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1. Introduction

1.1 The Antarctic Continent

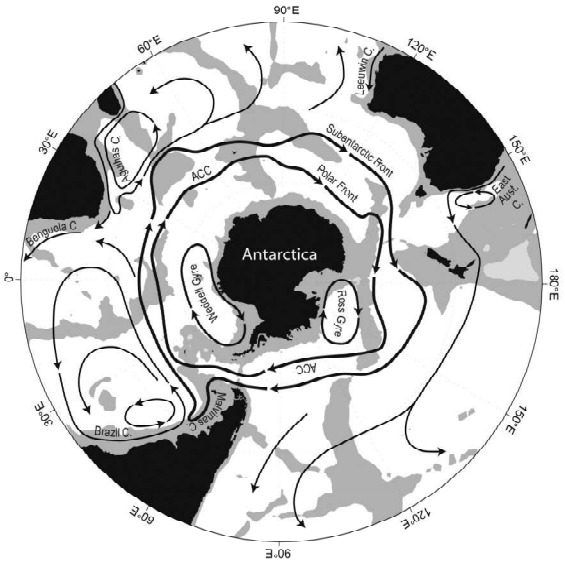

Fig. 1: Map of the Southern Hemisphere of Earth with indications of the major current systems in the Southern Ocean (Gray: depths < 3500 m; Arrows: Flow-direction of current). After Sloyan and Rintouhl (2001), modified by Leese (2008).

About 180 Myr ago, the supercontinent Gondwana broke up into Antarctica, Africa, Australia, South America and the Indian Subcontinent (Riffenburgh 2007). As the Australian and the South American plates slowly separated from Antarctica, causing the opening of the Drake Passage between South America and the Antarctic continents, the Antarctic Circumpolar Current (ACC) surged and triggered the accelerated temperature dropdown of the Antarctic around 37-33 Myr ago (Kennett 1977). The ACC are water masses between the Polar Front (PF) and the Sub-Antarctic Front, which drift in eastward direction driven by west winds. This current is fast and strong and most prominent on surface waters (Lawver and Grahagan 1998, 2003; Lawver and Muller 1994) (Fig. 1). Due to the ACC, the Southern Ocean is an independent circumpolar and thermally isolated ocean with constant low water temperatures from -1.86°C to +2.0°C (Leese et al. 2008). Radical change of climate conditions affected many species of the Antarctic, leading them to their extinction (Dayton 1990). The new climate conditions simultaneously provided an isolated environment, with a unique habitat, where some taxa (e.g. Isopoda, Amphipoda, Cumacea, Tanaidacea, Ascidiacea, Actiniaria, Holothuroidea, Polychaeta, Porifera, notothenioid fishes) experiences radiation, resulting in new endemic species (Andriashev 1965; Koltun 1969, 1970; Dell 1972;

Knox 1977; Knox and Lowry 1977; Sicinski 1986; Gallardo 1987; Gutt 1988, 1990 a, b; Eastman and Grande 1989; Arntz et al 1997). This endemism proves the fact of Antarctica having faced a long period of evolution under relative isolated conditions (Clarke and Crame 1989). Due to new speciation because of isolation, Antarctica is called an "evolutionary incubator" (Watling and Thurston 1989).

1.2 Speciation, Isolation and Distribution of Antarctic Species

When talking about both, speciation and species conservation, two of the most important factors that have to be taken into consideration are gene flow and genetic drift (Hellberg 1994). Limited gene flow accelerates divergence among populations mostly caused by natural selection and genetic drift within the isolated populations (Hellberg 1994). If the populations drifting genetically from each other are found to be separated by long geographic distances – so called “isolation by distance” (Wright 1943) – the degree of gene flow limitation directly depends on the number of migrants between populations and geographic connections between interacting populations (Hellberg 1994). Thus, genetic differentiation is highly correlated to migration (Hellberg 2002). Differentiation of populations may occur between populations with a wide spatial distribution, where only neighbor populations are able to exchange genes between each other (“stepping stone” model, Kimura and Weiss 1964). In order to understand how populations differentiate in absence of major physical barriers, as it is the case within marine habitats, one must consider whether there is “enough” and geographically unbiased gene flow between neighboring populations or not (Hellberg 1994). All in all, the equilibrium between gene flow, genetic drift and geographic range is one of the main factors, which determines how speciation occurs and how species are maintained. Hansen (1978) and Jablonski (1986, 2006) proposed that taxa with limited larval dispersal should have greater numbers of species compared to related taxa with high larval dispersal.

In general, gene flow between different marine localities around the Antarctic is supposed to be easily enhanced by the ACC thanks to the lack of major physical barriers. The ACC provides a water route for pelagic larval dispersal (Dauer et al. 1982). Thus, panmixia can be generally assumed for pelagic taxa and species with pelagic larval stages, commonly

resulting in circumpolar distribution (Dayton 1990; Robinson 1983; Scheltema 1986). Some examples of species with circumpolar distribution probably due to pelagic or planktonic larval stages are *Euphausia superba* (Antarctic krill; Tynan 1998; Nicol 2000; Mangel and Nicol 2000; Hofmann et al. 1996; Atkinson 2008; Everson 2000), *Dissostichus mawsoni* (Antarctic toothfish (Parker et al. 2002) with at least one year of pelagic larval phase (Clers et al. 1996)) as well as some species among Antarctic phytoplankton (Medlin et al. 2000) and planktonic Archaea (Murray et al. 1999). Circumpolar distribution is assumed for some caridean benthic shrimp as well, like *Notocrangon antarcticus* (no or very short pelagic larval stages (<48h)), *Chorismus antarcticus and Nematocarcinus lanceopes* (last two with planktotrophic larvae).

In 1972, Dell first suggested a circumpolar distribution for Antarctic benthic species. However, a benthic lifestyle means restricted distribution and most studies, which have assumed uniform and circumpolar distribution patterns for Antarctic benthic species only rely on morphological devices and have been in some cases recently rejected by new studies using mitochondrial DNA as well as nuclear markers like microsatellites. Among isopods for example, like *Glyptonotus antarcticus*, (see Held 2000; Held and Wägele 2005) and *Ceratoserolis trilobitoides* (see Held 2003), as well as pycnogonidae like *Nymphon australe* (see Mahon et al. 2008) and *Colossendeis megalonyx* (see Krabbe et al. 2009), cryptic speciation was detected. Cryptic speciation is when a morphologically defined species actually consists of different genetically defined species, which however are identical in their morphology. In those cases mentioned above, cryptic speciation might be also due to lack of pelagic larvae. The increasing discovery of cryptic species however, indicates that most reported circumpolar distributed species probably consist of a complex of cryptic species with smaller distribution ranges (Krabbe et al. 2009).

A phenomenon that must be also considered in terms of speciation is the progression of the grounded shelf ice during glaciations periods towards the outer continental shelf along most Antarctic coasts. Such events wiped out most shelf inhabitants as the impact of the shelf ice masses on the benthos scraped the first layers, destroyed the habitat and eliminated its inhabitants (Fig. 2; Leese et al. 2008a). Specimens that survived the mentioned events, probably hid in some sort of refugia and accordingly where separated during the glaciations periods from other populations from the same species either in deeper zones where the

glaciations had no big impact, or in some other refugia like caves or crevices, save from the grounded ice sheets (Thatje et al. 2008). Moreover, the onset of extreme climate conditions and the cyclic glaciation periods probably caused cycles of fragmentation of habitats, which resulted in reproductive isolation, followed by secondary contact of populations after a glacial maximum. This process was called “taxonomic diversity pump” by Clarke and Crame (1989) as it may have been the reason for new speciation. In addition, the extinction and recolonization of habitats by populations, in this special case due to transient changes in glaciations events, led to unexpected patterns of genetic differentiation between the populations (Slatkin 1993). Biodiversity in benthic taxa for example also indicates that the process of glaciations must occur gradually. An evidence for a gradual glaciation event is the high biodiversity of the benthos fauna. Benthic inhabitants must have had enough time to migrate or hide in sheltered regions to be able to survive such extreme periods in order to be nowadays represented by a diverse number of species high in representatives. Under mentioned climate conditions and fluctuations speciation or within shorter time frames a heterogeneous population structure may have been the result (Leese et al 2008).

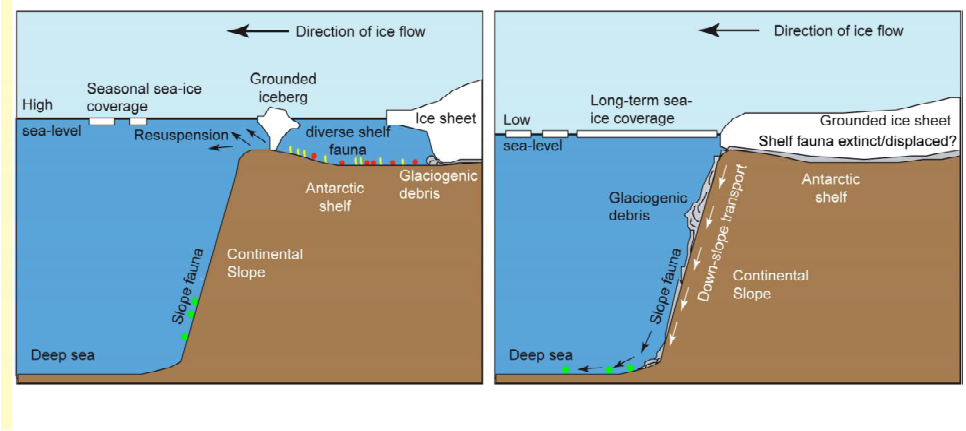

Fig. 2: Vertical profile of the Antarctic shelf. Left: interglacial periods with habitat for shelf fauna. Right: glacial maxima with ice sheets covering the habitat for shelf fauna. After Thatje et al. (2005), modified by Leese (2008).

Off shore, and in the concrete case of the Scotia Ridge between South America and the Antarctic Peninsula, a homogenous dispersal of populations along the Scotia Arc has been proposed by Knox and Lowry (1977). Their theory claims that the islands of the Scotia Arc are used as “stepping stones” between South America and the Antarctic, facilitating a continuous genetic exchange between populations along the migration routes. Nevertheless,

benthic taxa are mostly immobile at the adult stages and have rather no migration behavior and thus no dispersal (Leese 2008). For some species among crinoids (Wilson et al. 2007) and bivalves (Linse et al. 2007) a homogenous distribution of populations along the Scotia Arc has been rejected already.

1.3 Study Case

Herein presented research focuses on decapods, which particularly show a scarcity of representatives in Antarctic regions compared to other taxa. Scientists have been searching for reasons for the lack of decapod species in the Southern Ocean, as they are otherwise very common in many different habitats within boreal and subtropical regions (Thatje 2003; Barnes 1987). Over 130 benthic and pelagic decapod species are found in the Southern Ocean north of the Polar Front (PF), while just 27 south of it (Wittmann et al 2010). Low number of decapod species in the Antarctic (about twelve caridean shrimps after Thatje 2003) could be a result of lacking adaption to polar conditions (e.g. physiological processes ((Parnas et al. 1994; Richmond et al. 1995; Dunn and Mercier 2003; and references within Wittmann et al 2010)), high energetic costs in reproduction processes, lower nutrition sources in combination with seasonal food availability and constant low temperatures, which affects and slows down metabolic processes as well as reproduction (Brey and Clarke 1993). Due to all the special features mentioned above, species like *Notocrangon antarcticus*, which are highly represented along the Antarctic continental shelf, despite extreme living conditions, among only few other decapods become highly interesting for research (most of them as well caridean shrimps (as e.g. *Nematocarcinus lanceopes* and *Chorismus antarcticus*)) (Arntz & Gorny 1999).

This project investigates the population genetic structure of *Notocrangon antarcticus* (Fig. 3 and 4) with regard to distribution around the Antarctic continent and along the Scotia Arc, using different types of genetic markers – 16S (mtDNA) and microsatellites (nDNA). On the one hand, the slow evolving 16S rDNA molecular marker is faster evolving than 18S rDNA, yet, it is still not the best marker to detect signals at intraspecific level and has in general a haploid nature. On the other hand, microsatellites are fast evolving and provide additional

information due to the diploid nature of nuclear markers and thus, supplement the results by having a high resolution at intraspecific level and detect even minor signals of population structure (Leese at al. 2008; Held 2000, 2003; Held and Wägele 2005; Wilson et al. 2007).

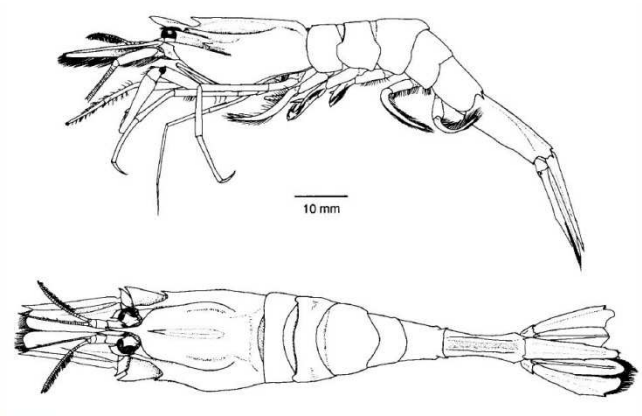

Fig. 3. *Notocrangon antarcticus* (Pfeffer 1887). After Komai and Segonzac (1996).

Fig. 4: *Notocrangon antarcticus* after preservation in 99% ethanol. Left: dorsal photograph. Right: lateral photograph of a brooding female with fertile eggs attached to its pleopods.

Notocrangon antarcticus is an Antarctic crangonid shrimp belonging to the decapods crustaceans, infraorder Caridea and family Crangonidae (Fig. 3 and 4). It was first described by Pfeffer (1887) and has a benthic lifestyle on the shelf, found in the entire Antarctic region including the sub-Antarctic island of South Georgia (Gorny et al 1993), which makes it a good model for population genetics. *N. antarcticus* is characterized by a significant reduction of the larval planktonic phase with probably missing or very short pelagic larval stage (<48h) (Arntz and Gorny 1991; Makarov 1970) since larvae hatch at a very advanced stage (Gorny et

al 1993). Little is known about the life history of *N. antarcticus* but its high occurrence, wide distribution and successful adaption all around the Antarctic benthos, makes it interesting to investigate the genetic structure of its population. First research projects on *N. antarcticus* with genetic markers have been started by Susannah Spieker (2009) within the framework of her Bachelors degree. However, she was looking for cryptic speciation within the *N. antarcticus* populations, as it has been proven to be more usual than expected throughout recent studies mostly among benthic species (see paragraphs above). Spieker (2009) used the mtDNA of the cytochrome oxidase unit CO1 but the genetic differentiation rejected a possible cryptic speciation event among the analyzed populations of *N. antarcticus*. The study indicated a possible population structure, although it was not explicitly tested as the sample size was small and from the two mitochondrial markers used (16S and CO1), only results from the CO1 marker where analyzed as the data for 16S was very little. Morever, both markers are not fast evolving enough for studies on population structure. Dealing with population genetics concerning distribution of genetic variability among populations of a single species requires more variable and preferably independent, co-dominant markers like microsatellites (Held & Leese 2007). These short tandemly repeated nuclear DNA motifs evolve faster than mitochondrial genes and thus provide high analytic power for studies on intraspecific level.

1.4 Objectives

The main objectives are the characterization of the previously isolated microsatellite markers (Agrawal et al. in prep.) in order to study the population structure of *N. antarcticus* combined with the additional 16S rDNA mitochondrial marker.

The study of the populations genetics of *N. antarcticus*, based on genetic markers, was performed with three isolated microsatellite markers and the ribosomal gene 16S to analyze intraspecific distribution patterns with a special focus on SGI in terms of population genetics, since the Scotia Arc is considered to be a key zone to study changes in decapods life story and distribution to evaluate evolutionary pathways and progress in terms of speciation (Crame 1999; Thatje et al. 2005). Herein, the hypothesis of the ACC as an important route for gene flow within the Antarctic, as well as a barrier to dispersal beyond the PF should be

tested. In this context, one should keep in mind that the exact location of the ACC and PF is still debated and probably oscillates somewhere between 47°S and 63°S (Kock 1992). Additionally, the probability of *N. antarcticus* using the Antarctic Peninsula and the islands of the Scotia Arc as “stepping stones” between populations will be discussed as well as the influences of climate and ecosystem change on the species populations.

2. Materials and Methods

2.1 Sample

Gentetic data of samples from various regions of the Antarctic (South Georgia and (SGI) South Orkney Islands (SOI); the Antarctic shelf (Larsen A, B and C (LA, LB, LC)); the East Weddell Sea Region (EWS) and Terre Adélie (TA)) was collected to evaluate the dimension of gene flow between the different Antarctic locations and test different hypotheses and their validity concerning the population of *N. antarcticus* (Fig. 5; App. 1, 2).

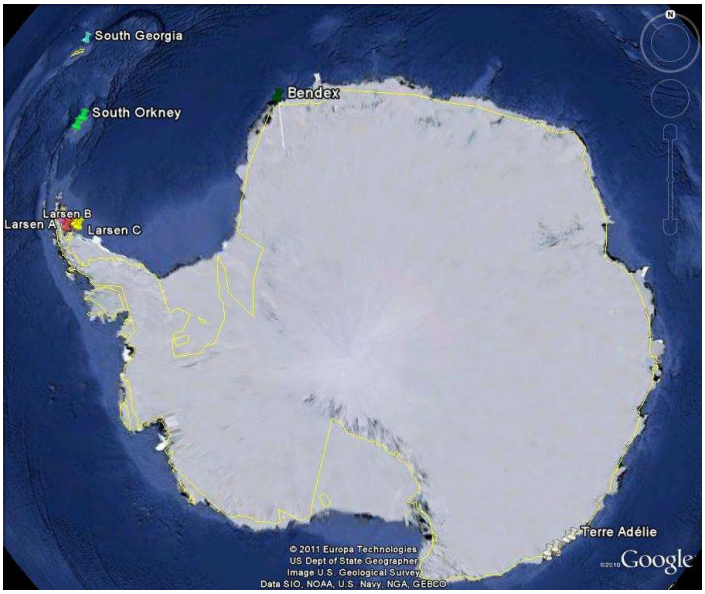

Fig. 5: Sample sites of the Antarctic decapod *Notocrangon antarcticus*: South Georgia Islands (SGI), South Orkney Islands (SOI), Antarctic Peninsula (Larsen A: LA, Larsen B: LB and Larsen C: LC), East Weddell Sea (EWS) and Terre Adélie (TA).

Specimens of *Notocrangon antarcticus* were collected during the "CAMBIO" (ANTXXVII/3, RV-Polarstern 2011 and "CEAMARC 2007" expeditions. Collection sites were South Georgia Island (SGI), South Orkney Island (SOI) the Antarctic Peninsula (AP) (Larsen A (LA A), Larsen B (LB B), and Larsen C (LC C)) and East Weddell Sea (EWS) during CAMBIO and Terre Adélie (TA) during CEAMARC (Fig. 5; App. 1, 2). The depths of sampled sites ranged from 321.0 to 566.7 m for CEAMARC/TA (App. 1) and 390.6 to 926.0 m for CAMBIO (App. 2). All samples were immediately fixed in ice-cold ethanol (97%).

For mitochondrial 16S rDNA gene and microsatellite analyses, DNA was extracted from aforementioned collection sites (32 from SGI, 30 from SOI, 90 from the AP (30 from LA, 30 from LB, 30 from LC), 5 from EWS and 23 from TA) shown in App. 1 and 2 (see Appendix).

2.2 DNA Extraction

DNA was extracted from samples of the CAMBIO expedition according to following extraction-protocol: „ DNA Purification from Tissues (QIAamp DNA Mini Kit) – QIAGEN". However, only 100µl of elution buffer (EB) were used to increase DNA concentration of the extraction. 2-3 legs of each *Notocrangon antarcticus* were removed with a pair of sterile forceps and DNA was extracted according to the manufacturer's instructions.

For the samples of the CEAMARC expedition (TA) DNA-extraction was performed using the Puregene DNA Purification Kit (Gentra Systems: Minneapolis, MN55447, USA; modified by C. D. Schubart in June 2009). DNA was extracted from muscle tissue dissected from the legs - with a sterile scalpel.

2.3 Mitochondrial DNA Markers

2.3.1 16S rDNA

2.3.1.1 Amplification and Sequencing

A subset of 27 specimens from SGI, 11 from SOI, 45 from the AP (15 LA, 16 LB, 14 LC), 5 from EWS and 10 from TA were analyzed for variation of the mitochondrial 16S rRNA gene. In some cases, DNA of samples used initially, had to be exchanged by DNA of other samples of the same locality, due to bad quality, and should be re-extracted in the future.

Amplification of a fragment of around 550bp of the 16S rRNA mitochondrial gene was initially carried out in 10 µl reactions containing 1× HotMaster reaction buffer, 0.2 mM dNTPs, 0.5 µM of each primer: 16Sa 5'-CGC CTG TTT ATC AAA AAC AT-3' and 16Sb 5'-CCG GTC TGA ACT CAG ATC ACG-3' (Palumbi et al (1991)), 1 µl of DNA template (50ng/µl), 0.02 U/µl HotMaster *Taq* (Eppendorf,5-Prime) and 0.5M Betaine, filled up to 10 µl with sterile

H_2O. The PCR temperature profile for the 16S amplification was: initial denaturation at 94°C (2 min); 35 cycles of denaturation at 94°C (20 s), annealing at 50°C (20 s), extension at 65°C (30 s); final extension at 65°C (8 min). PCR products were purified using the ExoSAP procedure (Hanke and Wink 1994), using 20 U ExoI (0,25µl) and 4 U SAP (1µl) (both Fermentas) per 5µl of PCR product with an incubation of 15 min at 37°C followed by inactivation at 80°C for 15 min. Before continuing with the sequencing procedure, some purified products were diluted 1:2 or 1:5, depending on the intensity of the bands visible on a 2% TBE agarose gel. Purified PCR products were bidirectionally sequenced after a cycle-sequencing process of: initial denaturation at 96°C (1 min) and 28 cycles of denaturation at 96°C (10 s), annealing at 50°C (5 s), extension at 60°C (7 min).Cycle-sequencing was performed in 10 µl reaction volumes using 1 µM of either 16Sa or 16Sb primer, 1µl of the purified template DNA/PCR-product and the BigDye Terminator Kit 3.1 chemistry (Applied Biosystems) according to the recommendations of the manufacturer. The cycle-sequencing PCR fragments were cleaned according to the "Dye-Ex 96 Protocol for Dye-Terminator Removal – Modified Protocol" from QIAGEN. Sequencing of the clean product was conducted on an ABI 3130xl sequencer.

2.3.1.2 Data Analysis

Quality of the sequences was checked and subsequently aligned and assembled with Codon Code Aligner version 3.7.1 (CodonCode Corporation 2007-2009) in order to create the Haplotype-network (Fig. 6).

2.3.2. 18S rDNA

2.3.2.1 Amplification and Sequencing

The 18S ribosomal gene was tested for a total of 16 samples from all sample sites using primers 18A1 5' – CCT AYC TGG TTG ATC CTG CCA GT – 3' and 1800 5' – GAT CCT TCC GCA GGT TCA CCT ACG – 3' designed by Trisha Spears (according to Vonnemann et al. 2004). Amplification was carried out in 10µl reactions (see above). The PCR temperature profile for

the 18S amplification was: initial denaturation at 94°C (2 min); 35 cycles of denaturation at 94°C (20 s), annealing at eight different temperatures from 39.9 to 50.3°C (20 s), extension at 65°C (30 s); final extension at 65°C (8 min) on an Epgrandient thermocylcer (Eppendorf). The PCR products were tested on a 2% TBA agarose gel. PCR products were purified and prepared for cycle sequencing under same conditions as mentioned above for 16S.

2.3.2.2 Data Analysis

Quality of the sequences was checked and subsequently aligned and assembled with Codon Code Aligner version 3.7.1 (CodonCode Corporation 2007-2009).

2.3.3 Cytochrome Oxidase CO1 mtDNA

2.3.3.1 Amplification

DNA from the SGI population was chosen to try the standard cytochrome oxidase (CO1) primers by Folmer et al (1994) LCO (5' GGT CAA CAA ATC ATA AAG ATA TTG G 3') and HCO (5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3'). Amplification was carried out in 10µl (see above). The PCR temperature profile for the CO1 amplification was: initial denaturation at 94°C (2 min); 38 cycles of denaturation at 94°C (20 s), annealing at eight different temperatures from 39.9 to 50.3°C (20 s), extension at 65°C (30 s); final extension at 65°C (8 min) on an Epgrandient thermocylcer (Eppendorf). The PCR products were tested on a 2% TBA agarose gel.

2.4 Microsatellites

2.4.1 Primer selection

Microsatellite primers for *Notocrangon antarcticus* were first isolated and designed within the Bachelor thesis of Susannah Spieker in 2010 after the reporter genome protocol (Leese et al. 2008) using *Mus musculus domesticus* as reporter genome DNA, yet never tested (Spieker et al 2010). Microsatellite markers Ncr1, Ncr2, Ncr3, Ncr4, Ncr5, Ncr6, Ncr7, Ncr8, Ncr9, Ncr10, Ncr11, Ncr12, Ncr13, Ncr14, Ncr15, Ncr16, Ncr17, Ncr18, Ncr19, Ncr20 (Agrawal

et al. in prep.; App. 3, Appendix) were herein tested on random individuals of all seven sample sites through gradient PCRs to determine the ideal annealing temperature (gradient: 40-60/62°C). Ncr1, Ncr3, Ncr6, Ncr12, Ncr14 were labeled (one of each pair, with either Hex or Fam) and applied to assess intraspecific genetic polymorphisms for all specimens from the seven sample sites (App. 1, 2)

2.4.2 Isolation, Amplification, Sequencing

Standard 10 µl reactions consisted of 1× PCR HotMaster Buffer, 0.2 mM dNTPs, 0.5 µM of each primer (one labeled (forward), one unlabelled (reverse)), 0.02 U/µl HotMaster *Taq* (Eppendorf, 5-Prime), 0.5 M Betaine (Sigma Aldrich) and 1-2µl of DNA with a concentration of 50ng/µl determined by (Nano-Drop). Cycling conditions on an Epgradient thermocycler (Eppendorf) were different depending on primers used (Tab. 1). A final extension step of 20 minutes at 65°C was performed to reduce *in vitro* artifacts due to incomplete adenylation of products (Leese and Held 2008). PCR products were visualized on 2% TBE agarose gels, diluted 1–10 fold with molecular grade water (CARL ROTH) and 1 µl of the diluted product was denatured in a mixture of 14.7 µl HI-DI formamide with 0.3 µl GeneScan ROX 500 size standard (both Applied Biosystems).

Tab. 1: Annealing temperatures for labeled primers isolated for microsatellite loci of the nuclear DNA of *Notocrangon antarcticus*.

	Ncr1	**Ncr3**	**Ncr6**	**Ncr12**	**Ncr14**
Annealing Temperature	50°C	50°C	54°C	50°C	45°C

In the case of Ncr20 and Ncr17, due to the fact that the primers amplified more than one product during the PCR, the resulting PCR products were separated by cutting the fragments out of an 2% TBE agarose gel according to the manufacturers protocol “5Prime PCR Extract and GelExtract Mini Kits Manual” (2007©) before sequencing. The cycle-sequencing was run under the same conditions as mentioned above.

2.4.3 Fragment Analysis and Genotyping

The fragments were analyzed on an ABI 3130xl; and allele length scoring was performed using the software GENEMAPPER 4.0 (Applied Biosystems). Samples were genotyped 4-7 times separately and results were compared to minimize genotyping errors. In addition, microsatellite fragments of random samples were amplified under same PCR conditions in separate PCRs, the fragments were analyzed 2-4 times and the results were compared, to calibrate the scoring criteria and to confirm scored genotypes. Samples with uncertain results were sorted out of further data analysis.

2.4.4 Cloning

In order to improve and redesign some of the existing microsatellite primers (Ncr and Mys by Agrawal et al. in prep.) for *Notocrangon antarcticus,* the PCR products of these primers were inserted in a plasmid pCR2.1-TOPO®TA vector from Invitrogen® (Lot no. 841084) and transformed in competent E.coli cells (Invitrogen® , Promega, Ch. 873292A) according to the heat-shock/ one-shot protocol manual from the Invitrogen kit: TOPO TA Cloning Kit. Cultures of positive colonies, identified by blue-white selection (IPTG/X-Gal), were grown overnight (ca. 17h at 37°C) on agar-LB-medium containing 100 µg/mL ampicillin. The competence of the cells was proofed before cloning the insert in a PUC 19 Vector (lot no. 837179) according to the recommendations of the manufacturer.

The PCR cycle profile for the cloning step was: initial denaturation at 94°C for 2min; 38 cycles of denaturation at 94°C for 20 secs, annealing for 20secs at different temperatures according to the primers used, and elongation at 65°C for 30secs followed by a single final elongation step of 20 min at 65°C. PCR products were tested on a 2% TBE agarose gel, cleaned with the QIAquick PCR Purification Kit according to the manufacturer's protocol for PCR products and frozen. Approximately four hours before cloning, the PCR products were thawed and 2 µl of each PCR product were used as template for a second PCR at aforementioned conditions to ensure adenylation of the PCR products for cloning. The new PCR products were cleaned with the same QIAquick PCR Purification Kit and tested on a 2% TBE agarose-gel. Purified

PCR products for Mys primers (Agrawal in prep.) were pooled and 2µl of the mixture were used for the one-shot cloning step with a single cell charge. A second cell charge was equally cloned but with 2µl of pooled PCR products for Ncr Primers (Agrawal et al. in prep.). For the transformation step, provided salt-solution (lot no. 804050) and water (lot. nr. 830136) were used. Each cell culture was equally divided on 6 plates with agar-medium to grow colonies over night at 37°C. 96 positive colonies of each cell culture (192 colonies) were chosen, placed separately on agar and sequenced by QIAGEN. Aforementioned 96 positive colonies were also grown over night at 37°C in liquid LB medium to provide an exact copy of the samples send to QIAGEN if needed. Additionally, some more positive colonies (672) were picked, grown at 37°C, precipitated and stored either in 10x HotMaster-PCR buffer (Eppendorf, 5-Prime) or in molecular grade water (CARL ROTH) at -20°C.

2.4.5 Data Analysis

The genotyping and allele scoring of the microsatellite fragments was performed using GENEMAPPER 4.0 (Applied Biosystems, 2004)

The GENEMAPPER Software generates genotypes from the raw spectra of prepared samples run on an electrophoresis instrument. The instrument performs electrophoric separation of the fluorescent labeled Fragments (due to the labeled primers used – “Hex” (hexachlorofluorescein phosphoramidite) or “Fam” (carboxyfluorescein) (Metabion int. AG.)). Thus, it monitors fluctuations in emitted light as the fragments migrate passing a laser. The Data Collection Software assembles the collected spectral signal for each fragment from each sample and stores the data for further analysis. GENEMAPPER Software separates the collective raw spectra for each sample into the component signals, corresponding to the emission wavelength of the fluorescent dyes used for the primers and size standard. Subsequently the software generates genotypes by processing the resulting dye “signals” (GENEMAPPER Software, User’s Guide, Copyright 2004, Applied Biosystems).
The resulting peaks were then genotyped manually from 4-7 times separately and results were compared to minimize genotyping errors.

After genotyping, the microsatellite allele size data from an excel sheet was changed in format using MSAT TOOL KIT, version 3.1.1 (12/2008; Park, 2001). The output file was converted into the required file formats, for further analysis, using CONVERT, version 1.3.1 (3/2005; Glaubitz, 2004).

To study the population structure with information from different microsatellite loci several statistical programs were employed, which are described briefly in the following paragraphs.

During the polymerase chain reaction (PCR) for microsatellites amplification some errors can occur, mostly among the annealing and amplification processes, as: one or more alleles do not amplify ("null-alleles"); biased changes in allele sizes occur due to stuttering of the polymerase while amplifying the repetitive motif, resulting in fragments with less base-pairs (bp) ("Stuttering"); large alleles are not amplified as efficiently as small alleles ("Large allele dropout"). MICROCHECKER 2.2.3 (Shipley 2003) helps to detect this type of errors to decrease bias during the interpretation and further analysis of the microsatellite allele data. This application is based on a Monte Carlo simulation (bootstrapping) method that generates expected homozygotes and heterozygotes allele size difference frequencies and compares this with the genotypes from the input allele size data. To calculate expected allele frequencies and frequency of any null alleles, the program uses the Hardy-Weinberg theory of equilibrium (HWE) (Van Oosterhout et al. 2003, 2004). Thus, this program was used to check the raw data for genotyping errors and for the presence of null alleles. The expected number of homozygotes for each class (allele size) is calculated based on the heterozygote frequency for that class. This number is then compared to the observed number of homozygotes. The probabilities of observed homozygote frequencies are computed using two methods: using the homozygote and heterozygote frequencies of each size class ("binomial based"); and by comparing the observed value to the mean rank position of that value in the simulated values ("rank based") (Van Oosterhout et al. 2003).

Null allele frequencies are shown by estimating allele frequencies and can be compared to the null allele frequencies obtained by using Chakraborty (Chakraborty et al. 1992) and Brookfield (Brookfield 1996) methods. However, no evidence was found for null alleles within the input data, thus, this function was not needed.

Population differentiation on *genic* differentiation level, as well as on *genotypic* differentiation level was performed for all population pairs with GENEPOP version 4.1 (Raymond and Rouset 1995). Both tests were run with following parameters using the same Markov Chain (by Gou and Thompson 1992) to assess p-values: 10000 burnin, 100 batches with 5000 MCMC steps each (MCMC: Markov Chain Monte Carlo; a class of algorithms that takes samples from probability distributions, based on the construction of a Markov chain). Genotypic differentiation is tested for following hypothesis H_0: "*genotypes are drawn from the same distribution in all populations*" related to the **distribution of diploid genotypes** in the different populations, while *genic* differentiation is tested for H_0: "*alleles are drawn from the same distribution in all populations*" concerning the **distribution of alleles** among the given samples. The p-value output was used to assign the significance of differentiation by using the calculated F_{ST}-values. The F_{ST}-values were calculated for all population pairs with GENEPOP, which follows standard ANOVA as in Weir and Cockerham (1984). The F_{ST} max value was computed by FSTAT (Goudet, 1995 (modified 2001)) after recoding the input file with RECODEDATA, version 0.1 (Meirmans, 2006). Hence, the standardized F'_{ST} value can be calculated dividing the F_{ST} value provided by GENEPOP by F_{ST} max, as it is recommended by Leese et al. (2008) and presented in the manual of RECODEDATA as it has become a common index for the magnitude of population structure. Moreover, diverse Hardy-Weinberg (HW) tests were performed with GENEPOP all with the same parameters and using the same Markov Chain (Dememorization: 10 000; Batches: 20; Iterations per batch: 5000) as well as for the computation of F_{IS} (inbreeding-coefficient).

In addition, STRUCTURE 2.3.3 2010 (Pritchard et al. 2003) supplied Bayesian multilocus-based clustering algorithm and was used to carry out individual assignment tests to populations. STRUCTURE was demanded with the Java front end and CONVERT transcribed the GENEPOP file with the genotype tables, into a STRUCTURE-compatible file-format. The clustering model of STRUCTURE, assigns individuals probabilistically to a population or jointly two or more populations from a *K* number of possible populations depending on their admixture level. Each *k* population is characterized by a number of allele frequencies at given loci. The program assumes that the loci within populations are at HWE and linkage equilibrium – in other words the parameters are set to group individuals together to populations in order to provide aforementioned priors (Structure 2.2 Manual). For the *N. antarcticus* data set, most likely number of populations was developed with prior

information on geographic origin of individuals and the maximum number of population was set to seven according to the number of sample sites (K from one to seven). The number of MCMS steps was set to 100000. Results were controlled as described in the manual-operating instructions to test the set up parameters and were found to be suitable. Hence, mentioned parameter sets were used to perform four independent iterations with a burn-in period of 1000 and a no. of MCMC steps of 100000 with and without using the population admixture model and with and without giving the sample location as a prior. Again, aforementioned tests were also performed with and without assuming correlation of allele frequencies. The number of populations was set from K=1 to K=7 according to the number of sample sites, in order to detect potential subpopulations. The final number of populations was determined by comparing the difference of calculated Bayes-factors for different number of assumed subpopulations and taking the corresponding and smallest “K” value (for the highest value of the differences between Bayes-factors) as the expected number of subpopulations for *N. antarcticus.*

3. Results

3.1 mitochondrial DNA markers

3.1.1 16S rDNA

A 550 base-pairs (bp) long fragment of the 16S rDNA was sequenced and a total of 98 sequences were aligned so as to investigate the possible existence of cryptic speciation in *Notocrangon antarcticus* within the sample sites around the Antarctic. Subsequently, two haplotypes were observed differing from each other by 5 bp within a total of 507 analyzed bp (1% mutation). One haplotype resulted for the sample region of SGI and the other haplotype for the rest of the sample regions around the Antarctic, as shown in Fig. 6.

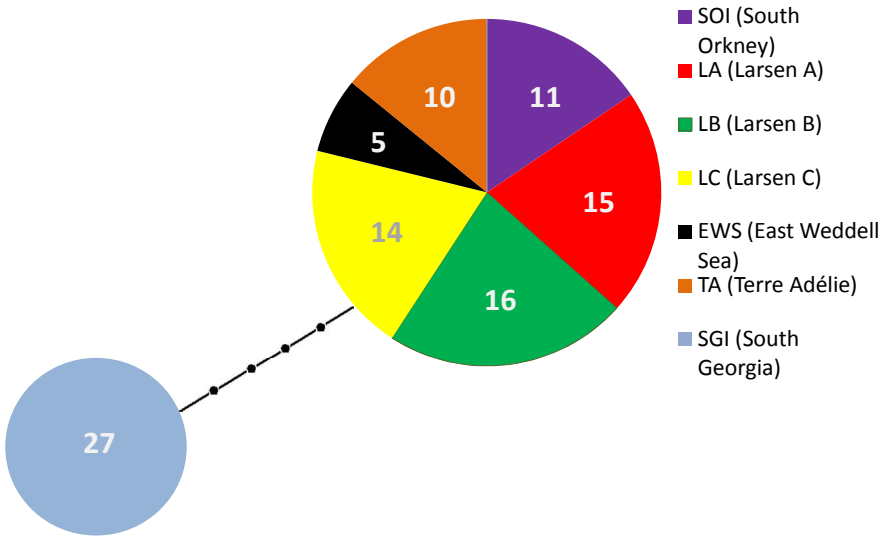

Fig. 6: 16S rDNA Haplotypes: network for a total of 98 sequences of the 16S rDNA of *Notocrangon antarcticus* from 7 localities of the Antarctic. The big circle represents one haplotype with each color representing one sample site and each number representing number of sequences and therefore, number of samples aligned for each region. The smaller light blue circle represents the second haplotype belonging to the sample site of SGI. The black line with dots connecting the two circles shows the base-pairs (bp) of difference between the two haplotypes, with each dot representing an additional single bp-mutation to the line.

Based on the 16S data, there is a clearly difference between the localities of SGI and the rest of the Antarctic *N. antarcticus*, probably due to lack of gene flow across the Polar Front. Results from the 16S rDNA haplotype network, surely confirm population differentiation

within *N. antarcticus*, which can be better investigated with help of faster evolving markers such as microsatellites.

Considering the fact that the sequences for sample sites SOI, LA, LB, LC, EWS, TA did all show the same haplotype, there is no need to increase the number of individuals tested to increase the reliability and significance of this clear 16S rDNA data set.

3.1.2 18S rDNA

Due to its length, an amplification of the whole fragment failed and thus mostly smaller fragments of either the "beginning" or the "ending" region of the fragment were successfully sequenced. Complete fragment of the whole 18S region were scarce and no mutations between different sample locations could be detected after the alignment of the sequences of the fragments. Considering as well that the 18S gene evolves slower than 16S and as the results of 16S did not show much variability, to continue and optimize the amplification of the 18S fragment for *Notocrangon antarcticus* did not seem to be necessary and of major importance for this study.

3.1.3 Cytochrome oxidase (CO1) mtDNA

The amplification PCR of the CO1 mitochondrial gene showed results for an annealing temperature from 39.9 to 44.3°C, but resulted in two PCR products for, as detected in the 2% TBE agarose gel - the bigger being approximately 800 bp long and the smaller one 200 bp long. Even though, the 200 bp long fragment is too small to be the sought fragment, it will interrupt the sequencing of the 800bp long CO1 fragments. Therefore, the sequencing process for CO1 could not be carried out within the framework of this study and the two fragments must be either cut out of the gel and purified before yielding more results, or different primers have to be used for this gene. These two PCR products were sighted for all tested temperatures, so, if the same primers shall be used and the protocol has to be modified to amplify only one fragment, the use of different annealing temperatures can be excluded. A possible explanation for the appearance of the small fragment (ca. 200 bp)

might be the presence of a pseudo gene of a region of the CO1 (originally mtDNA) located in the nuclear DNA or a totally different product unrelated to CO1. In this case a dilution of the template DNA might help to discard the smaller fragment, since more mtDNA as nDNA is expected in the DNA extracted (due to many mitochondria and only one nucleus per cell).

3.2 Microsatellites

3.2.1 Marker Selection

Within the 20 microsatellite primers designed for *Notocrangon antarcticus* in past research projects, five (Ncr1, Ncr3, Ncr6, Ncr 12 and Ncr14) were chosen and fluorescent-labeled for intraspecific population analysis (App. 3, Appendix). All 20 designed primers were tested and primers Ncr2, Ncr4, Ncr7, Ncr8, Ncr9 and Ncr11, were found to amplify a fragment without any repeat or variation and thus, were rejected for further analysis. However, the locus Ncr11 might be mutating to fast as the sequence showed many ambiguous peaks and therefore does not give a reliable signal, as no clear repeat was detected. Fragments for primers Ncr10, Ncr13 and Ncr16 have to be re-sequenced after only giving a result of 5bp during the sequencing process. Primers Ncr17 and Ncr20 showed 2-3 bands in the 2% TBE agarose gel and the amplified fragments were therefore treated separately through cutting and purifying in order to treat the different products separately. Since, latter primers were not specific enough to amplify only one fragment, new primers have to be designed and they had to be subsequently expelled from the fragment analysis within this project. Yet, the corresponding loci should be taken into consideration for further research projects as they show repeats in their sequences and might be, for instance, potential microsatellites. Locus Ncr5 showed a very complex repeat pattern over 25 bp length and was not used for the fragment analysis either but the function as genetic marker cannot be rejected. Locus Ncr15 showed a repeat motive and could be a good candidate for future analysis in order to expand the data used for this thesis. Ncr18 and Ncr19 primers did not amplify any fragment nor gave any other results (see App. 4).

In order to re-design primers which did not yield a clear sequence or amplified more than one region, the products of these primers were cloned into *E. col*i as described under 2.4.4. The products seemed to have been successfully cloned, detected by IPTG/X-Gal blue/white

selection; and the chosen colonies were stored appropriately in order to be analyzed in future studies.

3.2.2 Fragment Analysis

Out of the five labeled markers, three polymorphic and reliable microsatellite loci developed for *N. antarcticus* were applied to attain intraspecific genetic polymorphisms for all extracted specimens from all sample sites. Ncr6 was discarded because it seemed to only have monomorphic peaks over all sample sites, as well as Ncr12 because of its genotyping was not reliable, due to many stutter peaks. The genotyped alleles for each tested marker on each individual are shown in App. 5. The missing allele data from some samples in App. 5 has to be supplemented in future and is only missing because of lack of time and not because of the failure of fragment amplification.

Alleles for Ncr1, Ncr3, and Ncr14 (App. 3) were polymorphic in all tested populations. Screened alleles per locus for all specimen ranged from 3 (Ncr1) to 19 (Ncr3) and the number of genotypes from 6 (Ncr1) to 47 (Ncr3) (Tab. 2). Thus, locus Ncr1 appears to be less polymorphic compared to Ncr3 and Ncr14, despite having the highest number of scored individuals, so the small sample size is probably not the reason for small range of allele-types. The probability of observed homozygotes was only significant in the case of Ncr14 according to MICROCHECKER (App. 6). However, the number of expected and observed homozygotes does not differ drastically among the other loci Ncr1 and Ncr3 either. All in all, no evidence was found neither for scoring errors due to stuttering nor for large allele dropout, nor null alleles, in all three loci. Hence, the data were considered as reliable and allow further tests on population genetics.

Tab. 2: Microsatellite analysis of the species *Notocrangon antarcticus* containing number of scored samples (N_S) scored alleles (N_A) and inbreeding-coefficient (F_{IS}) for each loci and each population; observed heterozygosity (H_O) and expected heterozygosity (H_E) for each population over all loci. Populations represent sample sites off South Georgia Island (SGI), South Orkney Island (SOI), Antarctic peninsula (Larsen A, B and C (LA, LB, LC)), East Weddell Sea (EWS) and Terre Adélie (TA).

	N_S			**N_A**			**F_{IS}**			**H_O**	**H_E**
	Ncr1	*Ncr3*	*Ncr14*	*Ncr1*	*Ncr3*	*Ncr14*	*Ncr1*	*Ncr3*	*Ncr14*		
SGI	21	12	9	3	11	6	**0.3830***	-0.082	**0.3496***	0.6138	0.7090
SOI	14	11	9	3	10	4	-0.5838	-0.1	-0.098	0.8783	0.6926
LA	13	10	5	3	9	5	-0.4667	-0.0062	-0.1765	0.9154	0.7317
LB	13	10	6	3	11	3	-0.5349	-0.0843	-0.5789	0.9487	0.6775
LC	11	10	5	3	9	5	-0.2329	0.2317	-0.2121	0.8394	0.7518
EWS	4	5	5	2	7	7	-0.5	-0.1111	-0.1111	0.9167	0.7029
TA	14	12	8	3	10	5	-0.4649	-0.0168	-0.1395	0.8829	0.7217

Bold F_{IS}-values are significant with a * representing a p-value < 0.05. H_O and H_E are both in Hardy-Weinberg equilibrium with a p-value < 0.05.

After checking the data on its reliability, the genic differentiation between each possible population pair was computed with GENEPOP. The resulting analysis showed that the population of SGI clearly differed from the other six populations. Genic differentiation was significant between SGI and all other tested populations (SOI, LA, LC, TA (p-value < 0.01), LB and EWS (p-value < 0.05), see also Tab. 3). Different than expected were the results for the probability of genic differentiation between LC and the populations of SOI and LB, which showed significant probability of genic differentiation even though the sample site of LC and LB are geographically very close to each other (Tab. 3; Fig. 5). However, the magnitude and significance of the differentiation between populations can be only regarded considering the F_{ST} or standardized F'_{ST} values (see also following paragraphs and Tab. 4).

Tab. 3: Tests on genic and genotypic differentiation for the species *Notocrangon antarcticus*. Significance of the genic differentiation for all population pairs across all loci (upper diagonal) and of the genotypic differentiation for each population pair across all loci (lower diagonal) both calculated following the Fisher's method. Populations represent sample sites off South Georgia Island (SGI), South Orkney Island (SOI), Antarctic peninsula (Larsen A, B and C (LA, LB, LC)), East Weddell Sea (EWS) and Terre Adélie (TA)

	SGI	SOI	LA	LB	LC	EWS	TA
SGI		**	**	*	**	*	**
SOI	**		-	-	**	-	-
LA	*	-		-	-	-	-
LB	-	-	-		*	-	-
LC	*	**	-	**		-	-
EWS	-	-	-	*	-		-
TA	**	-	-	-	-	-	

-: p-value > 0.05; *: 0.05 > p-values > 0.01; **: p-values < 0.01. With H_0: "Alleles are drawn from the same distribution in all populations" for the genic differentiation probability and H_0: "Genotypes are drawn from the same distribution in all populations" for the genotypic differentiation probability.

The same test was thus carried out for the probability of genotypic differentiation between populations. This test showed respectively significant genotypic differentiation between the population of SGI and the populations of SOI, LA, LC and TA. Surprisingly, there was no significant probability of differentiation to be found between the population of SGI and the populations of LB and EWS as calculated for the probability of genic differentiation. The lack of significant genotypic differentiation between populations LB and EWS and SGI might be the consequence of a relatively recent separation of the SGI population from EWS and LB. However, it also depends on the H_0 hypothesis for the genotypic differentiation, which always considers the distribution of genotypes among populations. All in all, the genotypic differentiation test is congruent to the genic differentiation and confirms a significant differentiation of the LC population and the populations of SOI and LB as it was the case of the genic differentiation test. Furthermore, this test shows a significant probability of genotypic differentiation between the population of LB and EWS.

Results for Hardy-Weinberg (HW) exact tests showed a heterozygote deficit (H_1) for SGI (according to locus Ncr1 (p: 0.0053) and Ncr14 (p: 0.0450)), LC (according to locus Ncr3 (p: 0.0208)); and a heterozygote excess for populations SOI, LA, LB and TA (according to locus Ncr1 ($p<0.05$)). Regarding all populations together, Ncr14 did not show in any case a heterozygote deficit, nor did Ncr3, which, in addition, neither showed heterozygote excess. For the HW score (U) test, following the same parameters as in the aforementioned test, the

results for all loci and all populations, taken into consideration collectively, gave no evidence for neither excess nor deficit of heterozygotes. However, results by populations (for all loci) show a heterozygote excess for SOI and LB ($p < 0.01$). Heterozygote excess was as well computed for locus Ncr1 with the test by locus ($0.01 > p < 0.05$). The same test shows a heterozygote deficit for SGI (p: 0.045) among results by populations (pooled loci) and no kind of heterozygote deficit for any locus among results by locus (pooled populations). To sum up, the most important result to record of HW exact tests is the significant high heterozygote deficit among SGI, discussed later on.

F_{IS} (inbreeding coefficient) estimates show a significant inbreeding level for SGI as well as for LC, the latter being however, a bit lower (Tab. 2). In the case of SGI the high F_{IS} value can also be a consequence genetically isolation from all other populations tested (see Discussion)

H_E and H_O give expected and observed heterozygosity values and determine genetic diversity. H_O is practically the same among all populations and show equal distribution of genetic diversity and therefore a big effective population size (Tab. 2). Anyhow, the observed heterozygosity among the individuals of the SGI populations is lower than in all other populations, giving evidence of a lower effective population size than expected and thus, maybe lack of genetic exchange with the other populations.

The F_{ST} and the standardized F'_{ST} values were calculated for all loci pairs. According to the significance from Tab. 3, the F_{ST}/F'_{ST} values between SGI and the populations of SOI, LA, LC, TA are significant ($p < 0.01$) as well as the F_{ST}/F'_{ST} values between the LC and the population of SOI. However, after Bonferroni correction ($\alpha' = \alpha/k$; *k:* no. of independent significance tests at the α level (*k*= 7 (no. of populations) x 3 (no. of Loci) = 21); α=0.05; α'= 0.00238) only the F_{ST}/F'_{ST} values between SGI and the populations of SOI, LC and TA as well as between LC and SOI can be considered as highly significant ($p < 0.00238$) (Tab. 4, see bold F_{ST}/F'_{ST} values).
Bonferroni correction is an α-value you can compute to adjust the criteria of significance to the number of populations and test run for it (markers/loci used). This is a more strict operation to decide over significance of values. However, it is not always applied thus, the discussion of the results will be only based on the standard α-values of 0.05 or 0.01 or the α-value after Bonferroni correction for even higher "significance".
A similar procedure is done by computing the standardized F'_{ST} values but in a modified way. F_{ST} values give the magnitude/significance of the degree of population structure given by

Fisher's-test. However, in some cases the magnitude of F_{ST} values is very low and therefore gives less evidence of significance to the population's structure. Standardized F'_{ST} values are higher and more evident than F_{ST} values, but the proportion/ratio among standardized F'_{ST} values compared to the proportion/ratio among F_{ST} values stays more or less the same. As a consequence, one could get comparable results from the F_{ST} values than from standardized F'_{ST} values, which have become a common index and are thus used herein.

Tab. 4: Pairwise F_{ST} estimates for all loci (diploid) Pairwise as in Weir and Cockerham (1984) (lower diagonal) and standardized F'_{ST} (upper diagonal) for seven different populations of *Notocrangon antarcticus*. Populations represent sample sites off South Georgia Island (SGI), South Orkney Island (SOI), Antarctic peninsula (Larsen A, B and C (LA, LB, LC)), East Weddell Sea (EWS) and Terre Adélie (TA)

	SGI	SOI	LA	LB	LC	EWS	TA	
SGI		**0.2680****	0.2760**	0.1076*	**0.3252****	0.2752*	**0.3576****	
SOI	**0.0670****		0.0268	0.0420	**0.2032****	0.1644	-0.0188	
LA	0.0690**	0.0067		0.1168	-0.0596	-0.1136	-0.0552	**(F_{ST} max: 0.250)**
LB	0.0269*	0.0105	0.0292		0.2304*	0.2868	0.0816	
LC	**0.0813****	**0.0508****	-0.0149	0.0576*		-0.1280	0.0676	
EWS	0.0688*	0.0411	-0.0284	0.0717	-0.0320		0.0896	
TA	**0.0894****	-0.0047	-0.0138	0.0204	0.0169	0.0224		

F'_{ST} values were calculated with F_{ST} max. * and ** indicate significant F_{ST} and F'_{ST} values for standard α-values (0.05 and 0.01) without Bonferroni correction (*: 0.05 > p > 0.01; **: p < 0.01). Bolded F_{ST} and F'_{ST} values are still significant after Bonferroni correction (p-value < 0.00238).

Results from the F_{ST}/F'_{ST} values reinforce presumptions about a lack of gene flow between SGI and most of the other Antarctic *N. antarcticus* populations as well as for the LC population from some other Antarctic *N. antarcticus* populations (according to standard α-values of 0.05). Yet, the results for LC should be observed with caution and critically. For future analyses a bigger sample set should be tested for more than 3 nuclear markers to reinforce the significance of results herein and decrease possible bias. A higher number of tested individuals might also reject the presumption of a partly isolated population in case of LC. Furthermore, above mentioned procedure should be also applied all over the tested regions to assure yielded results more confidently.

In order to get the best result of the Bayesian cluster analyses of STRUCTURE all possible models were tested (with and without admixture model; with and without information about geographic origin; and with or without the assumption of allele frequencies being independent among populations (8 tests in total)). The best model for the populations of *N. antarcticus* with the highest likelihood of the data resulted when using information on the

geographic origin of individuals as prior and assuming admixture and independent allele frequencies among populations (Tab. 5). Herefore, the software calculated two populations (K=2) to be the most likely population distribution among the samples. The samples were divided into two clusters: one for the population of SGI and the other one for the populations of SOI, LA, LB, LC, EWS and TA, as expected from the 16S haplotype network under 3.1 (Fig. 7). If no admixture was assumed, the most likely distribution was as well into two clusters (K=2) and thus, affirms the possible existence of two different population one for SGI and the other one for the rest of the sample sites around the Antarctic (Fig. 8). Nevertheless, the Bayesian factor for K=3 was higher than expected and might give a hint on a second subpopulation. The Bar plot for the latter model with K=3 also shows a third population cluster for LC (Fig. 9). However, the difference of Bayesian factors (Tab. 5) as well as the Ln P(D) values (Tab. 6) for K=3 is too small to be significant X. Yet, the hypothesis of a third subpopulation for LC could be supported by results computed for F-statistics and could be tested by using more loci (see paragraph above).

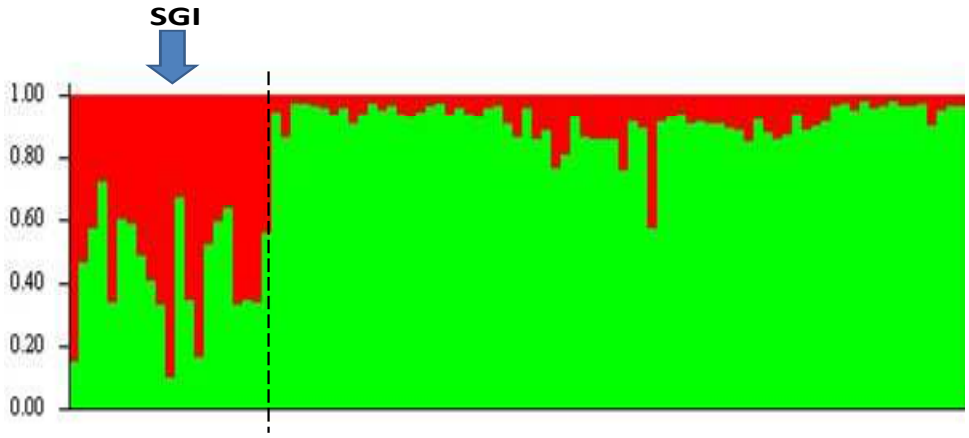

Fig. 7: Bar plot for Bayesian clustering using admixture model, sample location information and independent allele frequencies among populations as parameter settings for K= 2 (distribution of the samples into 2 populations). The figure shows two clusters; one belonging to the samples of SGI (*k2:* red, left side) and the other one to the samples from SOI, LA, LB, LC, EWS, TA (*k2:* green, right side). This model represents the best observed STRUCTURE model for *N. antarcticus.*

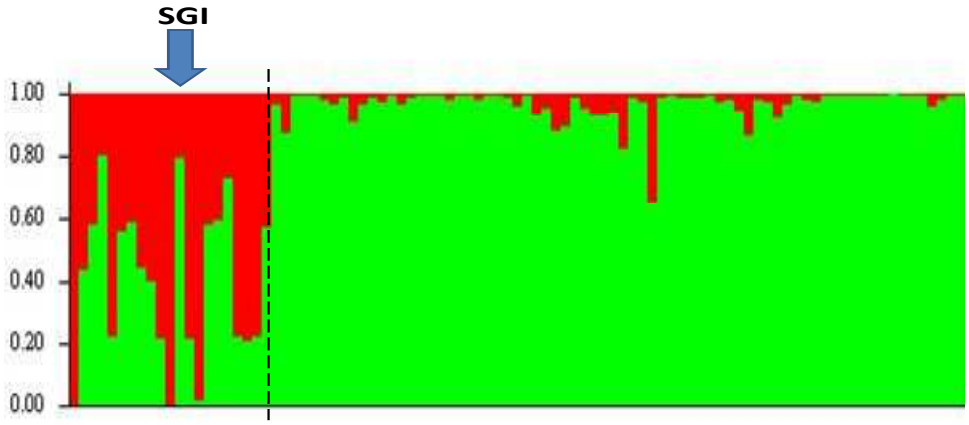

Fig. 8: Bar plot for Bayesian clustering using no admixture model, sample location information and independent allele frequencies among populations as parameter settings for K= 2 (distribution of the samples into 2 populations). The figure shows two clusters; one belonging to the samples of SGI (*k2:* red, left side) and the other one to the samples from SOI, LA, LB, LC, EWS, TA (*k2:* green, right side). This model represents the second best observed STRUCTURE model for *N. antarcticus*.

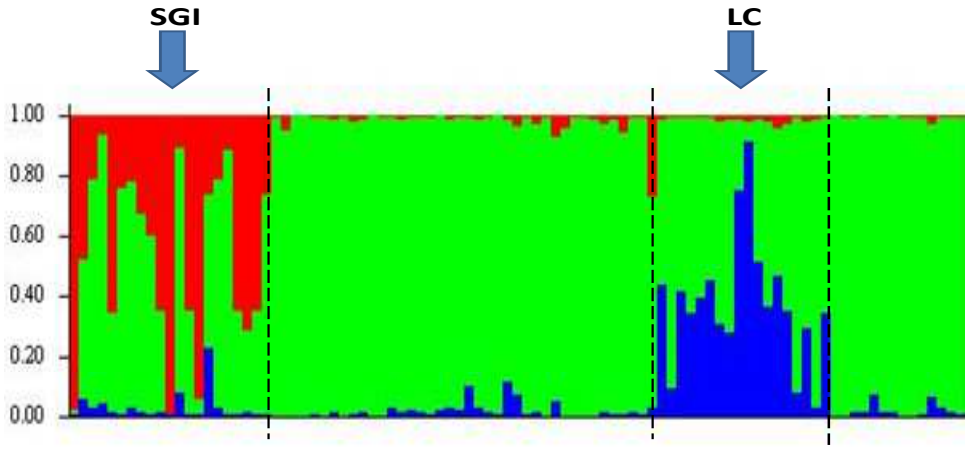

Fig. 9: Bar plot for Bayesian clustering using no admixture model, sample location information and independent allele frequencies among populations as parameter settings for K= 3 (distribution of the samples into 3 populations) from 2 different runs (run 11 and run 10). The 3 clusters are composed one by the SGI samples (*k1*, left side: blue; right side: red), the other one by LC samples (*k2*, left side: green; right side: blue), and the last one by samples from SOI, LA, LB, EWS and TA (*k3*, left side: red; right side: green).

Tab. 5: *Notocrangon antarcticus*; difference between Bayes-factors for different numbers of possible population clusters K (one to seven). The upper diagonal is for the STRUCTURE model assuming admixture and independent allele frequencies among the populations, using the sample location information as prior for distribution into possible population clusters. The lower diagonal is for a model with aforementioned parameters however without admixture.

	K=1	***K=2***	***K=3***	***K=4***	***K=5***	***K=6***	***K=7***
K=1		-0.999	7.67E-05	7.67E-05	7.67E-05	7.67E-05	7.67E-05
K=2	-0.502		**0.999**	**0.999**	**0.999**	**0.999**	**0.999**
K=3	-0.049	**0.900**		4.93E-08	4.93E-08	4.93E-08	4.93E-08
K=4	-4.10E-06	**0.950**	0.049		-2.97E-11	4.16E-13	3.25E-13
K=5	-7.65E-07	**0.950**	0.049	3.33E-06		3.01E-11	3.00E-11
K=6	1.16E-06	**0.950**	0.049	5.27E-06	-4.34E-06		-9.08E-14
K=7	-8.45E-06	**0.950**	0.049	1.93E-06	-7.68E-06	-9.61E-06	

Bold ciphers represent the best probable distribution within k populations (see also Tab. 6).

Results in Tab. 5 were generated by calculating the difference between the Bayes-Factors of each estimation of population numbers (K) among the samples (K= from one to seven). Therefore, average Ln P(D) values for each possible K (from one to seven according to the populations), computed during the different runs, were calculated from the project summary of STRUCTURE for the different models (Tab. 6). Next, average Ln P(D) were inserted in the below formula (equate for K=1) as explained by the STRUCTURE manual, to calculate the Bayes-factor for each possible population estimation (K= one to seven):

$$\frac{e^{\text{avg. Ln P(D), K=1}}}{e^{\text{avg. Ln P(D), K=1}} + e^{\text{avg. Ln P(D), K=2}} + e^{\text{avg. Ln P(D), K=3}} + e^{\text{avg. Ln P(D), K=4}} + e^{\text{avg. Ln P(D), K=5}} + e^{\text{avg. Ln P(D), K=6}} + e^{\text{avg. Ln P(D), K=7}}}$$

Once having the Bayes-Factors of each “K”, they were subtracted (Tab. 5) giving the difference between two different “K” assumptions and thus, the significance of assumed number of populations “K”. The higher the value, the higher the probability of the presumed number of populations “K”. If the results for two different “K” assumptions show similar values, the least number of populations “K” should be taken as the number of populations for the corresponding model. However, in this case, the results were unambiguous giving evidence of K=2 being the best setting for the assignment of individuals.

Tab. 6: Average Ln P (D) abstracted from the STRUCTURE project summary for the model assuming admixture and independent allele frequencies among populations, and using sample location as prior (first row) and for the model without assuming admixture but independent allele frequencies among populations as well as using sample location as prior (second row).

	K=1	**K=2**	**K=3**	**K=4**	**K=5**	**K=6**	**K=7**
avg. Ln P(D)							
admixture model	-721.1	**-711.925**	-728.75	-740.4	-736.15	-744.1	-741.875
avg. Ln P(D)							
no admixture model	-721.375	**-707.775**	-710.725	-719.875	-720.875	-725.55	-719.275

Bold Ln P(D) values are for the best K for used models.

All in all, the results of STRUCTURE indicate a clear population structure consisting of at least two subpopulations and therefore, reinforce the results obtained from the other tests on population genetics for *N. antarcticus.*

4. Discussion

According to the yielded results, three conclusions can be made. First (1), the Antarctic Continent including SOI shows a circumpolar distribution of the species *Notocrangon antarcticus* with more or less constantly recurring gene flow between mentioned populations. Second (2), the population of SGI seems to be an independent evolutionary significant unit (ESU) as it is significantly genetically isolated from the rest of the populations off Antarctica and SOI. Third (3), there are indications for genetically isolation of the LC population within the Antarctic continental shelf, situated in the Antarctic Peninsula.

(1) In former times, most Antarctic species were believed to have a circumpolar distribution with no or only little genetic differentiation. However, most of the initial studies were based on morphology alone and had no molecular/genetic evidence. Thanks to modern research with molecular markers cryptic speciation has been proven to be present for many species over different taxa (Held 2000, 2003; Held and Wägele 2005; Mahon et al. 2008; Krabbe et al. 2009).

Herein presented results for *N. antarcticus* give evidence for a more or less genetically homogenous and circumpolar distribution of the species along the continental shelf of the Antarctic (for LA, LB, EWS and TA) including SOI region all belonging to one single ESU. Reasons for this distribution pattern might be lack of major physical barriers, the strong ACC as a pathway for gene flow between specimen within geographically high distanced locations and drifting of larvae or adults on floating substrata or human engendered transport of (Robinson 1983; Scheltema 1986). It is interesting to find a homogenous distribution pattern for *N. antarcticus* as it probably has very short larval stage (Arntz and Gorny 1991), which would give less evidence to a homogenous distribution due to larval dispersal enhanced by water currents. The adult's benthic, less mobile life style and digging behavior would also reject migration events over long distances along the shelf. Other benthic species like *Chorismus antarcticus* and *Nematocarcinus lanceopes* (both benthic shrimps) also show a strict circumpolar distribution confirmed by studies based on molecular markers like mtDNA of the 16S ribosome gene and nuclear DNA of the 28S gene (Raupach et al 2010). Apparently, gene flow is still maintained between benthic species and their capacity of dispersal is sometimes underestimated. Moreover the presence of pelagic larval

stages does not automatically imply a circumpolar distribution. Modern research with molecular markers have brought cryptic speciation to light for many species over different taxa, such as Antarctic Cephalopoda (Brierley et al. 1993; Allcock et al. 1997; Sands et al. 2003), Teleostei (Patarnello et al. 2003; Kuhn and Gaffney 2006), and Euphausiacea (Patarnello et al. 1996; Jarman et al. 2002), even though all have pelagic life stages and should therefore be capable to disperse around Antarctica using the ACC as route for gene flow enhancement. It is important to consider more aspects in this context. Due to (not clear-causality?) high paternal energetic investment into larvae from *N. antarcticus* and the short pelagic larval stages; it is possible that larvae are able to drift with the ACC for short periods, overcome shorter distances and at the same time have enough energy left to successfully settle at the right time in the new habitat. After growth and reproduction genetic exchange between populations becomes possible. Environmental conditions of the new habitat might not differ much from their original environment as they are probably not so far, making it even easier to adapt. Due to the continuous distribution and high frequency of *N. antarcticus* along the continental shelf, populations are linked with each other making it easy for immigrant specimens (larvae or adults) to pass their genes on to the next population and so on. Thus, a subdivision along the shelf populations is missing. In contrast larvae from other species (see ascidians in (2)), which have long or median pelagic phases, might not survive the long voyages along the ACC nor maintain gene flow between populations. As a result, only larvae that developed in the original habitat might be able to successfully reproduce within its original population and thus, keep the genetic input only within the location of origin. Therefore, no homogenous circumpolar distribution can be established because of missing gene flow. Reasons for the high mortality of larvae drifted for long distances by the ACC might be the sharp temperature changes along the PF and the drift to inadequate environments for their development as well as predators and lack of nutrition sources in open waters, which would explain cryptic speciation within species with pelagic larvae.

Anyhow and probably the most plausible reason for a genetic homogenous and circumpolar distribution of *Notocrangon antarcticus* along the Antarctic continental shelf and SOI is the fact that the frequency of the species is relatively high and wide distributed, inhabiting more or less the whole benthos of the Antarctic shelf. In other words the existence of a continuous environment for *N. antarcticus* allows its specimen to be genetically linked

without having the need of migration over long distances and wide larval dispersal. Therefore, constant gene flow between locations is either disturbed by isolation by distance nor major physical barriers nor fragmentation of habitats.

(2)The population of SGI seems to be an isolated evolutionary significant unit (ESU) (see 16S haplotype-network, Fig. 6; and STRUCTURE population-clusters). One of the main reasons for this event might be found within the location of SGI. Even though, the Polar Front (PF) was postulated to be at 50°S (Hedgpeth 1969) and hence, north of SGI, recent cruises from the *RV* Polarstern have provided information indicating that the PF might be located south of SGI (Arntz 2005; Clarke and Barnes 2005). After all, since SGI is located somewhere in between the "border" of the PF, the climate conditions around SGI are being affected by it as the PF is said to be the barrier between sub-Antarctic and Antarctic waters, and thus evoke drastically differences of water temperatures between the two regions.

Temperature is a primary and probably the most important exogenous factor concerning oogenesis and growth in ectotherm invertebrates. Hence, temperature differences between SGI and other Antarctic regions might be responsible for the differences among others in maturity size and rate of development (Bergström 1992; Calcagno et al 2005). Previous studies have already shown physiological differences with regard to the population of *N. antarcticus* in SGI. Individuals from SGI did not only differ in their size at gonadal maturity - having a smaller average carapace length (CL) in SGI (females: 14.6 ± 1.2 mm; males: 12.2 ± 3.3 mm) than in SOI (females: 16.6 ± 1.3 mm; males: 13.3 ± 2.6 mm) (Lovrich et al 2005), but, also in fecundity - being higher in SGI than in SOI; and the timing of reproduction cycles - delayed off SOI (Lovrich et al 2005; Gorny et al 1993; Makarov 1970; see also following paragraph).

Similar results were also achieved in an investigation for the mysid crustacean *Antarctomysis maxima* and *Atarctomysis ohlini*, which had a reproductive cycle of two years in SGI and of four years in SOI (Ward 1984). The studies mentioned above only compared two populations, the SGI and the population of SOI, which could also be interpret as evidence for anomalies within the population of SOI. However, the results presented herein reject aforementioned assumption, as SOI does not differ genetically from the rest of the *N. antarcticus* populations from the Antarctic continent but only from the population of SGI.

Consequently, the *N. antarcticus* population off SGI is differing physiologically from the rest of the Antarctic.

As mentioned in the last paragraph, temperature might be the cause and answer for the differences between “Antarctic” and “SGI” populations of the species *N. antarcticus*. Temperature conditions between SGI and the Antarctic Continent (including SOI) are considerably different, with SGI having a surface water temperature of 2 to 4°C (Hunt et al 1991) while SOI and the Antarctic Continent stay around and below 0°C (Grabbert et al 2003; Murphy et al 2007). Besides, the current glaciations periods and the ice covered water surface (SGI: 57%; SOI: 90%) last longer and are respectively larger with longer periods of ice cover off SOI and Antarctica. Mentioned factors are, in conjunction with other abiotic factors like mineral enrichment and sun light disponibility, necessary for phytoplankton productivity (Atkinson 2001; Barnes et al 2005), resulting in a delayed food availability among Antarctic regions in comparison to SGI. All these factors are probably some reasons why Lovrich et al. (2005) and Gorny et al. (1993) have found out that the reproductive cycles of *N. antarcticus* at SGI start significantly earlier in the year compared to that of SOI; at a smaller maturity size, and with higher number of smaller eggs. The latter, probably because of lower necessity of high energetic input (e.g. egg mass) for the development of the egg in warmer regions (SGI) than in a colder ones (rest of Antarctic) and also due to a smaller size of hatching females in warmer habitat. Moreover, females at SGI are able to even breed annually, while females off Antarctica and Weddell Sea omit one year before starting a new reproductive cycle, being able to breed only every second year (Lovrich et al. 2005; Gorny et al. 1993). The pause of one year after a reproduction cycle for Individuals of the Antarctic region might as well be a result of the higher necessary energetic input per egg, which on the one hand, first has to be stored by the females before being able to start a new reproductive cycle and on the other hand, is harder to store and gain in colder regions with lower food sources. The fact that the reproductive cycles of the two distinguished populations of *N. antarcticus* are shifted within one cycle might already have drastical consequences on gene flow between populations. Gene flow between the Antarctic populations and the population of SGI would be impossible since a different reproductive rhythm with different times of mating (starting earlier in SGI) would separate populations reproductively from each other. Genetic drift will start to take place and might even lead to cryptic speciation if genetic drift and isolation keep augmenting.

Additionally, if the polar front is located southwards of SGI it might be a serious barrier for specimen dispersal between populations south and north of the PF and so between the SGI and the rest of the Antarctic populations. The PF was long time believed to be nearly impossible to overwhelm. Examples of bivalves, brittle stars and sea slugs show a separation after the ACC established 30-24 Myr ago (Lawver et al. 1992) probably because of lack of continuous migration between populations and thus, genetic discontinuity due to lack of gene flow from north to south across the ACC. There are also some studies, showing that warm water and cold water eddies can easily pass this barrier and therefore, serve as a transport system for species from north of the PF towards the Antarctic and vice-versa, connecting locations and helping fauna to preserve a collective biogeographical distribution along the Scotia Arc (Marshall and Pugh 1996; Bernard et al. 2007). Other possible means of transport across the PF might be floating substrata or even human engendered transport of species e.g. through fishering nets and ships (Robinson 1983; Scheltema 1986). Anyhow, the PF is still a strong barrier dividing ocean systems with different climate conditions north and south of the ACC, which fortifies its significance as barrier between ecosystems. Consequently, if the PF is located south of SGI, it would lead to a separation from the Antarctic habitat, making it rather part of the sub-Antarctic environment and automatically, providing best conditions for the origin of a new ESU, which is already the case and even speciation in long term.

Moreover, a SACCF (South Antarctic Circumpolar Current Front) has been registered north of SGI, which is supposed to wrap SGI in an anti-cyclonal way (Thorpe et al. 2002). The SACCF would, together with the changing position of the PF, lead to a constantly instable and more different environment off SGI than expected. Regarding these facts, the establishment of possible and potential recruits and migrant larvae or even unlikely adult-migrants of *N. antarcticus* as well as any other Antarctic species, will rather be impossible. A low range of migrant specimens would lead to a low effective population size and thus, be the cause for loss of genetic variability (see Tab. 2, H_E), which already seems to be the case for *N. antarcticus* off SGI. Aforementioned loss of genetic variability due to either pre-zygotic reproductive isolation (due to different spawning times) or physical isolation of the SGI population by the PF and SACCF would also explain the high F_{IS} obtained in the results under point 3.

Similar differentiation between the populations SGI and SOI are also found in some ascidians like *Aplidium falklandicum* (see Demarchi et al. 2010). Ascidians are completely sessile creatures, which could provide an explanation for the population differentiation within specimens from SGI. Nonetheless, ascidians have pelagic larvae that are easily drifted by ocean currents (Demarchi et al. 2010), which should allow gene flow between SGI and other populations. The fact that they still show population differentiation in SGI again reinforces theories of SGI being isolated in different ways from the Antarctic, perhaps even more than once thought to be. Moreover, other species even show morphometric differences among individuals from SGI. Hence, possible separations of the SGI populations have been proposed a several times through the last century (Borradaile 1926; Zarenkov 1968; Boschi et al. 1992).

In this regard, it might be worth mentioning that some other populations of Antarctic shrimps like *Chorismus antarcticus* and *Nematocarcinus lanceopes*, have similar reproductive cycles all over their habitats including subantarctic regions like SGI (Lovrich et al 2005; Gorny et al 1992). These two Antarctic shrimps have a benthic lifestyle as *N. antarcticus* and show a similar distribution. However, studies from Raupach et al. (2010), did not report any genetic differentiation among regions or populations for both 16S and 28S genes. However, the study from Raupach et al. (2010) did not include individuals from SGI. Hence, there might also be a hidden population differentiation/ESU within the specimens from SGI among these two caridean shrimps. A genetic population investigation for *C. antarcticus* from SGI in comparison to the data available from older studies off Antarctica would be very encouraging and might give theories about anomalies among SGI populations even more significance. Anyhow, If no population differentiation should be detected, possible reasons could be the existence of planktotrophic larvae (see Raupach et al 2010) – missing in the case of *N. antarcticus* – allowing the dispersal beyond and within the ACC; as well as high coexistence of *Chorismus antarcticus* with sponge communities, which are more likely to detach from the substrate and operate as mean of transport by rafting along the ACC. In the case of *N. lanceopes,* a possible dispersal through migration can also be more likely because of the species occuring at depth of even 4000 meters and thus may migrate across deep waters (see Fig. 10). *N. antarcticus* does not inhabit such depths (50-2031m, with highest frequencies between 227 and 831m depth after Arntz and Gorny 1991); impeding migration between areas separated by high profundities. Besides, *N. antarcticus* shows an intensive

digging behavior (Gutt et al. 1991) into solid substrata making dispersal by rafting on loose substrate components unlikely to occur, since it also seems to prefer substrata poorly covered by epifauna (Storch et al. 2001). *N. antarcticus* are mostly found buried (Gutt et al. 1991) into the substrata, which also makes a migration behavior implausible for this species.

All in all, one must consider that there are many factors deciding over gene flow and genetic drift between populations. Not only does the climate (temperature and currents) and geography (distance and depth) play eminent and highly impacting roles in population structure and speciation (see also Hellberg 1994), but also physiology and different life-styles of different species or even populations.

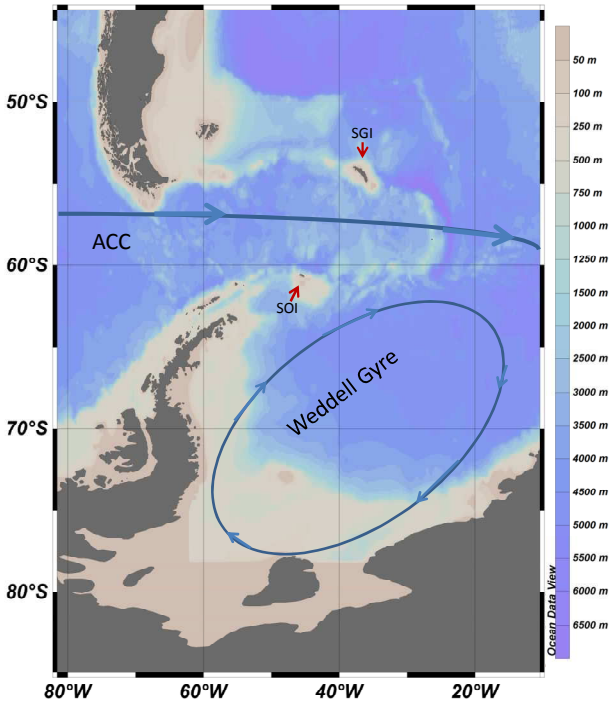

Fig. 10: Map by "Ocean Data View" showing the depths profile of the Scotia Arc between South America and Antarctica with the islands of South Georgia (SGI) and South Orkney (SOI) as landmarks and the Antarctic Circum-polar Current and the Weddell Gyre indicating the direction of the most striking currents for this area.

(3) The haplotype network for 16S rDNA clustered the populations of SOI, LA, LB, LC, EWS and TA all together. This arrangement was supported by the nuclear microsatellite data of the three polymorphic markers Ncr1, Ncr3 and Ncr14. However, there was a hint on a third subpopulation consisting of specimen only from LC within data from the microsatellite markers (see F_{ST} values and the probability of genic differentiation for LC). Reasons could be following factors: LC is located at the southernmost region of the Antarctic Peninsula and is

therefore the area less exposed to the ACC along the continent. The AP would act as a shield protecting this population from strong currents and thus, disrupting the pathway for gene flow through the ACC, which would provide a circumpolar distribution of the population's genes as well as income of genetic diversity. The microsatellite data also showed evidence for gene flow between populations LC and LA, EWS and TA. Existing genetic similarities between LC and LA can be easily explained due to the short distance between the populations, which can be overwhelmed simply by migration along the shelf without the need of larvae drift by water currents. Connection of LC with EWS and the genetic differentiation between LC and SOI can both be explained by the current of the Weddell Sea – the Weddell Gyre. This current, connects population off the Antarctic Peninsula (AP), acting as a route for gene flow. Though, it might as well exclude genetic input of the population of SOI towards the AP due to its eastward drift, which would simultaneously explain existing genetic input between LC (AP) and EWS (see Fig. 10). The northernmost region of the AP (LA and LB), however, might still be under constant gene flow with other regions due to the ACC (as they are geographically more exposed). The stronger ACC might also generate genetic input coming from LC to SOI and other populations around the Antarctic continent, but at the same time, a possible input of gene flow from e.g. SOI to LC might be drifted in the opposite direction (because of the eastward drift), leading all in all, to an isolation of the LC population.

Still, a genetic differentiation between LC and LB seem at first sight implausible. Both populations are situated relatively close to each other and migration as well as larval exchange between LC and LB could be easily assumed. This occurrence might be explained by the accelerated ice discharging events, which lead to the collapse of the Larsen B shelf (Rignot et al. 2004). The first collapse of ice shelf at the AP was 1995. 1995 the Larsen A shelf collapsed followed by the collapse of the Larsen B ice shelf 2002. The breaking of grounded shelf ice might lead to fragmentation of population over a certain period, as the impact of ice masses on the shelf after collapse could exterminate shelf inhabitants. The collapse of shelf ice has a deep impact on the shelf population up to some 100 meters of depth. If parts of the circumpolar population of *N. antarcticus* are wiped off by the collapse of the shelf ice, the continuity of distribution along the continental shelf would be thus disturb and linkage between locations would break until the number of specimen of the affected area increases again by reproduction of the individual, who survived the impact or by migrant individuals

from neighbor populations. The LB population might have been therefore wiped out or drastically reduced after the collapse 2002. The results concerning genetic differentiation between LC and LB might be due to the fact that LC still consists of specimen of the older population, while LB is a rather “new” population reestablished after the LB shelf ice collapse.

Anyhow, one must consider that there is not enough evidence yet, within this study for the acceptance of a genetically isolated LC population and thus, a further ESU within *N. antarcticus*. According, to the paragraph above, the subdivision into more ESU might be on the one hand enhanced, due to accelerated climate warming and ice-flow, which stretches and thus thins the ice shelves making them collapse (Rignot et al. 2004) and splitting populations within the Antarctic continental shelf. On the other hand if climate conditions stabilize, a subdivision into further ESUs might as well go back after the population has recovered from fragmentation due to aforementioned shelf ice collapse. Furthermore, increasing the sample size used for studies in population structure as well as the number of molecular markers might also bring some more clarity into herein discussed results and as well increase their credibility. It may be useful to compare the populations with nuclear markers under selection like genes for heat-shock-proteins as they might as well reveal some more information on population structure.

5. Conclusions and Perspectives

Desirably, samples from all around the Antarctic continent including the Scotia Arc should have been analyzed to document the exact distribution of genotypes and alleles within species of *Notocrangon antarcticus*. Moreover, confirming the existence of a separate ESU for SGI, a stepwise analysis of the population's structure along the Scotia Arc would be very interesting, mostly to determine where exactly the SGI ESU's geographic break is located or if it is rather the case of a stepwise differentiation of the population along the Scotia Arc. In this respect, an analysis with further programs like MIGRATE-N could give more exact information about the migration behavior of the individuals within the Scotia Arc, from and to Antarctica. This way the current gene flow among the locations. Migration estimates are eminent to understand processes which mould genetic variation within habitats and consequently also speciation itself (Hellberg 1994). Additionally, a further study considering the mitochondrial gene cytochrome oxidase (CO1) would provide intermediate levels of information in terms of population differentiation, since it is a faster evolving marker than 16S and not as fast as nuclear microsatellites. A haplotype network of the CO1 would supplement the information gained herein (see also Spieker 2009). CO1 primers (Folmer et al. 1994) have been tested within this project, however they amplified two bands, hence, the products should be analyzed separately and according to the results the procedure of the isolation process for this marker has to be modified in order to be up for further analysis.

It must also be taken into consideration that there are still samples from this study that have not been genotyped yet for the microsatellite loci and which would provide a bigger and thus, more reliable data set. The enlargement of the data set may clarify the unexpected hint on existence of a third population of. Clear results of the 16S gene, leave no doubt of SGI being an established and independent population.

Some microsatellite primers that have not been used within this study can be redesigned and optimized to widen the range of nuclear markers. Usually, when working with microsatellites, at least 8 polymorphic markers should be tested for all individuals among populations to obtain a reliable and convincing data. Thus, probably a repeat of the reporter genome protocol (Held and Leese 2007) should be performed again with *N. antarcticus,* maybe using more than one reporter genome (as in previous primer isolation with *Mus*

musculus domesticus) in order to isolate and use more nuclear markers. Nonetheless, some experiments in order to improve and increase the number of current nuclear markers have been started within this study and their results still have to be analyzed.

All in all, gene flow over long distances is suggested for the species of *N. antarcticus* around the Antarctic continent, as populations from AP and TA show no differentiation, though situated on opposite sides of Antarctica. However, gene flow through the Scotia Arc is restricted with a notable genetic (genic and genotypic) differentiation of the SGI population. Nevertheless, a further genetic differentiation of the species concerning the Antarctic Peninsula can be analyzed in more detail and deliver more significant results by using additional statistical programs, as well by enlarging sample sites and number of samples per sample sites and by using a larger number of nuclear polymorphic microsatellite markers and including a second mitochondrial gene – CO1. In addition, it might be interesting to establish a method to investigate a possible reproductive isolation between SGI and the rest of the Antarctic *N. antarcticus* by for example artificially providing an environment which is adequate for reproduction for both Antarctic and South Georgian specimen and looking at mating behavior between both populations to determine if there is still a chance of coupling though delay of reproductive cycles off Antarctica and SOI, mentioned before. Currently, however, genetic distance between *N. antarcticus* specimen of different locations is not high enough, so we still assume all *N. antarcticus* to belong to the same species.

It will be interesting to continue investigating this species and its adaption to new environmental conditions due to a probable shift of the ACC and PF towards the south and collateral increasing of temperatures by global warming. Antarctic benthic shrimp do not suffer from high competition as there are only few of these species in the Antarctic benthos and have been scarcely found in digesting tracts of bigger organisms like seals and whales. Thus they may not be much affected by predation, natural enemies or competition, as the habitat of the different benthic decapods barley overlaps. Hence, if global warming consequently provides a bearable habitat for northern species and thus, leads to a migration of these towards south intruding into *N. antarcticus'* habitat. As a consequence, regression of benthic decapods might occur, leading probably again to speciation due to regression of the population's size and thus restricting gene flow because of formation of niches. Furthermore, the accelerated collapse of massive shelf ice will continue fragmentizing

habitats and thus the population of *N. antarcticus* leading to further opportunities for speciation. New studies have shown an acceleration of this process with a current ice velocity of 500 meters per year at the AP (Rott et al. 2002). Former measurement of ice velocity along the shelf recorded only ten to fifty meter per year (Michel and Rignot, 1999), which means over tenfold of previous measurements. One should not forget however, that speciation takes thousands of years and can only be predicted. Anyhow, the collection of data on population structure and geographical distribution of "key" species like *N. antarcticus*, which happens in almost all benthic regions off Antarctica, can provide extra information about consequences of global as well as regional warming on biota and ecosystems within one of the most affected continents worldwide (Vaughan et al. 2003; Mereduth and King 2005).

6. Summary

The population genetic of the Antarctic benthic shrimp *Notocrangon antarcticus* was analyzed with 3 polymorphic microsatellite markers and with the 16S rDNA gene. Therefore, samples from 7 different locations: Antarctic Peninsula (Larsen A, Larsen B and Larsen C), South Orkney Island, South Georgia Island, East Weddell Sea and Terre Adélie were collected during the CEAMARC (2007) and CAMBIO (2011) expedition. The results of the mitochondrial 16S gene marker showed two clades within the haplotype network, one for South Georgian samples and the other one for the rest of the samples tested herein (Antarctic Peninsula, South Orkney Island, East Weddell Sea and Terre Adélie). The microsatellite data reinforced the results from the 16S haplotype network showing as well genetically differentiated population clusters – one for SGI and one for the rest of specimen of the continental shelf and the South Orkney Island (SOI). In addition, the microsatellite data gave some hints on a further genetically partially differentiated population for the samples of the Larsen C shelf of the Antarctic Peninsula. Last mentioned however, still needs to be further analyzed.

All in all, the population of SGI shows a clear genetic isolation from all other tested populations and can be considered as an independent evolutionary significant unit (ESU). Furthermore *N. antarcticus* seems to have a circumpolar distribution with more or less constant gene flow around the Antarctic continental shelf including the island of South Orkney. Nevertheless, further microsatellite primers should be isolated in order to give higher evidence to the data. Usually, six to eight polymorphic markers are recommended for such tests. In addition, the number of individuals tested per location should also be increased.

7. Zusammenfassung

Die Populationsgenetik der antarktischen Garnele *Notocrangon Antracticus* wurde mit Hilfe dreier polymorphischen Microsatelliten Markern und des 16S rDNA Genes analysiert. Demnach, wurden Individuen aus sieben verschiedenen Stellen um die Antarktis während der CEAMARC (2007) und CAMBIO (2011) Expedition gesammelt. Die untersuchten Sammelstellen waren: die antarktische Halbinsel (Larsen A, Larsen B und Larsen C), die Inseln South Orkney (SOI) und South Georgia (SGI), die östliche Region des Weddell Meeres und Terre Adélie. Ergebnisse des Haplotypennetzwerkes für das 16S Gen zeigten eine Aufteilung der Individuen in zwei genetisch unterschiedliche Haplotypen – ein Haplotyp für die Individuen um SGI und der andere für die restlichen Individuen aus den Regionen um den antarktischen Kontinent und SOI. Der Datensatz der nuklearen Microsatelliten Marker bestätigte die oben genannten Ergebnisse für die 16S rDNA indem sie auch eine Einteilung der Individuen in zwei genetisch voneinander unterschiedliche Einheiten sogenannten „evolutionary significant subunits“ (ESU) erzielten – eine Einheit bestehend aus Individuen um SGI und eine aus den restlichen Individuen um Antarctica und SOI. Der Microsatelliten-Datensatz wies auf eine weitere womöglich genetisch differenzierte Population der Individuen aus Larsen C auf der antarktischen Halbinsel hin. Letzteres muss jedoch noch weiter analysiert werden.

Alles in allem, sind die Individuen um SGI eindeutig genetisch von den anderen Individuen isoliert und somit genetisch unterschiedlich und können deshalb als unabhängige ESU angesehen werden, während sonst bei Individuen von *Notocrangon antarcticus* um den antarktischen Kontinent und die Insel von SOI von einer homogenen Verteilung ausgegangen werden kann. Trotzdem sollten weitere Primer für Microsatelliten isoliert werden um die gesammelten Individuen genauer untersuchen zu können. Eine Erhöhung der Anzahl an untersuchten Individuen pro Sammelstelle sollte genauso in Erwägung gezogen werden. Allgemein wird es empfohlen mindestens sechs bis acht polymorphische Microsatelliten-Marker für populationsgenetische Tests zu verwenden.

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10. Appendix

App. 1: Information about *N. antarcticus* samples from the CEAMARC cruise …….

#Lot	Station	Locat.	Date	Gear Type	Start Lat.	Start Lon.	Depth (m)	Code
298	CEAMARC-30	TA	24/12/07	Beam Trawl AAD	-66.003943	-143.716085	439.793777	R478-4
298	CEAMARC-30	TA	24/12/07	Beam Trawl AAD	-66.003943	-143.716085	439.793777	R478-14
298	CEAMARC-30	TA	24/12/07	Beam Trawl AAD	-66.003943	-143.716085	439.793777	R478-15
388	CEAMARC-38	TA	24/12/07	Beam Trawl AAD	-66.333198	-143.357078	719.658279	R478-13
388 (2)	CEAMARC-38	TA	24/12/07	Beam Trawl AAD	-66.333198	-143.357078	719.658279	R478-12
388 (3)	CEAMARC-38	TA	24/12/07	Beam Trawl AAD	-66.333198	-143.357078	719.658279	R478-10
897	CEAMARC-37	TA	28/12/07	Beam Trawl AAD	-66.570203	-143.377362	834.547195	R478-21
897	CEAMARC-37	TA	28/12/07	Beam Trawl AAD	-66.570203	-143.377362	834.547195	R478-22
897	CEAMARC-37	TA	28/12/07	Beam Trawl AAD	-66.570203	-143.377362	834.547195	R478-23
1319	CEAMARC-51A	TA	30/12/07	Beam Trawl AAD	-66.324275	-145.534688	553.193618	R478-5
1319 (1)	CEAMARC-51A	TA	30/12/07	Beam Trawl AAD	-66.324275	-145.534688	553.193618	R478-2
1319 (2)	CEAMARC-51A	TA	30/12/07	Beam Trawl AAD	-66.324275	-145.534688	553.193618	R478-6
1319 (3)	CEAMARC-51A	TA	30/12/07	Beam Trawl AAD	-66.324275	-145.534688	553.193618	R478-7
1725	CEAMARC-55	TA	03/01/08	Beam Trawl AAD	-66.750233	-144.981717	406.47264	R478-20
1725 (1)	CEAMARC-55	TA	03/01/08	Beam Trawl AAD	-66.750233	-144.981717	406.47264	R478-9
1725 (2)	CEAMARC-55	TA	03/01/08	Beam Trawl AAD	-66.750233	-144.981717	406.47264	R478-11
2009	CEAMARC-62	TA	04/01/08	Beam Trawl AAD	-66.143585	-143.295548	549.650527	R478-18
2009	CEAMARC-62	TA	04/01/08	Beam Trawl AAD	-66.143585	-143.295548	549.650527	R478-19
2725	CEAMARC-71	TA	14/01/08	Beam Trawl AAD	-66.388780	-140.428852	791.48223	R478-16
2725	CEAMARC-71	TA	14/01/08	Beam Trawl AAD	-66.388780	-140.428852	791.48223	R478-17
3083 (1)	CEAMARC-19	TA	15/01/08	Beam Trawl AAD	-66.170640	-139.353133	656.641555	R478-8
3083 (5)	CEAMARC-19	TA	15/01/08	Beam Trawl AAD	-66.170640	-139.353133	656.641555	R478-3
3083 (6)	CEAMARC-19	TA	15/01/08	Beam Trawl AAD	-66.170640	-139.353133	656.641555	R478-1

Sample region was only Terre Adélie (TA)

App. 2: Information about *N. antarcticus* samples of the CAMBIO Polarstern ant XVII 3 cruise 2011

Station	Locat.	Date	Gear (type)	Start Lat.	Start Lon.	End Lat.	End Lon.	Depth (m)	Cont. No.	Cont. Type	Vail No.	Samples
PS77/260-6	EWS	20/3/11	AGT Bottem	70° 50,24' S	10° 35,65' W	70° 50,71' S	10° 36,56' W	259.5	117	2° jar		4
PS77/292-2	EWS	31/3/11	Trawl						147	1° jar		1
PS77/226-7	LA	26/2/11	AGT+RD Bottem	64° 54,87' S	60° 37,26' W	64° 54,83' S	60° 36,32' W	231	69	4° jar	181	~15
PS77/228-3	LA	27/2/11	Trawl Bottem	64° 55,85' S	60° 34,33' W	64° 54,11' S	60° 29,25' W	279.7	70	5° jar	182	~30
PS77/228-3	LA	27/2/11	Trawl	64° 55,85' S	60° 34,33' W	64° 54,11' S	60° 29,25' W	279.7	71	5° jar	184	~20
PS77/228-4	LA	27/2/11	AGT+RD	64° 55,86' S	60° 34,18' W	64° 55,41' S	60° 33,05' W	315.7	75	5° jar	191	20
PS77/228-4	LA	27/2/11	AGT+RD	64° 55,86' S	60° 34,18' W	64° 55,41' S	60° 33,05' W	315.7	76	5° jar		30
PS77/228-4	LA	27/2/11	AGT+RD Bottem	64° 55,86' S	60° 34,18' W	64° 55,41' S	60° 33,05' W	315.7	77	5° jar		35
PS77/231-3	LA	28/2/11	Trawl Bottem	64° 55,54' S	60° 32,89' W	64° 53,63' S	60° 28,10' W	314.2	82	5° jar	206	18
PS77/231-3	LA LA	28/2/11	Trawl	64° 55,54' S	60° 32,89' W	64° 53,63' S	60° 28,10' W	314.2	83	5° jar	207	20
PS77/252-7	North LB	10/3/11	AGT	64° 41,64' S	60° 30,57' W	64° 42,27' S	60° 31,76' W	327	122			1
PS77/250-6	Seep LB	8/3/11	AGT	65° 23,60' S	61° 32,59' W	65° 22,38' S	61° 33,46' W	566.7	121			4
PS77/248-3	South LB	7/3/11	AGT+RD	65° 55,12' S	60° 19,67' W	65° 55,88' S	60° 20,15' W	433	103	5° jar	248	22
PS77/248-3	South LB	7/3/11	AGT+RD	65° 55,12' S	60° 19,67' W	65° 55,88' S	60° 20,15' W	433	104	4° jar	249	17
PS77/233-3	West LB	1/3/11	AGT+RD Bottem	65° 33,69' S	61° 37,40' W	65° 32,88' S	61° 37,10' W	324.2	87	3° jar	212	9
PS77/235-8	West LB	2/3/11	Trawl Bottem	65° 30,82' S	61° 36,09' W	65° 32,69' S	61° 28,85' W	448.7	89	5° jar	219	20
PS77/235-8	West	2/3/11	Trawl Bottem	65° 30,82' S	61° 36,09' W	65° 32,69' S	61° 28,85' W	448.7	91	4° jar	221	9
PS77/237-2	LC	3/3/11	Trawl	66° 13,87' S	60° 11,02' W	66° 11,10' S	60° 8,55' W	382.7	92	4° jar	223	18
PS77/239-3	LC	4/3/11	AGT+RD Bottem	66° 11,56' S	60° 8,42' W	66° 12,07' S	60° 10,37' W	362	95	5° jar	235	50
PS77/214-1	SGI	16/2/11	Trawl Bottem	54° 24,42' S	35° 36,85' W	54° 25,68' S	35° 39,41' W	274.7	28	4° jar	92	>20
PS77/214-2	SGI	16/2/11	Trawl Bottem	54° 24,42' S	35° 36,85' W	54° 25,68' S	35° 39,41' W	274.7	29	5° jar	95	~20
PS77/214-2	SGI	16/2/11	Trawl Bottem	54° 24,42' S	35° 36,85' W	54° 25,68' S	35° 39,41' W	274.7	30	5° jar	96	~20
PS77/214-2	SGI	16/2/11	Trawl	54° 24,42' S	35° 36,85' W	54° 25,68' S	35° 39,41' W	274.7	31	5° jar	97	~20
PS77/214-5	SGI	16/2/11	AGT+RD	54° 25,99' S	35° 41,63' W	54° 26,33' S	35° 41,43' W	266.2	36	5°jar	100	~20
PS77/214-5	SGI	16/2/11	AGT+RD	54° 25,99' S	35° 41,63' W	54° 26,33' S	35° 41,43' W	266.2	37	3°jar	101	1
PS77/214-5	SGI	16/2/11	AGT+RD	54° 25,99' S	35° 41,63' W	54° 26,33' S	35° 41,43' W	266.2	38	5°jar	102	~20
PS77/214-5	SGI	16/2/11	AGT+RD	54° 25,99' S	35° 41,63' W	54° 26,33' S	35° 41,43' W	266.2	39	5°jar	102	50
PS77/214-5	SGI	16/2/11	AGT+RD	54° 25,99' S	35° 41,63' W	54° 26,33' S	35° 41,43' W	266.2	40	5°jar	102	17
PS77/214-5	SGI	16/2/11	AGT+RD	54° 25,99' S	35° 41,63' W	54° 26,33' S	35° 41,43' W	266.2	41	5°jar	102	53
PS77/217-5	SOI	19/2/11	AGT+RD	61° 8,74' S	43° 58,15' W	61° 8,91' S	44° 0,24' W	398.2	47	5°jar	109	~20
PS77/217-5	SOI	19/2/11	AGT+RD Bottem	61° 8,74' S	43° 58,15' W	61° 8,91' S	44° 0,24' W	398.2	48	5°jar	109	~30
PS77/217-6	SOI	19/2/11	Trawl Bottem	61° 8,61' S	43° 59,22' W	61° 10,52' S	44° 4,91' W	354	52	5°jar	127	28
PS77/218-2	SOI	20/2/11	Trawl	61° 10,36' S	45° 44,62' W	61° 10,67' S	45° 38,03' W	336.7	56	4°jar	137	10

Sample regions were South Georgia Islands (SGI), South Orkney Islands (SOI), Antarctic Peninsula (Larsen A: LA, Larsen B: LB, Larsen C: LC) and Easter Weddell Sea (EWS)

App. 3: Microsatellite primers for studies on population genetics, isolated with the reporter Genome protocol after Leese et al. (2007), by Agrawal et al. for *N. antarcticus* (*Mus musculus domesticus* as reporter genome)

Primer name	**5'-Primer sequence-3'**	**Primer name**	**5'-Primer sequence-3'**
Ncr1 (E03) F	AACTTGTTGGACCTTCAT	Ncr10 (F01) R	TTTGTTTCTGCGTTTGTTGA
Ncr1 (E03) R	GCGAGGAGGATGATTTGTGG	Ncr11 (D04) F	GCTCTAAGGTGGGTCTAATA
Ncr2 (H08) F	TCCACCAAGTAATAACA	Ncr11 (D04) R	AAATCAGCAAACTTAGGC
Ncr2 (H08) R	TAGAAACCAGACCTTGTA	Ncr12 (H01) F	CAAGACGCAAAGTGCCATAAG
Ncr3 (A03) F	ACACCTACTTTCTGTGACCA	Ncr12 (H01) R	TATGATGGGTGTCAAAATG
Ncr3 (A03) R	AGAACTAGATAACTAAAGGG	Ncr13 (F02) F	CAGTGTCTCTAACTACAA
Ncr4 (G06a) F	TCGGTTCAAGTTACGGTTCT	Ncr13 (F02) R	GGGTCAAGGGATGAAGTC
Ncr4 (G06) R	GACCTATCACCTGCCAGAGA	**2nd Order:**	
Ncr4 (G06a) F	TCGGTTCAAGTTACGGTTCT	Ncr13 F02 R	GGGTCAAGGGATGAAGTGC
Ncr5 (G06a) R	GTAACTTGAGCTAGGACGCTT	Ncr14 B08 F	GAATCTACAACGTCGTCAT
Ncr4 (G06b) F	AAGCGTCCTAGCTCAAGTTAC	Ncr14 B08 R	AGGAAGGGTTCTTTATTCAA
Ncr5 (G06b) R	GAATCGGCTACAAACTCAGG	Ncr15 C11 F	CAGATAAAAGCAGAAAAACAGTC
Ncr5 (G06) F	TCTCTGGCAGGTGATAGGTC	Ncr15 C11 R	CATTAGGAAAGGAATGATTTCGC
Ncr5 (G06b) R	GAATCGGCTACAAACTCAGG	Ncr16 B11 F	GAATACAATGCAATCACTACA
Ncr6 (C03) F	GGATCACCTATCAATGAAGC	Ncr16 B11 R	CATCGCTCGATGATCTATAA
Ncr6 (C03) R	ACACCTCCAATCGCTTCAT	Ncr17 C01 F	AGCCATGGACGTATCAAGA
Ncr7 (B10) F	CGTATTTCCACCAAGTAA	Ncr17 C01 R	AAATACATCTAGACCAAA
Ncr7 (B10) R	TCTTTCCAACGACATCCT	Ncr18 G04 F	ACTTCATATATTTTTATGC
Ncr8 (B02) F	ATTGATAAGCACTAACAT	Ncr18 G04 R	TGAAATAAAAGACCTTGTC
Ncr8 (B02) R	TCCTATGAGGTAGATTGA	Ncr19 D02 F	TTTCTGTGTGCAACTGAAGC
Ncr9 (E09) F	ATACCGACCTAACAAATCCA	Ncr19 D02 R	TTAGGTAAAGGAAACAACTGGGC
Ncr9 (E09) R	TACACTTATTCATAGGTTTG	Ncr20 A02 F	ATCATTCCATCCATACAT
Ncr10 (F01) F	TACCAGCACAATCTCTCCAA	Ncr20 A02 R	AAATTCAAACTTGTTGGAC

App. 4: characterization of the microsatellite primers designed for Notocrangon antarcticus by Agrawal et al. (in prep.)

Primer Name	Repeat Motive	Label	Features
Ncr1	(TG)23	Hex	*polymorphic repeat*
Ncr2	(TTG)13	-	no notable mutation
Ncr3	(GTT)19	Hex	*polymorphic repeat*
Ncr4	-	-	no microsatellite
Ncr5	(GTTTGTT…)n	-	komplex 24bp long repeat; transposon?
Ncr6	(CCT)n	Fam	monomorphic repeat
Ncr7	-	-	no microsatellite
Ncr8	-	-	no microsatellite
Ncr9	(GT)~5 (TA)~4 ?	-	redesign primer/no microsatellite
Ncr10	?	-	no sequence
Ncr11	(CAAA)n ?	-	Mutation probably in very high rates; design better primer; too much stuttering
Ncr12	(TG)3CA(TG)2TT(TG)2CAC(GT)12G(GT)2	Fam	*polymorphic repeat*
Ncr13	?	-	no sequence
Ncr14	(GTCTT)n	Fam	*polymorphic repeat* but too many stutter peak in fragment analysis
Ncr15	(GACA)n	-	order labeled primer
Ncr16	?	-	no sequence
Ncr17	?	-	three fragments
Ncr18	?	-	no amplification
Ncr19	?	-	no amplification
Ncr20	?	-	two fragments

App. 5: Genotyping of scored alleles with GENEMAPPER 4.0 for each locus over all seven populations

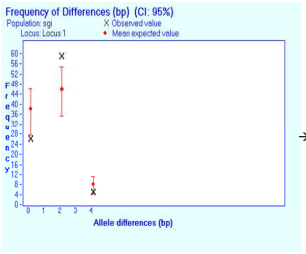
Sample	ncr1	ncr3	ncr14	Sample	ncr1	ncr3	ncr14	Sample	ncr1	ncr3	ncr14
SGI1	231	191	142	**SOI6**	229	194	133	**LC14**		175	136
	231	203	142		231	200	146			194	146
SGI10	231	188	149	**SOI7**	229	197	146	**LC2**	229		
	231	197	149		231	200	146		229		
SGI11	229			**SOI8**	229	175	133	**LC20**	229	185	133
	231				231	182	144		231	213	149
SGI12	229	185	138	**SOI9**	229			**LC23**		175	136
	229	203	146		231					188	144
SGI17	231			**LA12**	229	185		**LC24**	229	175	136
	231				231	203			231	213	149
SGI18	229	191		**LA14**	229	175		**LC26**	229		
	231	197			231	197			231		
SGI19	229			**LA18**	229	197		**LC27**	231	203	133
	231				229	200			233	203	149
SGI2	229	178		**LA20**	229	194	133	**LC28**	229	182	
	231	200			229	203	149		233	182	
SGI20	231	178	136	**LA22**	229			**LC29**	229	206	
	231	194	144		231				233	206	

SGI21	231			**LA23**	229	175	136	**LC5**	229	166	
	231				231	175	146		229	203	
SGI22	231	163	142	**LA27**	231	203	133	**LC7**	231	175	
	231	185	142		233	213	144		233	185	
SGI24	233	194	146	**LA28**	229			**LC9**	231		
	233	203	149		231				233		
SGI25	231			**LA30**	229	175		**EWS1**		188	136
	231				231	207				213	144
SGI27	231	178	142	**LA5**	229	194	133	**EWS2**	229	175	133
	231	210	146		231	203	144		231	200	149
SGI28	233			**LA6**	229	188	136	**EWS3**	229	175	131
	233				231	203	149		231	203	149
SGI30	229			**LA8**	231			**EWS4**	229	200	136
	231				233				231	210	146
SGI32	231	185	146	**LA9**	229	200		**EWS5**	229	175	136
	233	194	146		231	207			229	185	151
SGI4	231			**LB10**	229			**TA1**	229	185	133
	231				231				231	203	146
SGI5	231	182		**LB11**	229	185	133	**TA11**	229	185	133
	231	191			231	191	146		231	200	144
SGI8	231			**LB14**	231			**TA12**	229	185	133
	231				233				231	188	146
SGI9	231	185	136	**LB16**	231	194		**TA14**	229	175	
	231	200	144		231	203			229	194	
SOI1	229	191	136	**LB2**	229	197	133	**TA17**	229	207	
	231	203	146		231	207	146		233	207	
SOI10	231	194		**LB21**	229	185	133	**TA18**	229	182	133
	231	210			231	210	146		231	203	146
SOI14	229	197	144	**LB24**	229	194	136	**TA19**	229	175	
	229	200	146		231	210	146		229	194	
SOI2	229	175	133	**LB25**	229			**TA20**	229	194	
	231	207	146		231				231	200	
SOI22	229	191	133	**LB27**	233	194	136	**TA3**	229	194	136
	233	200	144		231	203	146		231	207	146
SOI23	229	194		**LB28**	229	178		**TA5**	229	175	144
	231	203			231	194			231	194	144
SOI29	229			**LB3**	229	175		**TA6**	231		
	231				231	203			233		
SOI3	229	191	133	**LB4**	229	207	133	**TA7**	229		
	231	203	144		231	219	146		231		
SOI4	229	191	146	**LB6**	231	185		**TA8**	229	191	133
	231	209	146		231	216			233	197	144
SOI5	229			**LC13**	229	175		**TA9**	229	194	133
	231				231	182			231	197	149

Blanks represent unamplified alleles. Sample regions were South Georgia Islands (SGI), South Orkney Islands (SOI), Antarctic Peninsula (Larsen A: LA, Larsen B: LB, Larsen C: LC), East Weddell Sea (EWS) and Terre Adélie (TA)

Ncr1-Locus (MICROCHECKER-Analysis)

Frequency of Differences (CI: 95%)



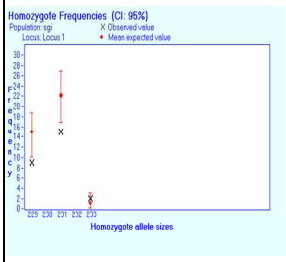
Probability of observed homozygot class frequency

Allele length	Binomial based	Rank based
233	0.1845	0.1285

→Combined probability for all classes: >0.05 (not significant)

Ncr1-Locus (MICROCHECKER-Analysis)

Homozygote Frequency (CI: 95%)

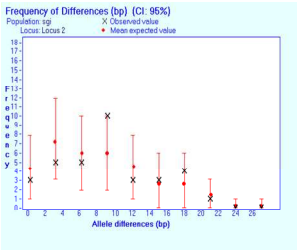


-**Total expected homozygots:** 37.705
-**Total observed homozygots:** 26

→No evidence for:
-scoring errors due to stuttering
-large allele dropout
-null alleles

Ncr3-Locus (MICROCHECKER-Analysis)

Frequency of Differences (CI: 95%)



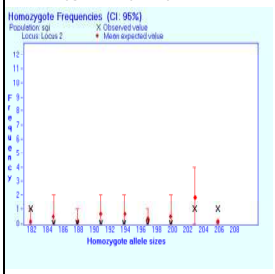
Probability of observed homozygot class frequency

Allele length	Binomial based	Rank based
182	0.067	0.0485
185	0.9876	1
188	0.6718	1
191	0.9834	1
194	0.9785	1
197	0.9911	1
200	0.9876	1
203	0.8934	0.7295
206	0.0074	0.001

→Combined probability for all classes: >0.05 (not significant)

Ncr3-Locus (MICROCHECKER-Analysis)

Homozygote Frequency (CI: 95%)

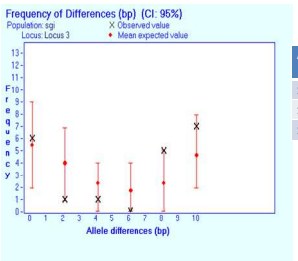


-**Total expected homozygots:** 4.735
-**Total observed homozygots:** 3

→No evidence for:
-scoring errors due to stuttering
-large allele dropout
-null alleles

Ncr14-Locus (MICROCHECKER-Analysis)

Frequency of Differences (CI: 95%)



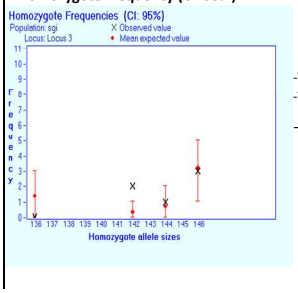
Probability of observed homozygot class frequency

Allele length	Binomial based	Rank based
142	0.0066	0.001
144	0.4177	0.2935
146	0.6718	0.519

→Combined probability for all classes: <0.05 (significant)

Ncr14-Locus (MICROCHECKER-Analysis)

Homozygote Frequency (CI: 95%)



-**Total expected homozygots:** 5.65
-**Total observed homozygots:** 6

→No evidence for:
-scoring errors due to stuttering
-large allele dropout
-null alleles

App. 6: Output of MICROCHECKER: The red bars represent the range of simulated values within the selected confidence interval. The mean value is represented as a red circle. The observed value of the input file is marked as a black cross. The probability of observed homozygote class frequency for each allele is given in the right sided table. **Left figures**: Probability of observed homozygote class frequency over all loci. The output data is based on a 95% confidence interval. **Right Figure**: Homozygote frequency of total expected and total observed homozygotes. The output data is based on a 95% confidence interval.