the Antarctic Peninsula, West Antarctica (Diaz et al. 2012). Thus the question arises as to why East Antarctic

Abatus do not appear to have recovered during the present interglacial period (i.e., $N_0 < N_1$)? Causative explanations for the relatively small current effective population sizes (N_0) inferred for the three studied species can only be speculative at this point. One possibility is that the effects of glaciations caused particularly drastic population bottlenecks in the studied area of East Antarctica, and that the time since recolonization of the region has not been long enough for these populations to recover effective sizes similar to the ones that ancestral populations had before the massive disturbance caused by glaciations. Another possibility raises regional environmental and ecological causes that should be further investigated. For example, Poulin et al. (2002) proposed that there is a relationship between the impact of ice disturbance and the relative abundances of broadcasters and brooders in Antarctic benthic echinoderms, with ecological dominance of broadcasters when the shallow habitats are more heavily disturbed. Reductions in effective population sizes can thus be expected by competitive exclusion, e.g. by the more abundant broadcaster, regular urchin, *Sterechinus neumayeri* (Brey 1991), and under a scenario of regional strong disturbance. These possibilities are not mutually exclusive and may explain in combination the low inferred effective population sizes of the three *Abatus* species studied.

The relationship between a species' life history strategy and its population structure is complex in marine invertebrates (e.g., Miller & Ayre 2008). Population structure was generally weak in the three species we surveyed, suggesting gene flow is possible at distances of 5 km and that life history is not a good predictor of their fine-scale population structure. Individuals collected at the sites located furthest apart (non-impacted sites: OF3 and OF4) lacked genetic differentiation in all three species, suggesting geographic distance does not play a significant role, at least at the present spatial scales. This was unexpected given that the congeneric brooding species *A.*

46 *cordatus* in the Kerguelen Islands is genetically structured over spatial scales as little as 10 m
47 (Ledoux et al. 2012). In contrast to these subantarctic islands, seasonal sea-ice dynamics on the
48 shallow Antarctic shelf could have an important role promoting dispersal at fine spatial scales
49 (Dayton et al. 1969). Likewise, passive rafting on floating substrata including icebergs, anchor
50 ice and kelp, has been proposed to explain long-distance dispersal in Antarctic and subantarctic
51 brooding species (Baird et al. 2011, Nikula et al. 2013).

52 It can be assumed that sympatric species are affected by the same environmental factors, and
53 therefore interspecific differences in spatial patterns of population structure suggest species-
54 specific causes (see Fig. 2 and 3). These may involve highly individualistic responses to glacial-
55 interglacial climate variation, as it has been proposed for species in the Northern Hemisphere
56 (Stewart et al. 2010). Different patterns of spatial genetic structuring can also arise from
57 differences in reproductive behaviour. *Abatus ingens* and *A. shackletoni* have both been reported
58 to exhibit pluriannual brooding cycles (Pearse & McClintock 1990, Chenuil et al. 2004) but the
59 reproductive behaviour of these Antarctic species is not completely understood yet. Additionally,
60 in the Antarctic benthic ecosystem our understanding of the factors affecting species' fine-scale
61 population genetic structure is very limited. In summer, millions of icebergs of different sizes
62 move embedded in ocean currents and wind drifts causing massive disturbance in benthic
63 communities (Barnes & Conlan 2007), and could have an important role for passive dispersal of
64 brooding species resulting in spatially complex patterns of genetic connectivity that are
65 inconsistent among species.

66 No conclusive evidence of an impact of pollutants from Davis station was found in the genetic
67 diversity, population structuring or effective population size of any species. However, limitations

68 inherent to our study to detect such impact should be noted. Previous applications of the Msvar
69 method have failed to date known recent population declines when the genetic signature of a
70 longer-term demographic change was present in the population (Beaumont 1999a, Storz et al.
71 2002). Thus, although the method is in fact able to detect contemporary human-induced
72 demographic changes (Goossens et al. 2006), it is possible that a recent decline would not be
73 correctly dated for the presently studied populations. In addition, the selective effects by
74 anthropogenic pollutants may be too recent to be detected (Davis Station was opened in 1957),
75 especially considering the long generation time of these species (ca. 20 years). Moreover, the
76 observed connectivity between sites with different degrees of exposure to pollutants from the
77 station could counteract any impact on genetic diversity due to increased mortality in impacted
78 areas. Hierarchical AMOVA analysis indicates a significant percentage of the genetic population
79 differentiation in *A. shackletoni* can be explained by the exposure to wastewater effluent from
80 Davis Station. However, this seems unlikely since our results obtained with STRUCTURE for
81 the species (Fig. 2) show that diverging individuals correspond to OF1 and OF2 but not to the
82 OF, which is the closest site to the pollution source and likely the most impacted. It should be
83 noted that the genetic differentiation of OF1 and OF2 could reflect differences in depth or
84 temporal variation, since these sites are located at slightly higher depths than the rest of the sites,
85 and were sampled two years before the other three sites.

86 In summary, our results suggest that the three *Abatus* species studied in the Vestfold Hills, East
87 Antarctica, have suffered a long-term and remarkable population decline and that their current
88 reduced effective population sizes could make them vulnerable to demographic, environmental
89 or genetic stochasticity. Although the estimated time when these species' populations started to
90 decline precedes any potential effects from anthropogenic factors, they may be concealed by the

strength of this earlier demographic decline and anthropogenic impacts should not be completely dismissed. The patterns of genetic structure observed in *A. ingens* and *A. shackletoni* seem to reflect historical signatures of survival in two different refugia during glacial cycles and secondary contact after recolonization of the study area. Contrary to previous evidence of fine-scale population structure in *A. cordatus* from the Kerguelen Islands, population connectivity at spatial scales of 5 km for these three brooding heart urchins species suggest that conditions in the Antarctic may promote dispersal, perhaps involving rafting on floating sea ice.

Acknowledgements

The authors thank the Davis Station summer marine teams of 2009/10 and 2012/13 for assistance in field collections and laboratory processing of samples, particularly Jake van Oosterom, Jonathan Stark, Ian Aitkenhead and Patricia Corbett. This study was supported by Australian Antarctic Science Grants awarded to K. Miller (Project 3051) and C. King (Project 4100)

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General Discussion and Conclusions

4.1 *Genetic structure of Abatus populations in East Antarctica*

The spatial scale at which gene flow (genetic exchange) occurs among populations has an important role in the evolution of a species (Slatkin 1987). The broad aim of this study was to characterize the genetic diversity and structure of Antarctic heart urchin populations at different spatial scales (hundreds of meters to thousands of kilometers) in order to elucidate past and contemporary processes affecting the evolution of these species in East Antarctica. Populations of *Abatus nimrodi* and *A. ingens* were found to be isolated or semi-isolated (infrequent long distant dispersal cannot be discarded) at spatial scales over 1000 km. At distances of ca. 5km, gene flow is evident for *A. ingens*, *A. shackletoni* and *A. philippii* in Vestfold Hills (VH), although populations are not panmictic. Furthermore, the importance of historical cyclic glaciations influencing population structure and genetic diversity of *Abatus* species in East Antarctica is clear at both spatial scales studied, and across the nuclear and mitochondrial markers utilized. The brooding life history strategy of these species may prevent gene flow across large scales, but it does not seem to result in predictable fine-scale population structure at least to the same extent as other brooding taxa (Ledoux et al. 2012). Moreover, varying patterns of population differentiation found among the three species studied in VH, despite their common life-history, suggest that environmental factors (e.g. sea-ice dynamics) could facilitate passive dispersal (e.g. floating on icebergs or anchor ice) of these brooding species, creating complex patterns of population connectivity.

4.2 *The influence of glaciations on the genetic population structure of Abatus*

The genetic diversity and structuring patterns inferred both with mitochondrial and nuclear markers for *Abatus* populations suggest these benthic species endured glacial periods in isolated and small ice-free refugia on the East Antarctic shelf. Evidence pointing to survival in this kind of refugia includes: (i) Low genetic diversity found in extant *Abatus* populations, likely reflecting pronounced bottlenecks during glaciations and the founding of contemporary populations by a small number of individuals (ii) statistical parsimony haplotype networks obtained for *A. nimrodi* and *A. ingens* are consistent with the network patterns observed in other Antarctic brooding species proposed to have survived in shelf refugia (Allcock & Strugnell 2012), and (iii) a unique and shared haplotype between *A. nimrodi* individuals from WI and DDU suggests common ancestry and recolonization from the same refugial population, possibly in the DDU area (Post et al. 2011). In addition, the deep divergence between the WI-DDU mitochondrial lineage and the VH *A. nimrodi* mitochondrial lineage suggests they may have diverged in different isolated refugia, the latter possibly in the VH area (Domack et al. 1998, O'Brien et al. 1999). Finally, the Bayesian inference of two genetic clusters and their genetic admixture (using microsatellite markers) in *A. ingens* and *A. shackletoni* individuals from VH suggest long persistence and secondary contact after population differentiation in glacial refugia.

Alternative explanations for the genetic patterns found in East Antarctic *Abatus* populations, i.e. deep sea or subantarctic refugia, are unsatisfactory. For example, deep-sea refugia have been proposed for a sea spider (Arango et al. 2011) with extreme eurybathy (depth range of over 4000m). Genetic signatures in species that could have found refugia in the deep-sea are different than the ones reported in this

study for *Abatus* populations, i.e. high haplotype diversity and parsimony networks with large numbers of haplotypes each restricted to an area reflecting local diversification, and no major bottlenecks during past glaciations. On the other hand, recolonization by a small number of founders coming from subantarctic islands would have produced similar genetic patterns to the ones observed in our studied *Abatus* species. For example, COI sequence data from *Abatus agassizii* in the Antarctic Peninsula, suggest that this population corresponds to a recent re-colonization from refugia in subantarctic islands located to the north of the peninsula (Diaz et al. 2012). Subantarctic islands might have provided refugia for benthic populations near the Antarctic peninsula but this is less likely to be the case in East Antarctica where the distances between subantarctic islands and between them and the mainland is very large. Further, the species under investigation in this study are all presently restricted to the Antarctic continent.

Once the present interglacial climate was established and habitat became available, recolonizing populations are expected to have expanded rapidly. However, here, I present evidence of a remarkable long-term population decline in three *Abatus* species in VH inferred by a Bayesian method using microsatellite markers. This method has proved to be more efficient than other methods available (e.g. BOTTLENECK and M-ratio) to detect population size changes, provided these changes are not too weak or too recent (Girod et al. 2011). It should be mentioned that the same simulation study by Girod et al. (2011) showed that while the method is robust to moderate departures from the SMM model (Girod et al. 2011), it can be sensitive to severe departures from a strict SMM model. This is also a practical limitation of microsatellite markers since their evolutionary dynamics are difficult to infer and can vary among loci. Finally, mismatch analyses based on the sequences of two

concatenated mitochondrial markers (COI and 16S) indicate a similar declining trend for *A. ingens* in DDU.

Founders of *Abatus* populations might have had to compete with an already established community, which may have affected their population radiation. It has been proposed that interglacial periods characterized by frequent ice-disturbance are unlikely to be favorable to brooding species in the shallow subtidal zone (Poulin et al. 2002). On the contrary, the conditions during glacial periods were disadvantageous to species with a planktotrophic developmental mode. Such differential selective pressures would explain the ecological dominance of planktotrophic species, including echinoderms such as the regular sea urchins *Sterechinus neumayeri* (Brey & Gutt 1991), particularly in heavily ice disturbed areas (Poulin et al. 2002). It is therefore possible that the reduced effective size of *Abatus* populations in East Antarctica is explained by a combination of highly ice impacted areas and competitive exclusion with species with a planctotrophic larval phase.

4.3 *The influence of life history in the population structure of Abatus*

Fine-scale population structure and low dispersal abilitiy in three Antarctic heart urchin species (*A. ingens*, *A. shackletoni* and *A. philippii*) were predicted as a consequence of their shared brooding life history strategy. Moreover, those patterns were expected to be similar among these closely related species based on their sympatry and the absence of methodological bias since the same set of microsatellite markers was used across species. However, evidence of population connectivity across distances of up to 5 km, as well as differences in the spatial genetic strucutre among species, showed that it was not possible to generalize about dispersal based only on life history. These results highlight the importance of comparative

multispecies approaches, and the complex relationship between life history and genetic population structure in benthic marine invertebrate species. Similarly, in temperate waters of Tasmania and South Australia, different patterns of genetic population structure have been inferred between congeneric and sympatric haliotids (abalone), which share life history characteristics (Miller et al. 2009, Miller et al. 2014). Although predictions based on life history regarding a species dispersal capability, and spatial population structure, may work for some taxa, e.g. octopus (Higgins et al. 2013), for conservation purposes, the idiosyncratic responses to population processes imply that species-specific management reflective of a species population structure remains important (Miller et al. 2014).

4.4 *Implications for conservation*

The Antarctic marine ecosystem is facing increasing anthropogenic pressures, e.g. climate change, localized pollution from numerous research stations, overfishing, impacts from tourism, and invasive species (Aronson et al. 2011). Antarctic benthic species are considered especially vulnerable because they are highly isolated and adapted to extremely cold environments (Barnes & Peck 2008). In particular, heart urchins are predicted to be negatively impacted by ocean acidification (Sewell & Hofmann 2011). In addition, the long term decline and reduced effective population sizes estimated in my study for *A. ingens*, *A. shackletoni* and *A. philippi* in VH suggest that these populations may be even more vulnerable to demographic, environmental or genetic stochasticity. In theory, a small effective population size can lead to loss of genetic diversity, short-term loss of fitness, inbreeding depression and therefore increased extinction risk (Frankham et al. 2009). Despite the population decline inferred in VH, at a large spatial scale, levels of mitochondrial genetic diversity were higher in VH than in WI or DDU. Mitochondrial diversity is correlated

to effective population size in some animal groups (Piganeau & Eyre-Walker 2009) indicating populations at WI and DDU may have small effective population sizes. Moreover, mismatch analysis indicates that the *A. ingens* population in DDU might be declining as well.

Genetic analysis of marine population structure can inform the design of Marine Protected Areas (MPAs) for the preservation of biodiversity (Palumbi 2004). Different reserve designs are needed with respect to whether a species has restricted of high dispersal capability, and also depends on the conservation and management objectives for a MPA. Populations with low dispersal could be "trapped" and so not serve to replenish populations far from reserves, while expansive unprotected zones could reduce the effectiveness of a MPA to protect highly dispersive species (Palumbi 2003). Thus, it has been proposed that MPAs should be as large as the mean larval dispersal distance of a species (Botsford 2003).

Despite the expectation of direct development hindering connectivity, and evidence of fine scale (less than 1 km) population structure from *Abatus cordatus* in the Kerguelen Islands, I found evidence of population connectivity at distances of 5 km for *A. ingens*, *A. shackletoni* and *A. philippii* in VH. Thus, my results suggest that in order to preserve *Abatus* spp. in East Antarctica MPA's should be designed with a size at least as big as 5km. An alternative is a coordinated system of MPAs to serve as stepping-stones to allow recruits from distant populations to be connected. However, the complex patterns of population structuring found for the *Abatus* spp. studied (e.g., different patterns of geographical differentiation among species) suggest that stochastic environmental factors have a strong role in the dispersal abilities of these brooding species. Therefore, future research is needed at intermediate scales (>5km

and <1000 km) in order to determine the dispersal capabilities of *Abatus* in East Antarctica and to accurately inform MPA design.

There are some limitations regarding the direct application of molecular results in the context of conservation biology. Ideally, molecular methods should be accompanied by other approaches, such as demographic analysis (Lande 1988). This is because small amounts of gene flow (one migrant per generation) can prevent the accumulation of genetic differences among local breeding populations (Slatkin 1987), and thus inferences of genetic population connectivity may not necessarily represent dispersal at ecological spatial or temporal scales, nor reflect the capability of a species for replenishment of populations outside a marine reserve, at a time scale of ecological interest. However, effective management planning should recognize and aim to maintain genetic diversity in order to ensure the long term persistence of a species (Frankham et al. 2009) and enhance the ecosystem resilience to environmental change (Reusch et al. 2005) .

The first step to assess and protect biodiversity at the organismal (species) diversity level is the correct taxonomic identification of species (Pante et al. 2015). The high level of genetic divergence (2.6%) between *A. nimrodi* mitochondrial lineages from VH and WI plus DDU suggest potential cryptic speciation. Further investigation, e.g. morphological and reproductive isolation tests, are necessary to determine if such divergences represent geographic intraspecific structuring or allopatric speciation. Finally, the phylogenetic analysis among *Abatus* spp. and *Amphipneustes lorioli* suggest that *A. nimrodi* is more closely related to *A. lorioli* than to the rest of the *Abatus* species included in the analysis (*A. ingens*, *A. shackletoni*, *A. philippii* and *A. cavernosus*). This provides a framework to further investigate the species composition of these genera.

4.5 *Conclusions*

The present study provides an important insight into the processes that have shaped patterns of genetic diversity and structure of Antarctic heart urchin populations at different spatial scales in East Antarctica. This research has implications for the management and preservation of Antarctic marine biodiversity. The main findings can be summarized as follows:

- Historical climate variation has left a strong signature on the genetic diversity and structure of contemporary populations of *Abatus* species in East Antarctica. Populations appear to have survived glacial cycles in shelf refugia, however they do not show signs of expansion in either VH or DDU. On the contrary, evidence of a long-term population decline was found for *A. ingens*, *A. shackletoni* and *A. philippii* in VH, and possibly for *A. ingens* in DDU.
- The brooding life history of the Antarctic heart urchin species studied is not a good predictor of the spatial scale of genetic structuring. Gene flow is possible at distances of ca. 5 km in VH. However, this mode of development may play a role in the isolation of populations at scales of ca. 1000 km. Patterns of fine-scale (<5 km) population connectivity were inconsistent across three sympatric *Abatus* species, highlighting the importance of a multispecies approach to understand the factors driving population structure in the Antarctic marine environment. Antarctic environmental conditions may promote passive dispersal, e.g. rafting on icebergs or anchor ice, creating these complex population structuring patterns.

- Vulnerability of *Abatus* populations in East Antarctica was suggested by their reduced effective population sizes and population isolation at a large scale, which can contribute to a higher risk of local extinction.

- Recommendations for conservation involve a minimum size of 5 km for MPAs implemented in the VH area, and future research at intermediate scales in order to refine estimates of dispersal capabilities for these species.

- The phylogenetic analysis revealed a paraphyletic relationship of *Abatus* species with respect to *Amphipneustes lorioli* indicating that further taxonomic investigation is needed to determine the species composition of these genera.

- Finally, a new set of microsatellite markers has been developed that can be potentially used to study populations of *A. ingens*, *A. shackletoni* and *A. philippii* across the distribution of these species, including the entire Antarctic continent.

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Appendices

Appendix C2, Chapter 2

Table 1. Accession numbers for the sequences included in the Bayesian analysis that were obtained in Gene Bank.

Species	COI Access. no.	16S Access. no.
Spatangus raschi	AJ639911	AJ639811
Paleopneustes cristatus	AJ639908	AJ639808
Brisaster latifrons	HM542115	AJ639806

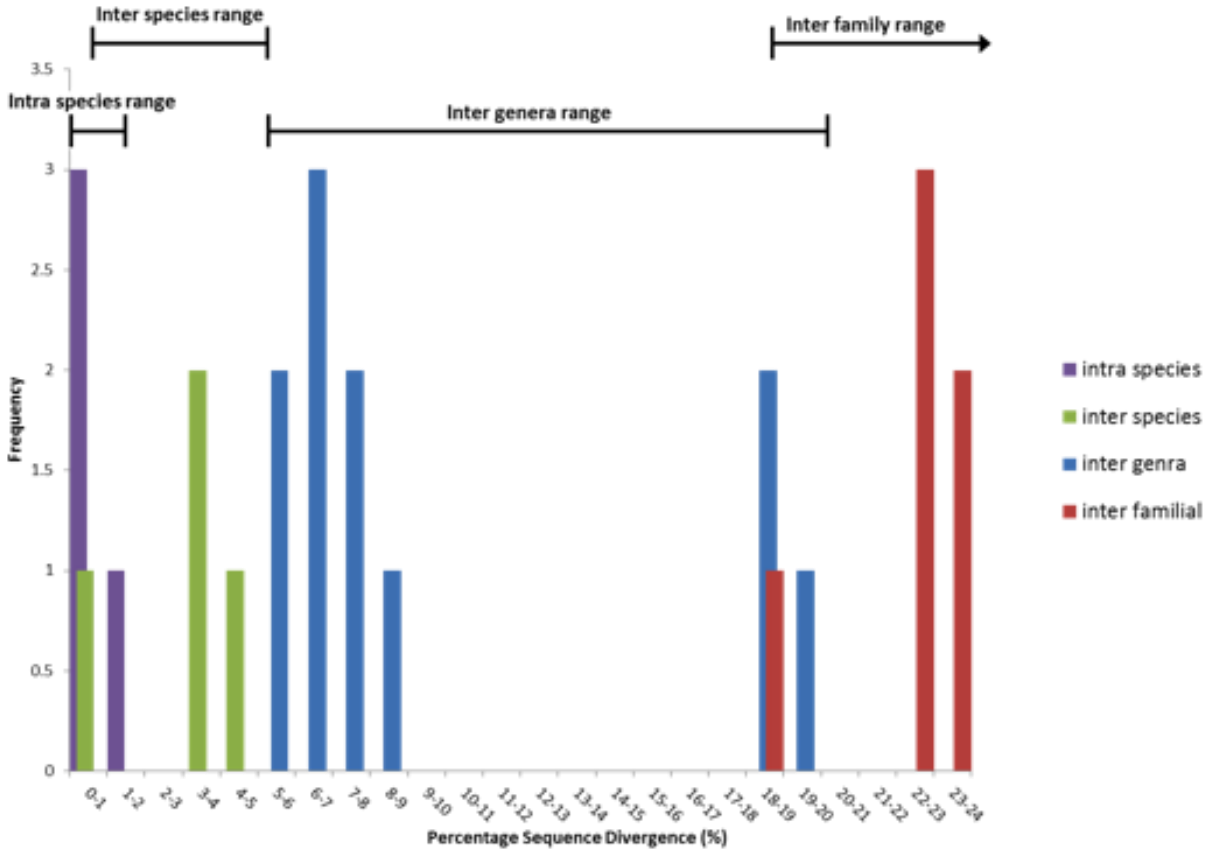

Fig. A1 Frequency distribution of percentage sequence divergence in Spatangoid urchins based on pairwise comparisons of CO1 sequence data. This graph was taken from van Oosterom (2013) for comparison with the percentage of sequence divergence calculated in Table 4, Chapter 1 of this thesis. Comparisons were made by van Oosterom (2013) within species, among species within the same genus, among genera, and among families based on data from the following morphological species: *A. ingens, A. cavernosus, A. philippii, A. shackletoni, Brisaster similis, Brisaster latifrons, Paraster doederleini, Amphipneustes lorioli* and *Echinocardium cordatum.*

Appendix C3, Chapter 3

Table A1. Cross-species amplification success in three *Abatus* species.

Locus	*Abatus ingens*			*Abatus shackletoni*			*Abatus philippii*		
	N $_{ind.}$	***Na***	**Size range**	***N*** $_{ind.}$	***Na***	**Size range**	***N*** $_{ind.}$	***Na***	**Size range**
Ab_07	**104**	**14**	**201-315**	**93**	**7**	**195-216**	**43**	**5**	**201-303**
Ab_15	**108**	**3**	**150-156**	**77**	**4**	**132-156**	**44**	**2**	**153-156**
Ab_16	**107**	**8**	**66-105**	**93**	**6**	**90-105**	**44**	**5**	**78-102**
Ab_17	**108**	**3**	**154-157**	**92**	**16**	**157-259**	**44**	**4**	**157-229**
Ab_18	**108**	**3**	**195-207**	**93**	**7**	**195-216**	**44**	**5**	**189-207**
Ab_29	**108**	**1**	**234**	**93**	**4**	**228-243**	**44**	**1**	**234**
Ab_31	**108**	**5**	**223-235**	**93**	**3**	**229-235**	**43**	**2**	**229-232**

Table A2. Sampling sizes and results for three *Abatus* species by locus and site for:

Allelic richness (AR), H-W exact-tests (H-W), and Fis values

Locus	*A. ingens*					*A. shackletoni*					*A. philippii*		
	STP1	**STP4**	**Hbay**	**Ofall**	**Abeach**	**STP1**	**STP4**	**Hbay**	**Ofall**	**Abeach**	**Hbay**	**Ofall**	**Abeach**
07	NA	OK	OK	NA	NA	OK	OK	OK	OK	OK	OK	OK	NA
N	35	14	19	21	16	31	7	18	17	19	16	12	15
AR	7.71	3.00	7.53	6.73	7.73	3.970	4.00	2.63	2.89	2.97	3.00	3.00	4.6
H-W	0.00*	0.02	0.02	0.00	0.01	0.71	0.66	0.11	0.20	0.03	0.24	1.00	0.02
Fis	0.20	0.44	0.01	0.2	0.3	0.03	0.00	0.37	0.28	0.33	0.14	0.08	0.39
15	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	NA	OK	OK
N	35	15	19	22	17	30	7	16	17	16	17	12	15
AR	2.04	1.00	1.00	1.00	1.00	3.03	3.00	2.00	2.65	2.00	2.00	2.00	2.00
H-W	1.00	-	-	-	-	0.31	0.63	0.31	0.05	0.1	0.01	0.03	0.00*
Fis	-0.02	-	-	-	-	0.05	0.25	-0.35	0.23	-0.5	0.66	0.68	0.87
16	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
N	34	15	19	22	17	31	7	18	17	19	17	12	15
AR	5.48	4.93	7.12	4.69	5.47	3.81	4.00	3.35	3.35	3.71	3.64	3.91	2.99
H-W	0.57	3.93	0.76	1.00	0.70	0.12	0.44	0.09	0.93	0.50	1.00	0.26	0.86
Fis	-0.32	0.16	0.26	-0.16	-0.17	-0.31	0.31	-0.09	0.07	-0.08	0.66	0.68	0.87
17	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
N	35	15	19	22	17	31	7	17	17	18	17	12	14
AR	2.00	2.00	2.73	2.64	2.97	6.97	4.00	6.81	5.92	6.76	3.57	3.92	2.99
H-W	0.00	0.00	0.03	0.03	0.10	0.88	0.63	0.37	0.23	0.33	0.36	0.83	0.27
Fis	-0.51	0.86	0.26	-0.23	-0.15	-0.12	-0.1	0.05	0.1	0.08	0.06	0.21	-0.08
18	OK	NA	OK	NA	OK	NA	OK	NA	NA	NA	OK	OK	OK
N	35	15	19	22	17	31	7	18	17	19	17	11	15
AR	3.00	3.00	3.00	3.00	3.00	4.71	4.00	4.70	4.75	3.22	3.85	3.00	2.94
H-W	0.02	0.02	0.40	0.00*	0.76	0.00*	0.51	0.00*	0.00*	0.00*	0.47	1.00	0.55
Fis	0.14	0.51	0.15	0.47	0.13	0.6	0.05	0.5	0.69	0.65	0.19	0.05	-0.02
29	OK	OK	OK	OK	OK	OK	OK	NA	OK	OK	OK	OK	OK
N	35	15	19	22	17	31	7	18	17	19	17	12	15
AR	1.00	1.00	1.00	1.00	1.00	2.93	4.00	2.99	2.99	2.95	1.00	1.00	1.00
H-W	-	-	-	-	-	0.46	0.44	0.00*	0.00*	0.06	-	-	-
Fis	-	-	-	-	-	-0.15	0.18	0.43	0.23	0.01	-	-	-
31	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
N	35	15	19	22	17	31	7	18	17	19	17	12	14
AR	3.77	2.99	3.73	2.87	2.82	1.45	2.00	1.00	1.00	1.00	2.00	2.00	2.00
H-W	0.15	0.03	1.00	0.12	0.14	1.00	-	-	-	-	1.00	0.59	0.14
Fis	-0.20	0.42	0.02	0.35	-0.36	-0.01	-	-	-	-	-0.1	0.04	0.45

OK/NA = No evidence /evidence of null alleles detected by Microchecker, **AR** = Allelic richness (FSTAT) rarefaction (adjusts to sample size differences), **N**= sample size, **H-W** = P-value for H-W Exact test (Guo and Thompson, 1992) implemented in Genepop, * indicate significant P-values after Bonferroni correction. **Fis=** Weir & Cockerham's (1984) inbreeding coefficien

Table A3. Biased corrected mean and 95% confidence intervals for the diversity partitioning and differentiation statistics: G_{ST} (Nei, 1973) and G'_{ST} (Hedrick, 2005) and D_{est} (Jost, 2008) calculated in R with the package DiveRsity (Keenan et al., 2013).

Site Pair	*Abatus* species	G_{ST}	L95%CI	U95%CI	G'_{ST}	L95%CI	U95%CI	D_{EST}	L95%CI	U95%CI
OF1 vs. OF2	*A.ingens*	0.008	-0.008	0.033	0.020	-0.017	0.075	0.003	-0.009	0.025
OF1 vs. OF4	*A.ingens*	-0.002	-0.011	0.012	-0.007	-0.03	0.027	0.000	-0.005	0.013
OF1 vs. OF	*A.ingens*	0.002	-0.009	0.020	0.005	-0.022	0.047	0.000	-0.007	0.014
OF1 vs. OF3	*A.ingens*	0.000	-0.011	0.017	0.000	-0.027	0.044	0.000	-0.008	0.017
OF2 vs. OF4	*A.ingens*	0.012	-0.009	0.044	0.029	-0.019	0.102	0.005	-0.011	0.036
OF2 vs. OF	*A.ingens*	0.009	-0.013	0.043	0.021	-0.027	0.091	0.002	-0.009	0.024
OF2 vs. OF3	*A.ingens*	0.011	-0.012	0.047	0.027	-0.026	0.104	0.008	-0.008	0.038
OF4 vs. OF	*A.ingens*	0.006	-0.010	0.031	0.014	-0.025	0.073	0.002	-0.011	0.024
OF4 vs. OF3	*A.ingens*	0.000	-0.012	0.019	0.002	-0.031	0.049	0	-0.011	0.024
OF vs. OF3	*A.ingens*	0.004	-0.015	0.034	0.01	-0.035	0.075	0.000	-0.008	0.019
OF1 vs. OF2	*A. shackletoni*	0.004	-0.019	0.042	0.015	-0.062	0.122	0.000	-0.021	0.053
OF1 vs. OF4	*A. shackletoni*	0.027	0.006	0.055	0.098	0.028	0.187	0.024	-0.015	0.081
OF1 vs. OF	*A. shackletoni*	0.006	-0.008	0.026	0.019	-0.027	0.086	0.000	-0.018	0.033
OF1 vs. OF3	*A. shackletoni*	0.032	0.013	0.054	0.113	0.051	0.185	0.022	-0.012	0.071
OF2 vs. OF4	*A. shackletoni*	0.015	-0.008	0.054	0.056	-0.019	0.167	0.008	-0.032	0.081
OF2 vs. OF	*A. shackletoni*	0.000	-0.023	0.035	-0.002	-0.074	0.104	0.000	-0.023	0.059
OF2 vs. OF3	*A. shackletoni*	0.015	-0.009	0.052	0.053	-0.024	0.171	0.007	-0.033	0.081
OF4 vs. OF	*A. shackletoni*	0.002	-0.016	0.028	0.007	-0.052	0.088	0	-0.024	0.047
OF4 vs. OF3	*A. shackletoni*	0.000	-0.016	0.023	0.000	-0.053	0.073	0	-0.012	0.03
OF vs. OF3	*A. shackletoni*	0.011	-0.010	0.041	0.038	-0.031	0.129	0.015	-0.020	0.076
OF4 vs. OF	*A. philippii*	0.044	0.004	0.094	0.115	0.018	0.225	0.009	-0.009	0.041
OF4 vs. OF3	*A. philippii*	0.016	-0.014	0.057	0.043	-0.033	0.138	0.002	-0.013	0.034
OF vs. OF3	*A. philippii*	0.011	-0.017	0.052	0.03	-0.041	0.126	0.005	-0.014	0.045