Universität Bremen

Local adaptation in the presence of gene flow in Patagonian and Antarctic *Nacella* limpets

-a multimarker genetic and physiological approach-

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Lokale Anpassung in der Gegenwart von Genfluss in patagonischen und antarktischen Napfschnecken der Gattung *Nacella*

-ein genetischer und physiologischer Ansatz-

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Chapter 1

General introduction

1. Introduction

Speciation is one of the most investigated areas in evolutionary research because it is considered to be the major key in understanding the processes of evolution (Turelli et al. 2001; Coyne and Orr 2004). The main drivers of speciation processes are geographical separation and local adaptation. During geographical separation random neutral processes, such as genetic drift, are sufficient to drive population divergence due to the lack of gene flow. In contrast, local adaptation is promoted by natural selection (Lenormand 2002). With respect to the marine system, it has been assumed that the high potential for gene flow hampers adaptive population divergence and makes local adaptation in marine populations rare and unlikely. That has raised the question how the huge amount of biodiversity can be explained in the ocean, which is characterized by the absence of strict gene flow barriers over large geographical scales (Palumbi 1992).

Many advances have been made and recent theoretical approaches showed how speciation is possible when locally differing selective pressures are strong enough to overcome the homogenizing effects of gene flow (Palumbi 1992, 1994; Hewitt 2000; Conover et al. 2006; Nosil 2008; Fitzpatrick et al. 2008, 2009; Mallet et al. 2009). However, the actual roles of selection and local adaptation have only seldom been tested directly. The vast majority of population genetic studies applies neutral molecular markers which do not respond to selective forces.

That leads to the important question “How to set-up a comprehensive molecular study to reveal historical and contemporary evolutionary forces responsible for the population structure and speciation in marine organisms?“. The present thesis aims at contributing to the current research on modes of speciation. The main goal is to demonstrate how the combination of different molecular markers, both neutral and under selection and with varying powers of resolution, allow to unravel species‘ evolutionary histories from evolutionary to ecological time scales.

Shallow-water limpet species from the genus *Nacella* from Antarctica and South America were chosen as study species, providing an excellent case to investigate impacts of geographical barriers and horizontal and vertical environmental gradients of various stress factors on the evolution of marine organisms.

1.1 The theory of speciation: Geographical isolation and natural selection

The common understanding of speciation is described as the spatial separation of a once continuous group of individuals into two new populations, where the appearance of any kinds of geographical barriers prevents gene flow between them (allopatric speciation, Mayr 1963). In the absence of the homogenizing effects of gene flow, local mutations and random effects of genetic drift increase genetic differentiation between two populations proportional to time (Kimura 1983). Locally differing environmental factors that cause different selective regimes further promote divergence. These major evolutionary forces ultimately drive speciation.

This understanding of how speciation works has always challenged marine biologists because the marine environment is characterized by the absence of strict barriers to gene flow over large geographical scales. At the same time many marine species exhibit developmental stages with pelagic larvae allowing for dispersal over long distances, which in turn further results in only little genetic differentiation among populations over large geographical scales (Palumbi 1992, 1994). Therefore, one superordinate question has motivated marine evolutionary scientists over the past decades: „How can we explain the huge amount of existing species in the ocean?“. In higher latitudinal regions glacial periods during Pleistocene glaciations, 2.6 million years (Ma) to 12 thousand years (kyears) before present (BP), are believed to be one reason for speciation, where temporal habitat fragmentation and temporal isolation caused by advancing glaciers promoted speciation processes (Clarke and Crame 1992; Held 2000, 2003; Hewitt 2000; Held and Wägele 2005; Thatje et al. 2005, 2008). The high biodiversity of other ecosystems, such as coral reefs, is, in contrast, not easily explainable with allopatric speciation, as there are no obvious known barriers preventing gene flow.

Alternative speciation concepts have been developed to account for the species richness problem in the ocean, which can by summarized in the term sympatric speciation. Sympatric speciation is a process taking place in the background of gene flow, where barriers to genetic exchange between diverging populations are absent (Fitzpatrick et al. 2008). In a strict biogeographical sense, sympatry describes the distribution of two groups of a species to be completely spatially overlapping. In a more demographic view the prerequisite of sympatric speciation is defined as panmixia in the initial state when divergence starts and does not necessarily require spacial overlapping (Fitzpatrick et al.

2008, 2009; Mallet et al. 2009). The driving force of sympatric speciation is natural selection establishing and increasing a genetic disparity between genotypes, where negative, post-zygotic selection acts on hybrid offspring, or where selection is pre-zygotic acting on hybridizing mating itself (reinforcing selection, Servedio and Noor 2003).

The impact of natural selection as a source of speciation in the open marine system has long been considered to be low because of the very high gene flow overriding the effects of locally varying selective pressures (Conover et al. 2006). However, the potential roles of selection and local adaptation have not been profoundly studied so far. The vast majority of population genetic studies applies neutral markers, which should per definition not respond to selective forces. Emerging evidence, provided by recent molecular studies with genetic markers under selection, encourage a re-evaluation of the common belief that adaptive divergence plays only a minor role in marine evolution. A study on the flounder *Platichthys flesus* by Hemmer-Hansen and co-workers (2007) showed strong population differentiation between panmictic populations with different salinity regimes when analyzing heat-shock protein genes under selection (the heat-shock cognate Hsc70). Larmuseau and colleagues (2010) proved adaptive divergence in rhodopsin genes among panmictic populations of the sand goby *Pomatoschistus minutus* inhabiting waters of different water turbidity. These two examples describe the impact of adaptive divergence driven by divergent natural selection between environments (ecological selection, Schluter 2009). That happens on scales that are much finer than previously believed, thus highlighting the potential of natural selection to overcome high gene flow (see Conover et al. 2006 and references therein).

1.2 The study case *Nacella*

South American and Antarctic members of the patellogastropod genus *Nacella* provide an excellent study case for research addressing evolutionary processes on various time scales. They allow for testing the importance of present physical barriers and varying selective regimes on horizontal and vertical scales in speciation processes.

The phylogenetic divergence between Antarctic *Nacella concinna* and South American *N. magellanica* and *N. deaurata* limpets is well-resolved in two different clades with complete lineage sorting (González-Wevar et al. 2010). This is in accordance with the central assumption that the large geographical distances and the presence of the Antarctic Circumpolar Current (ACC) represent biogeographical barriers for biological exchange between South America and Antarctica (Crame 1999; Clarke et al. 2005; Thatje et al. 2005). The creation of the ACC is believed to be the major reason for isolating Antarctica for the last 35 Ma, having led to the high degree of endemism on the Antarctic shelf (e.g. Munilla 2001; Clarke and Johnson 2003; Clarke et al. 2005). In 2005, however, Clark and colleagues raised the question „How isolated is Antarctica?“ due to growing evidence that the ACC might not represent the strict and insurmountable barrier to migration. Reports on mesoscale warm and cold core rings (eddies) crossing the fronts of the ACC and transporting organisms out of the Southern Ocean or into it represent only one of several potential ways for migration over the circumpolar current system (Clark et al. 2005; Bernard et al. 2007). Initial molecular estimations of South American and Antarctic sibling species revealed much younger speciation dates, leaving the true isolating force of the ACC and the geographical distance between South America and the Antarctic Peninsula still as an open question (Medlin et al. 1994; Patarnello et al. 1996; Bargelloni et al. 2000; Page and Linse 2002; Hunter and Halanych 2008; Thornhill et al. 2008; Wilson et al. 2009). The rapid climate change caused the sea surface temperature of the Antarctic ocean to rise about 1°C over the past 50 years (Meredith and King 2005). Consequently, that justifies speculation and concern about potential invasions of South American species into Antarctica threatening the uniqueness of the Southern Ocean fauna.

Antarctic and South American *Nacella* provide an ample opportunity for a comprehensive test study on the isolating function of the ACC. These limpets exhibit

pelagic larval stages with duration times of up to one month (Picken 1980) equipping them with the potential for long distance dispersal.

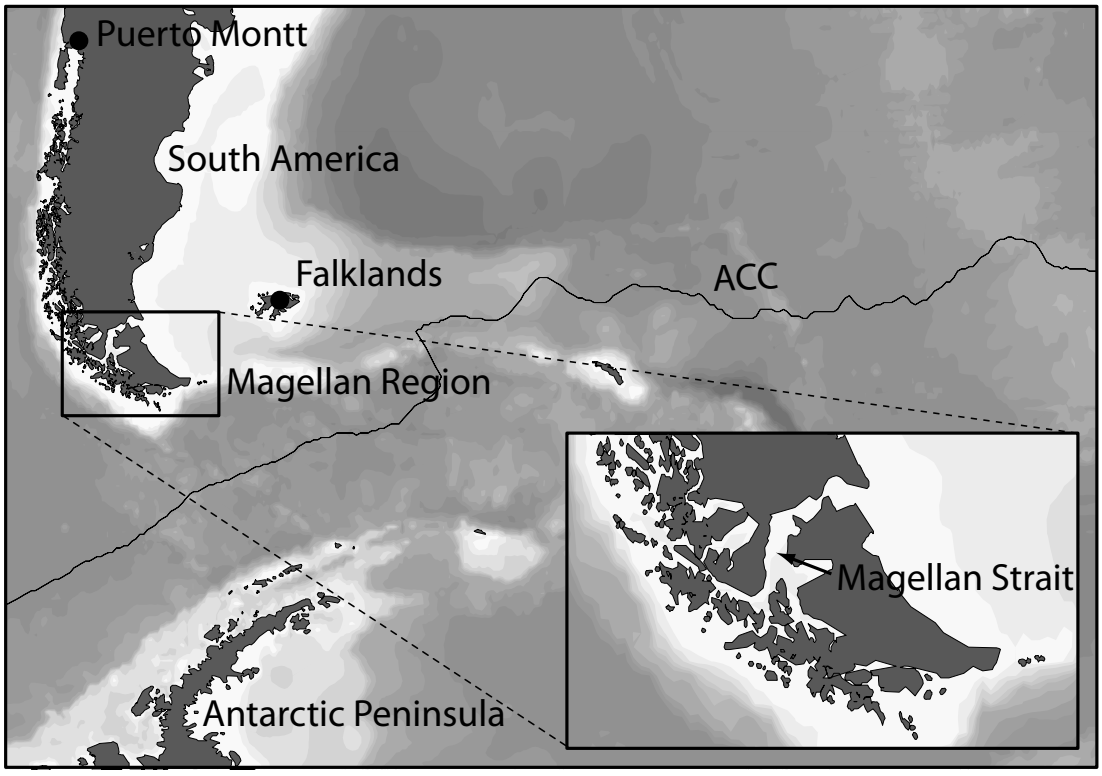

Fig. 1: Map showing the distribution area of South American *Nacella magellanica* and *N. deaurata* and Antarctic *N. concinna.* Puerto Montt represents the northern distribution area of *N. magellanica*, *N. deaurata* is only found in the Magellan region and on the Falkland Islands. ACC, Antarctic Circumpolar Current.

The South American *Nacella* species complex represents an abundant taxon inhabiting the shallow waters of Chilean and Argentinian Patagonia and Tierra del Fuego as well as on the Falklands/Malvinas. A closer look into this species complex highlights the problem of species categorization according to morphological criteria and provides a suitable study for recent speciation processes. *N. magellanica* exhibit the broadest distribution area of the described South American Nacellids, comprising the coasts from 42°S to the Southern end at Cape Horn (Fig. 1). The distribution range of *N. deaurata* is considerably narrower, restricted to the Magellan province and Tierra del Fuego. Both species are described to inhabit the coasts of the Falkland Islands which are situated approximately 500 km east of the Atlantic opening of the Magellan Strait on the South American shelf. *N. magellanica* is characterized by bigger shells with a centered apex and higher shell heights to shell lengths ratios, compared to *N. deaurata* whose apex is shifted towards the anterior (Fig. 2, see also Valdovinos and Rüth 2005). Both species

occur in sympatry in the Magellan Strait and Tierra del Fuego but show a distinct microhabitat zonation, with *N. magellanica* inhabiting the shallow intertidal and *N. deaurata* occupying the subtidal.

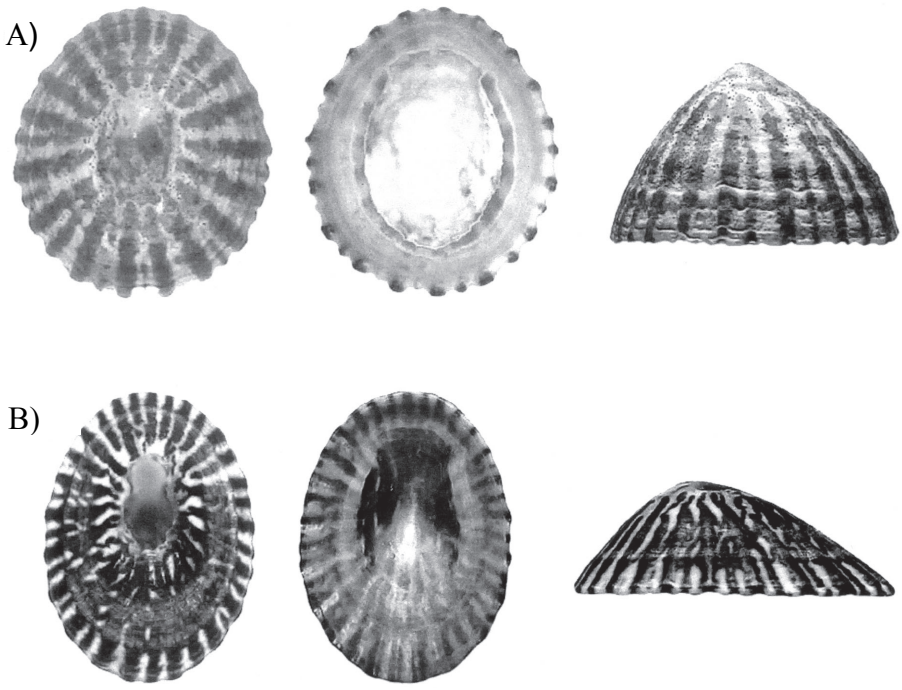

Fig. 2: Dorsal, ventral and lateral views on the shells of A) *Nacella magellanica* and B) *N. deaurata*. Pictures taken from Valdovinos and Rüth (2005).

Both nominal species have been subject to several scientific studies to disentangle their genetic status. Despite clear morphological distinctiveness in shell shape and profound differences in physiological adaptation (Malanga et al. 2004, 2005; González et al. 2008; Pöhlmann et al. 2011), it is controversial whether the classification of the two different morphotypes truly reflects reproductively isolated species. Genetic analyses using mitochondrial and nuclear genes failed to demonstrate reciprocal monophyly of specimens assigned to the two species on the grounds of their morphology (de Aranzamendi et al. 2009; González-Wevar et al. 2010). Fast evolving ISSR markers (Inter simple sequence repeats) on the other hand revealed significant amounts of genetic differentiation between *N. magellanica* and *N. deaurata* (de Aranzamendi et al. 2009). These controversial results raise the question whether or not there is still ongoing gene flow between both morphotypes and which forces could drive a genetic divergence between them, as indicated by the ISSR study?

An elegant suggestion how speciation in Patagonia could have been promoted is provided by Leese and co-workers (2008). They state that survival in Pacific and

Atlantic refugia could have led to population differentiation. Patagonia was repeatedly influenced by glaciers for about 85-95% of the last 800 kyears (McCulloch et al. 2000). During the glacial maxima, the Patagonian Pacific coastal areas were completely covered with ice, thus largely uninhabitable for marine species. The Magellan Strait, today connecting the Pacific and the Atlantic, was covered with glaciers on the western side and dry land on the eastern sector because global sea levels were more than 120 m lower than today (Fairbanks 1989). It is believed that it was not until 9 kyears BP that the sea level was high enough again to penetrate the Strait of Magellan from the Atlantic side and allow for a water passageway between the Pacific and the Atlantic oceans (McMulloch et al. 2000). The study on serolid isopods conducted by Leese et al. (2008) demonstrated how the glacial maxima supported an increase of genetic diversity caused by independent evolution in Atlantic and Pacific refugia. These allopatric phases, however, did not suffice to establish a complete reproductive barrier in the case of *Serolis* (Leese et al. 2008).

On the other hand, intertidal zones are among the highest stress environments. Their inhabitants are exposed to both, marine and terrestrial stress factors. Depending on the tidal cycle the organisms are periodically exposed to air during low tides, which means desiccation, strong shifts in temperature, UV radiation and physical stress caused by wave action and predation (Hofmann 1999; Somero 2002; Tomanek 2002). Several studies have demonstrated that closely related intertidal species or ecotypes with sympatric distribution patterns did show considerable genetic differences. Presumably, such a genetic differentiation occurred as a cause of sympatric speciation by ecological selection and local adaptation. One intriguing example for sympatric speciation mediated by pre-zygotic selection is given by the galician marine snail *Littorina saxatilis* that occurs in two ecotypes, which are largely sympatric but show slightly different vertical intertidal zonation patterns. Hybridization is possible, but restricted due to assortative mating driving the observed genetic divergence (Rolán-Alvarez 2007). Small-scale habitat stratification in three sympatric broadcast-spawning Hawaiian limpets (genus *Cellana*) further underlines the importance of ecological factors along vertical intertidal gradients in non-allopatric speciation processes (Bird et al. 2011). In the Caribbean Sea three sympatric broadcast-spawning corals of the genus

Montastraea serve as an example, how little differences in spawning time can result in the establishment of a reproductive barrier (Levitan et al. 2004).

Nacella magellanica and *Nacella deaurata* occur in sympatry and display high dispersal barriers making it unlikely that there is any physical barrier to gene flow between the two nominal species. Nevertheless, there is evidence in the literature that genetic divergence between both morphotypes could have been established by glacial maxima and/or ecological selection.

1.3 Molecular markers to investigate evolution

The basic question how to test the roles of geographical barriers on the divergence of species and how to investigate the contemporary and recent processes that lead to population divergence, display the fundamental problem of evolutionary science: The process of speciation itself can not be observed. In order to explain the processes which have led to separated gene pools and new species formation in the past, it is important to understand the processes leading to the structuring of the present-day distribution of genetic polymorphisms among the populations of a species (Held and Leese 2007). Biological markers are applied to meet these goals by serving as evidential criteria upon which individuals, populations or species can be distinguished and their population structure can be described. Traditional markers, which have been used in order to build species trees and infer systematic relationships of the earth‘s fauna, have been morphological traits, suitable to describe well-defined clades of species. However, phenotypic traits such as morphology do not allow for a distinction whether differences among populations are based on genotypic differences, thus based on a genetic level, or are a consequence of phenotypic plasticity. In order distinguish between genotypes that are causing differences in traits of interest like morphology and behavior, a study needs to be based on informative marker systems (Schlötterer 2004). Recently invented molecular genetic markers display an excellent tool to study evolutionary processes and to reconstruct population and species histories on the basis of genotypic variation (Zhang and Hewitt 2003). One of the strongest features of genetic markers is the varying resolution depending on their evolutionary rates. The differing mutation rates of available markers enable us to resolve evolutionary processes in certain time windows.

Macroevolutionary time scales: Phylogenies and divergence times

Slowly evolving genes like the 18S subunit of eukaryotic cytoplasmic ribosomes give insights into deeper phylogenies and reveal relationships among orders or families. Faster evolving genes with finer resolutions, e.g. the common mitochondrial genes cytochrome oxidase subunit I (COI) or the 16S subunit of mitochondrial ribosomes, allow for species identifications and give insights into more recent phylogenetic relationships. Gene sequence variations can not only be used to investigate the genetic relationships and phylogenies of orders, families and species, but also to calculate the times when the divergences into the different lineages occurred. The initial idea about the „molecular clock hypothesis“ started in the early 60´s. Zuckerkandl and Pauling (1965) proposed that mutation rates of genes are relatively constant, thus suitable to calculate species divergence times as a function of genetic distance and rate of change. However, since then many studies have proven that considerable rate heterogeneities exist both across the branches of a phylogenetic and even along single lineages. These findings make the application of constant mutation rates highly vulnerable to miscalculations of divergence times (Bromham et al. 1996; Yoder and Yang 2000; Thomas et al. 2006; Drummond et al. 2006). Newest advances in the field of molecular dating have accounted for that by the development of relaxed molecular clocks, which allow mutation rates to vary between different branches and also along each lineage (Drummond et al. 2006; Drummond and Rambaut 2007). These methods provide true confidence intervals reflecting the stochastic effect of the applied clock and describing the variance of the molecular clock estimate, which is indispensable if we want to accept or reject hypotheses. Molecular dating provides a technique to investigate the processes that have led to speciation in much greater detail by determining divergence times of groups of interest. Calculated divergence times can be compared with the occurrence of geological or climatic events, such as the establishment of a physical barrier like the ACC, to determine the reason for divergence.

Microevolutionary to ecological time scales: Genetic structure of local populations

Processes leading to population divergence in an interbreeding species are usually too recent to be revealed by gene sequence data. The lack of genetic differentiation between South American *N. magellanica* and *N. deaurata* based on mtDNA markers (de Aranzamendi et al. 2009; Gonzalez-Wevar et al. 2010) cannot reliably be interpreted as an evidence for ongoing gene flow between both morphotypes, as the applied markers might simply be too slowly evolving to detect genotypic variation. In order to get more in-depth understanding about the recent evolutionary history of populations of one species, fast evolving markers are necessary. Microsatellite markers have become the most popular and versatile marker system in population genetic studies in recent years (Selkoe and Toonen 2006). Microsatellites are certain genomic regions that occur in high frequencies on the DNA and are characterized by short tandem repeats of nucleotide motifs, each unit comprising 2-6 base pairs (Jarne and Lagoda 1996; Ellegren 2004; Selkoe and Toonen 2006). Most microsatellites display high mutation rates of $5x10^{-4}$ mutations per locus per generation, generating large allelic diversities allowing to perform genetic analyses on ecological time scales (Schlötterer 2004). They are furthermore biallelic, codominant markers following mendelian inheritance and are therefore appropriate for research regarding heterozygosity, paternity and individual relatedness.

In population genetic studies the geographical distribution of genetic variability is used to make inferences on the gene flow among local populations. Furthermore, it can be used to determine the genetic diversities and demographic histories of single local populations belonging to one species. The genetic diversity of a population gives insight into its fitness and adaptability (Booy et al. 2000; Reed and Frankham 2002). The higher the genetic diversity of a given population is, the better are the chances to survive environmental changes through adaptation. Patterns of allele frequencies are furthermore widely applied to make inferences on the demographic history of species. When populations or whole species go through a phase of strong reductions of population sizes, caused by e.g. habitat fragmentation, habitat loss or mass extinctions, the amount of genetic variability gets drastically reduced. That phenomenon is called “bottleneck event“ (Hewitt 2000). Species that went through such historical bottleneck events show a typical pattern of high amounts of low frequency polymorphisms

indicating population expansion, most likely directly connected to the availability of habitat (Hewitt 2000; Thatje et al. 2005).

Ecological time scales: The roles of selection and local adaptation in speciation

The application of neutral genetic markers does not allow for inferences on local adaptations in marine organisms (Hemmer-Hansen et al. 2007). In order to get additional information on the presence of adaptive divergence caused by natural selection, the analysis of genes under natural selection has been demonstrated to be very informative (Canino et al. 2005; Conover et al. 2006; Hemmer-Hansen et al. 2007; Larmuseau et al. 2010).

Additionally, physiological traits like enzyme activities and gene expression profiles can help to identify and characterize local selective pressures and allow to observe reactions to changing environments on even finer ecological scales (Conover et al. 2006). Physiological traits alone are not a very suitable means to study the adaptive divergence of populations. The biggest problem with such traits is the uncertainty whether differences are truly caused by adaptation or a consequence of high phenotypic plasticity. It is possible that even strong changes in a physiological trait across local populations only reflect its scope of reactions to varying environmental conditions. However, in combination with gene flow studies based on neutral markers and selection studies with selected markers the investigation of physiological traits provides further useful information on the role of adaptive divergence.

In this thesis I investigated two stress defense systems, the heat-shock response (HSR) and the enzymatic antioxidant defense. The HSR comprises the translation of several groups of heat-shock proteins (Hsps). Stressful situations like elevated environmental temperature increase the risk of cellular damage through unfolding and misfolding of proteins and enzymes that are crucial for cellular maintenance. It has been reported in a wide range of studies that such disadvantageous situations lead to an induction of the expression of heat-shock proteins to stabilize and refold damaged proteins. The HSR is a suitable tool to study physiological adaptations to locally varying environmental conditions and to understand the effects of climate change (Hofmann 1999; Tomanek 2010).

During aerobic metabolic processes oxygen derived molecules, so called reactive oxygen species (ROS; Boveris and Chance 1973; Abele and Puntarulo 2004; Murphy 2009), are naturally generated (e.g. superoxide, $O_2^{\bullet -}$; hydroxyl radicals, $^{\bullet}OH$; hydrogen peroxide, H_2O_2). They contribute to mitochondrial and cellular damage of proteins, lipids and DNA because of their high oxidative potential (Halliwell and Gutteridge 2007). During phases of oxidative stress, where organisms have to cope with elevated levels of ROS production, animals are capable of regulating the activity levels of antioxidant enzyme (superoxide dismutase, SOD; catalase, CAT) to reduce cellular damage (Weihe et al. 2010) making the antioxidant defense a suitable system for investigations on stress reactions.

1.4 Thesis strategy and research aims

The present thesis´ superordinate aim was to obtain new insights into the general understanding of the processes leading to the evolution of species in the marine realm. In a top-down approach I analyzed i) large-scale impacts of emerging geographical barriers and reoccurring glacial periods, and ii) small-scale influences of biogeographical patterning and local adaptations on the evolutionary history of Antarctic and South American *Nacella* limpets. The principal strategy of this thesis was to establish a comprehensive analytical framework to shed light on the various aspects of macro- and microevolutionary processes. A large set of genetic and physiological, neutral and selected markers was applied to address the following major tasks.

Task 1: Developing a comprehensive multimarker approach to reliably study the evolution of Antarctic and South American limpets species of the genus *Nacella*

Background: Each of the many different genetic markers available comes with certain advantages but to date not a single one exists that is free from any caveats and limitations (Avise 1989; Zhang and Hewitt 2003; Schlötterer 2004). Mitochondrial genes are susceptible to pseudogenes (Bensasson et al. 2001), to forces like selective sweeps and hitchhiking (Ballard and Kreitman 1995; Hurst and Jiggins 2005), and to biases stemming from their uniparental inheritance (Birky 1995). Fast-evolving multilocus markers such as RAPDs (random amplified polymorphic DNA), AFLPs (amplified fragment length polymorphism) or ISSRs (inter simple sequence repeats) are fundamentally restricted by their dominant mode of inheritance (Müller and Wolfenbarger 1999) Microsatellite studies can be biased by homoplasious mutations, which basically lead to character identity that is not related to a common genealogical history (Estoup et al. 2002; Selkoe and Toonen 2006). Single-marker studies are therefore prone to reconstruct the genealogy of the marker of choice but not the species genealogy (Avise 1989).

Therefore, I performed a multimarker study to get deeper insights into the evolutionary history of *Nacella* covering neutral processes but also those that are mediated by natural selection.

Strategy: Development of a set of fast evolving microsatellite markers (Chapter 2.1). Comparative analyses of presumably neutral microsatellites, mitochondrial gene sequences (COI) and nuclear gene sequences under selection (Hsp70) to understand neutral and adaptive evolutionary processes in marine species (Chapter 3.2). Analysis of stress reactions of the heat-shock response and the antioxidant stress system to investigate the contemporary stress on ecological scales in intertidal marine limpets (Chapter 2.2).

Task 2: Testing the importance of known physical barriers to gene flow in marine organisms with high dispersal capacities

Background: Today the Antarctic Ocean represents a unique environment, isolated from the rest of the world by large distances of deep ocean. It is believed that the large geographical distances to circumjacent continents and the ACC are the major reasons for this isolation. Recent molecular studies indicated speciation of South American and Antarctic sister taxa to be much younger than the proposed onset of the ACC. These conflicting results render the ACC a suitable test case for the actual role of gene flow barriers in marine speciation processes.

Strategy: The reconstruction of the phylogeny and divergence time of South American and Antarctic limpets with state-of-the-art statistical models using the mitochondrial genes COI and 16S and the nuclear gene 18S (Chapter 3.1). Calculation of genetic diversities as a proxy for potential adaptability to changing environmental conditions, in order to make inferences on the adaptive potential of Antarctic limpets to the rapid climate change (Chapter 4.1).

Principal questions:

- Is the ACC really a strict barrier to gene flow and migration between South America and Antarctica?
- What are the alternative explanations for the high endemic rates in the Antarctic Ocean in case the ACC alone did not suffice to prevent gene flow
- Will an ongoing global warming lead to the invasion of South American species into the Antarctic Ocean?

Task 3: Establishing a high quality population genetic and physiological analysis to reconstruct the recent evolutionary history of the Patagonian limpets

Background: In the recent past, several papers have tried to reveal a genetic differentiation between the two most conspicuous Patagonian limpet species *N. magellanica* and *N. deaurata.* Despite clear morphological differences and different habitat preferences a definite answer whether both morphotypes represent true isolated species or are a cause of phenotypic plasticity is still lacking.

Strategy: Performing a combined genetic and physiological study with neutral markers and those under selection, established in task 1, to reveal the true state of shallow-water limpets from Patagonia. Application of neutral markers, COI and microsatellites, as a baseline study to reveal gene flow patterns across the distribution area. Application of genetic markers under selection, Hsp70 genes, to test for a possible genetic differentiation between *N. magellanica* and *N. deaurata* caused by adaptive divergence (Chapter 3.2). Accomplishment of physiological stress experiments to unravel the impacts of ecological stress during air exposure on vertical and horizontal gradients (Chapter 2.2).

Principal questions:

- Can evidence be found for a genetic divergence between the two nominal species *N. magellanica* and *N. deaurata* when analyzing multiple, independent genetic markers or are the observed morphological and physiological differences merely caused by phenotypic plasticity?
- What role did the glacial periods, which repeatedly affected Patagonian coasts during the Pleistocene, play for the population structure of Patagonian limpets?
- Is there evidence that the steep vertical gradients in abiotic conditions in the intertidal have promoted disruptive ecological selection between both morphotypes?

Chapter 2

Publications

Chapter 2.1

Isolation and characterization of eight polymorphic microsatellite markers from South American limpets of the *Nacella* species complex

Kevin Pöhlmann and Christoph Held

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Abstract

In this study we provide eight polymorphic microsatellite markers for the two South American patellogastropods *Nacella magellanica* and *N. deaurata*. Allelic diversity ranged from 5 to 57 alleles per locus. Observed heterozygosity varied between 0.1 and 0.98. Three of the four loci designed for *N. magellanica* cross-amplified also with *N. deaurata*, and two loci vice versa. Six of the microsatellites successfully cross-amplified with the sister taxon *N. mytilina*. This set of microsatellites provides a suitable tool for population genetic and phylogeographic studies.

Keywords Nacellidae · Microsatellites · Population genetics · Multiplex PCR · Patagonia

South American members of the patellogastropod genus *Nacella* inhabit coastal areas of Patagonia and Tierra del Fuego as well as on the Falklands/Malvinas. Based on morphology nine different species are described to comprise this South American *Nacella* complex (see: Valdovinos and Rüth 2005). However, recent molecular studies raised the question whether this classification truly reflects reproductively isolated species or whether it is caused by phenotypic plasticity (de Aranzamendi et al. 2009; González-Wevar et al. 2010). We developed eight polymorphic microsatellites for the two most conspicuous species *Nacella magellanica* and *N. deaurata* to provide a tool for comprehensive studies on population structure, gene flow and demographic and evolutionary history of Patagonian limpets from the genus Nacella.

Microsatellite isolation was carried out with three individuals of *N. magellanica* and three of *N. deaurata*. Genomic DNA was isolated from muscle tissue preserved in ethanol using spin columns (QIAGEN DNeasy Mini Kit). For each species enriched microsatellites genomic libraries were produced using the reporter genome protocol by Nolte et al. (2005), modified by Held, Leese and Mayer (Leese et al. 2008; Held and Leese 2007). Single stranded DNA from *Mus musculus* bound to hybridization chips (Hybond N+, Healthcare) served as reporter genomes. Enriched fragments were PCR-amplified, purified using the QIAGEN Qiaquick Kit, cloned into pCR2.1-TOPO vector and transformed into chemically competent TOP10F0 *Escherichia coli* (Invitrogen). Positive clones, grown overnight in LB media, were sent to GATC-Biotech (Konstanz, Germany) for sequencing.

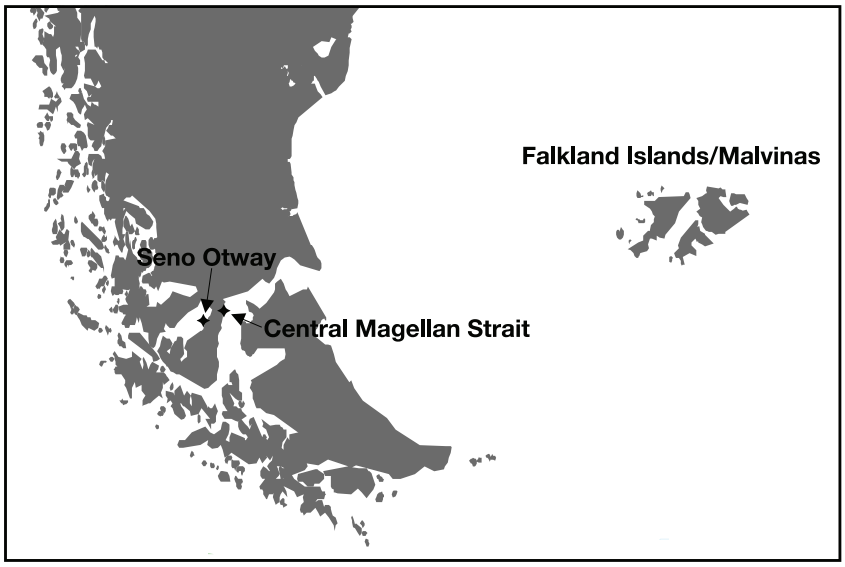

Fig. 1: Map showing the sampling sites of the investigated *Nacella* species

Sequence analysis and primer design were carried out with PHOBOS (Mayer 2008) and PRIMER3 (Rozen and Skaletsky 2000) both integrated into STAMP (Kraemer et al. 2009), a program pipeline based on the STADEN package (Staden 1996).

For *N. magellanica* twelve suitable candidate loci were found in 79 sequenced clones. For *N. deaurata* the analysis of 87 clones also resulted in twelve suitable loci. Primers for the candidate loci were designed using the multiplex option and a melting temperature of 55°C.

Primer pairs were tested on a gradient PCR with annealing temperatures from 48°C to 65°C. PCRs were carried out in total volumes of 25 µl, containing approximately 10 ng genomic DNA, 0.2 mM dNTPs, 0.5 µM primer, 0.5 M Betaine, 2.5 mM MgCl, 0.03 U/µl Hotmaster Taq (Eppendorf). Following PCR conditions were applied: 2 min at 94°C, 32 cycles of 20 s at 94°C, 10 s at different annealing temperatures, 60 s at 65°C and a final extension of 45 min at 65°C to minimize premature termination of amplification.

For *N. magellanica*, ten of the twelve loci produced distinct PCR products, for *N. deaurata* eleven out of twelve. These remaining 21 loci were amplified using fluorescently labeled primers carrying the dyes HEX and FAM, purified with ExoSAPit (Fermentas) and analyzed on an ABI 3130 x l sequencer using ROX GS500 size standard (ABI). Genotyping was performed using the software GENEMAPPER 4.0 (Applied Biosystems).

For each species four microsatellite loci could be reliably genotyped and the variability was assessed with specimens from three different populations (Fig. 1, Tables 1 and 2).

Table 1: Genetic characterization of four polymorphic microsatellite loci isolated from individuals of *Nacella deaurata* collected on the Falkland Islands and the Central Magellan Strait region. N_a, Number of alleles; T_a, annealing temperature; H_o / H_e, observed and expected heterozygosity; PI, probability of identity. Violations of Hardy Weinberg Equilibrium are shown in bold numbers (Bonferroni corrected 5% significance level = 0.00833)

							Seno Otway			Central Magellan	
Locus	**Primer sequence (5´-3´)**	**Dye**	**repeat motif**	**N_a**	**Size range (bp)**	**T_a (°C)**	**n**	**H_o / H_e**	**PI (unbiased)**	**n**	**H_o / H_e**	**PI (unbiased)**
Nma3	F: ATG AAT CAA AAC TGT TGG CT R: TGC GCT ATG ACA TAC ACA TT	Hex	$(C)_{14}(CA)_{14}$	25	189 - 220	57	31	0.81 / 0.88	1.854×10^{-2}	46	0,83 / 0.87	$2,348 \times 10^{-2}$
Nma4	F: ATC TCC GCA GAT ACA AAC AA R: GGG TAT TGG TGA GAT GTG TT	Fam	$(CA)_7CG(CA)_3$	17	184 - 202	57	31	0.77 / 0.89	1.827×10^{-2}	46	0,83 / 0.89	$1,682 \times 10^{-2}$
Nma6	F: CTT TAG CAA AAT TGG TTT CG R: GGC AGG TTT GAC AGC TAA T	Hex	$(CT)_5/(CT)_2GT(CT)_3GT(CT)_2/(CT)_6TG(CT)_{21}/(CT)_5$	57	192 - 324	57	31	0.84 / 0.97	3.036×10^{-4}	45	**0,73 / 0.95**	$2,975 \times 10^{-3}$
Nma12	F: TGT CAT CCG TCA AAA TGT TA R: TCT TCA ATG AGA CAA AAC CC	Fam	$(GA)_{31}$	28	177 - 235	57	29	0.83 / 0.95	2.948×10^{-3}	44	**0,75 / 0.94**	$4,400 \times 10^{-3}$

Table 2: Genetic characterization of four polymorphic microsatellite loci isolated from individuals of *Nacella magellanica* collected from Seno Otway and the Central Magellan Strait region. N_a, Number of alleles; T_a, annealing temperature; H_o / H_e, observed and expected heterozygosity; PI, probability of identity. Violations of Hardy Weinberg Equilibrium are shown in bold numbers (Bonferroni corrected 5% significance level = 0.00714)

							Falklands			Central Magellan	
Locus	**Primer sequence (5´-3´)**	**Dye**	**repeat motif**	**N_a**	**Size range (bp)**	**T_a (°C)**	**n**	**H_o / H_e**	**PI (unbiased)**	**n**	**H_o / H_e**	**PI (unbiased)**
Nde1	F: TAT CAA CGC ATC TTT CAT CA R: CAC GAT GTG TTG AGG TGT AG	Hex	$(GA)_{18}$	22	213 – 234	57	52	0.92 / 0.89	1.960×10^{-2}	39	0.97 / 0.95	2.973×10^{-3}
Nde2	F: TAG GTG TTA CGA GGA CGT TT R: GAT CAA GAT TCA TCA GTG GC	Fam	$(CT)_{18}(TC)_7$	25	154 - 218	57	52	0.98 / 0.92	7.703×10^{-3}	40	0.85 / 0.92	6.515×10^{-3}
Nde8	F: TGT TGA TGA TGA AGG TGA TG R: AGA GAG GAG CTA AAC CCA AT	Hex	$(GAG)_6(GAA)_2(GAG)_3$	19	108 - 151	57	46	**0.48 / 0.81**	4.133×10^{-2}	41	0.78 / 0.82	4.471×10^{-2}
Nde3	F: TGA TTT AGA TAG GAG AGC GG R: AGG CTA AAT AAG CAT TGT CG	Hex	$(AGAC)_5$	5	260 - 276	57	52	0.1 / 0.13	7.583×10^{-1}	42	0.12 / 0.11	7.788×10^{-1}

No evidence for scoring errors caused by large allelic dropout or stuttering could be found using MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004). Diversity measures and deviations from Hardy–Weinberg equilibrium (HWE) were tested using ARLEQUIN 3.11 (Excoffier et al. 2005). Allelic diversity ranged from 5 (Nde3) to 57 (Nma6) alleles per locus. Observed heterozygosities varied between 0.1 (Nde3) and 0.98 (Nde2). After Bonferroni correction (Rice 1989) the locus Nde8 deviated from HWE in the Falklands/ Malvinas Population of *N. deaurata* and Nma6 and Nma12 in the Central Magellan population of *N. magellanica*. These deviations could be caused by the presence of null alleles as suggested by MICROCHECKER although inbreeding and population expansion are also possible explanations of the observed reduction of heterozygosity. Global linkage disequilibrium analyses revealed no linkage between investigated loci as tested in GENEPOP 4.0.6 (Rousset 2008).

The unbiased probability of identity (PI) calculated in GIMLET 1.3.3 (Valiere 2002) revealed high PI values for all loci (minimum 7.788×10^{-1} for Nde3).

Cross-amplification tests resulted in successful amplification of the loci Nma4 and Nma6 in *N. deaurata* and *N. mytilina* that were originally developed for *N. magellanica* (Table 3). The three loci Nde1, Nde3 and Nde8 developed for *N. deaurata* successfully amplified in *N. magellanica*.

In total we developed and provide here eight polymorphic loci appropriate for population genetic studies with the South American limpet species *N. magellanica*, *N. deaurata* and *N. mytilina*. These markers enable us to develop a more profound classification of the genus *Nacella* and to study their evolutionary and demographic history.

Tab. 3: Cross-amplification of the microsatellite loci isolated from *N. magellanica* with *N. deaurata* and vice versa. All loci were tested for cross-amplification with *N. mytilina* (n=9)

Locus	*N. magellanica*	*N. deaurata*	*N. mytilina*
Nma3	189-220	not amplified	not amplified
Nma4	184-202	187-205	191-203
Nma6	192-324	186-318	190-198
Nma12	177-235	not amplified	not amplified
Nde1	210-246	213-234	210-235
Nde2	not amplified	154-218	168-194
Nde3	260-280	260-276	260
Nde8	108-147	108-151	125-150

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Chapter 2.2

Heat-shock response and antioxidant defense during air exposure in Patagonian shallow-water limpets from different climatic habitats

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Abstract

Climate warming involves not only a rise of air temperature means, but also more frequent heat waves in many regions on earth, and is predicted to intensify physiological stress especially in extremely changeable habitats like the intertidal. We investigated the heat-shock response (HSR) and enzymatic antioxidant defense levels of Patagonian shallow-water limpets, adapted to distinct tidal exposure conditions in the sub- and intertidal. Limpets were sampled in the temperate Northern Patagonia and the subpolar Magellan region. Expression levels of two Hsp70 genes and activities of the antioxidants superoxide dismutase (SOD) and catalase (CAT) were measured in submerged and 2- and 12-h air-exposed specimens. Air-exposed Patagonian limpets showed a tiered HSR increasing from South to North on the latitudinal gradient and from high to low shore levels on a tidal gradient. SOD activities in the Magellan region correlated with the tidal rhythm and were higher after 2 and 12 h when the tide was low at the experimental site compared to the 6 h value taken at high tide. This pattern was observed in intertidal and subtidal specimens, although subtidal individuals are little affected by tides. Our study shows that long-term thermal adaptation shapes the HSR in limpets, while the oxidative stress response is linked to the tidal rhythm. Close to the warm border of their distribution range, energy expenses to cope with stress might become overwhelming and represent one cause why the limpets are unable to colonize the shallow intertidal zone.

Keywords Patagonia · Heat-shock protein · Oxidative stress · Intertidal · *Nacella*

Introduction

Thermal tolerance and its impact on the horizontal and vertical distribution of species have received growing interest during the past decade, as biologists have looked more intensively for observable effects of climate change. Of all factors under change, aerial warming is believed to have the most immediate effect also in marine coastal environments (Somero 2005; Harley et al. 2006). Intertidal zones are high-stress environments and further characterized by steep vertical gradients in abiotic conditions and stress levels during tidal cycles. Especially in the high intertidal, marine fauna and

flora are periodically exposed to desiccation, warming or freezing depending on latitude, and also to osmotic stress caused by precipitation and evaporation (Hofmann 1999; Helmuth et al. 2006a, b; Tomanek 2002; Denny et al. 2006). Many marine animals living temporarily above the waterline have behavioral adaptations that help them avoid the most stressful conditions. Either they retreat to tidal pools during low tides, or they change their behavior to establish locally confined conditions under which they can survive, e.g. by hermetically closing their shells during aerial exposure. When conditions stray too far from the physiological optimum of the organism, this behavior can involve a state of transient hypoxia, especially if the animals are additionally warmed by solar irradiation. Organisms experiencing periodically recurring stress situations (e.g. during tidal cycles) often develop a state of heat hardening, which improves their chance of survival during severe stress, but is attained at the cost of significantly increased energetic investments into cellular protection and maintenance (Somero 2002; Hofmann 2005; Dong et al. 2008). As a consequence of the steep vertical gradients in exposure, the marine intertidal is often characterized by a pronounced faunal zonation. Distinctly adapted species typically occupy different positions on the environmental gradient, but also conspecific specimens can choose microhabitats at different shore heights of rocky intertidal zones, according to their individual tolerance towards tidal exposure (Tomanek and Sanford 2003; Weihe and Abele 2008; Weihe et al. 2010).

One important protective mechanism against a variety of stress conditions during tidal emersion such as temperature stress and oxygen deficiency is the heat-shock response (HSR). It describes the activation of so-called heat-shock proteins (Hsps) which act as chaperones stabilizing and salvaging denatured proteins and, in so doing, prevent formation of cytotoxic aggregates (Parsell and Lindquist 1993; Hartl 1996; Fink 1999). The most abundant molecular chaperones are from the Hsp70 class, which comprises several proteins of a molecular weight of 68-74 kDa (Lindquist 1986). The HSR thresholds in different marine organisms correlate with habitat temperature and the stress levels normally experienced by the organisms (Feder and Hofmann 1999), and it has been suggested that frequent expression of Hsps may be part of the physiological strategy of intertidal organisms to occupy ecological niches close to their thermal limits (Tomanek 2010).

Another important anti-stress reaction is the antioxidant defense system. Antioxidant enzymes such as superoxide dismutase and catalase I (SOD and CAT; see Abele and Puntarulo 2004) are central constituents of the inducible systems that control the detrimental effects caused by reactive oxygen species (ROS; e.g. superoxide, H2O2, OH·) produced in cells under physiological strain (Boveris and Chance 1973; Jones 2006; Murphy 2009). ROS damage DNA, proteins and lipids and thereby jeopardize cellular and organism fitness and function, forcing animals to invest more energy into cell repair. Heat stress in ectotherms generally induces mitochondrial ROS formation and, in less heat-tolerant animals, thermal inactivation of antioxidant enzymes enhances the oxidative stress condition (Abele et al. 2001, 2002; Heise et al. 2003).

We studied shallow-water limpets from the genus *Nacella* that inhabit different shore levels along the Patagonian coast of Southern Chile, with the objective to obtain new insight into the importance and plasticity of the HSR and the antioxidant defense in natural limpet populations. Ecologically, the study was aimed at understanding how thermal tolerance influences the distribution in our species of interest. The approach includes a large-scale comparative investigation of populations of the intertidal limpet *Nacella magellanica* from the northern edge (Puerto Montt, PM, ~42°S) and from the centre (Punta Arenas, PA, ~55°S) of its distribution range, representing two different temperature regimes. Mean summer air temperatures at PM are around 15°C compared to 8°C at PA. In a second step, we performed a small-scale comparison of the stress response between the shallow intertidal *N. magellanica* and the deep intertidal/shallow subtidal *Nacella deaurata*. Both nominal species occur in sympatry in Punta Arenas, but are differentially exposed to aerial and marine environmental conditions.

A full evaluation of the taxonomic status and genetic distinctness of *N. deaurata* and *N. magellanica* is beyond the scope of this study. Their status is controversially discussed based on morphological and genetic analyses (Powell 1973; Valdovinos and Rüth 2005; De Aranzamendi et al. 2009). Despite their clearly distinct morphologies (*N. magellanica* have larger shell heights and a centered apex, *N. deaurata* comparably flat shells and the apex shifted towards the anterior), the mitochondrial markers cyt b and COI did not distinguish both species (De Aranzamendi et al. 2009). The application of two different fast evolving molecular marker systems produced ambiguous results. An inter-simple sequence repeats analysis revealed considerable differences between *N.*

magellanica and *N. deaurata* (De Aranzamendi et al. 2009), but eight microsatellite loci showed no differentiation between the two morphotypes (Chapter 2.1). Difficulties in determining whether morphological disparities reflect species level differentiation or are caused by phenotypic plasticity are also known for other limpets such as the Antarctic sister taxon *Nacella concinna* (Hoffman et al. 2010b, Morley et al. 2010). Since the true genetic relationship of South American *Nacella* is not fully resolved, we consider them as two subpopulations between which gene flow cannot be fully ruled out.

Laboratory-based heat-shock experiments under controlled conditions do not necessarily reflect the natural anti-stress response of air-exposed intertidal limpets in the field (Clark et al. 2008a, b). Therefore, the experiments in the present work were conducted in the field, to mimic the combination of naturally occurring stressors that threaten intertidal organisms when air exposed during phases of tidal uncovering and, yet, be comparable between different study sites.

The aim was to investigate how the intensities of inducible HSR and antioxidant defense levels differ between limpets adapted to differentially exposed conditions in the sub- and intertidal. The HSR and antioxidant response of animals taken from their natural habitat at different positions in the tidal zone were measured after experimental air exposure for 2, 6 and 12 h, to simulate air exposure during tidal uncovering as a natural stressor. In addition to previous studies that focused on the temperature threshold at which the HSR response is triggered in organisms with vertical zonation patterns (for reviews see Hofmann 1999, 2005 Feder and Hofmann 1999), our experimental approach also determines the timing of the onset of the HSR (Clark et al. 2008b, Dong et al. 2008).

Specifically it allows us to test whether the onset of the HSR parallels previous results of the limpet antioxidant response. In the previous study, intertidal Antarctic limpets (*N. concinna*) had a delayed response to air exposure stress compared to their subtidal conspecifics, indicating adaptation to periodical air exposure in the intertidal specimens (Weihe et al. 2010). In addition, we performed parallel experiments in which animals were kept immersed during the whole low tidal cycle, to investigate whether either the Hsp expression or the antioxidant defense systems feature intrinsic patterns adaptive to tidal periodicity, as recently observed in a microarray study of intertidal *Mytilus* mussels (Gracey et al. 2008).

We hypothesize the following: (1) Limpets near their distribution edge at PM should show a more pronounced stress response to air exposure than limpets from the center at PA due to stronger temperature shifts between states of immersion and tidal uncovering at PM. (2) Limpets from the high intertidal should be better adapted to air exposure than limpets from the subtidal showing delayed onsets and less pronounced anti-stress reactions, because they endure phases of tidal uncovering more regularly.

Material and methods

Sampling sites and experimental design

Sample collection of *N. magellanica* and *N. deaurata* and experiments were carried out at two different sites in Southern Chile, in PM in Northern Patagonia and in PA in the Strait of Magellan (Fig. 1). Two to four hours is the normal time span that *N. magellanica* specimens experience tidal emersion twice a day at PA. By contrast, PM individuals of *N. magellanica* fall dry only during spring tides when the tidal range reaches its maximum of 6 m. At PA, *N. deaurata* suffer tidal effects when the shallow coastal waters warm up on sunny days, or when the animals become air exposed during extreme low tides. At PM, *N. deaurata* are absent.

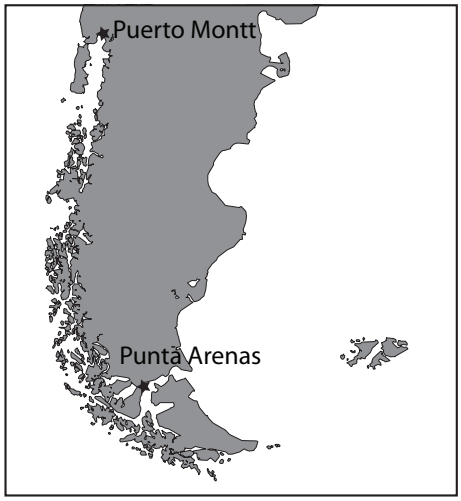

Fig. 1 Map showing the two different experimental sites at Puerto Montt, the northern distribution boundary, and Punta Arenas in the center

Samples of all subpopulations were collected by hand 1 to 2 h before low tide just before emersion of the intertidal specimens (see Table 1 for an overview of collection sites, time and conditions). For control values of gene expression and enzyme activities, control animals were dissected immediately and samples of foot muscle (gene expression) and gills (enzyme activities) collected. Foot muscle samples (100-300 mg) were stored in 1.5 ml of RNAlater (Qiagen), and gill tissues were snap frozen and kept in liquid nitrogen. Shells were kept for morphological examination.

For simulation of tidal air exposure during phases of low tides, experimental specimens were placed in three separate dry plastic tanks directly after collection, and were maintained on location over three different time spans of 2, 6 and 12 h. Each treatment group consisted of six animals of each subpopulation (two individuals of each treatment group and subpopulation per tank). Additional control experiments were conducted with submersed animals, simultaneously for each treatment group and subpopulation in plastic tanks filled with 1 l of fresh seawater. Seawater in the control was fully oxygen saturated throughout the experimental period.

Limpets from all treatments were dissected on location as described for untreated controls. In Puerto Montt, all the experiments were conducted on the same day, but in Punta Arenas the 12-h experiment was conducted on day 1, while the 2- and 6-h experiments were conducted on day 2 to ensure accurate sample handling under difficult weather conditions. Air temperatures, water temperatures and oxygen concentrations of the seawater in the experimental tanks were recorded in 30-min intervals throughout the experiments. Temperature and oxygen concentration in the sea surface water at the experimental sites were recorded every 2 h. Figure 2 shows the temperature protocols of both experimental sites. Oxygen concentration never decreased below 90% and can therefore be regarded as stable throughout.

Tab. 1 Overview of experiment sites, date and time as well as water temperatures and low tide time at the beginning of the experiments

Collection site	Coordinates	Experiment date	Water temperature	Experiment start time	Low tide time
Pta Arenas/Bahia Laredo	52°56’56”S 70°47’44”W	12h: 13.02.2009	9.4°C	07:30	09:30
		2h+6h: 14.02.2009	10.8°C	08:20	10:00
Puerto Montt/Chinquihue	41°30’42”S 73°00’55”W	26.02.2009	16.9°C	08:30	09:30

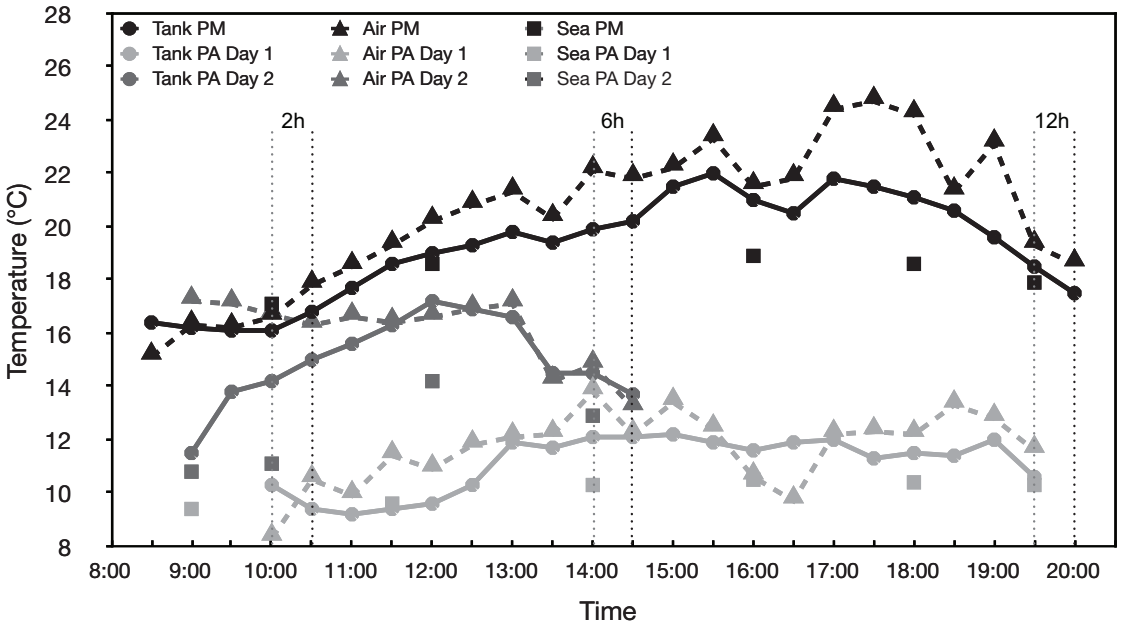

Fig. 2 Temperature profiles during the experiments carried out in Puerto Montt (PM, black lines) and Punta Arenas (PA, light grey lines = experimental day 1; dark grey lines = experimental day 2). Solid lines represent the water temperatures in the experiment tanks, dashed lines represent air temperature and squares describe the temperature of sea surface water at the experimental sites. Vertical dotted lines mark time points of sampling (black, Puerto Montt; grey, Punta Arenas)

Primer design

Degenerate primers reported by Clark et al. (2008a) were used to amplify fragments of the target heat-shock proteins (Hsp70A, Hsp70B, and Grp78) and of β-actin by a standard PCR in a total volume of 25 µl, containing approximately 10 ng genomic DNA, 1× HotMaster reaction buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 0.03 U/µl HotMaster™ Taq (Eppendorf). The following PCR conditions were applied: 2 min 94°C, 35 cycles of 20 s at 94°C, 20 s at 54°C and 40 s at 65°C, and a final extension of 5 min at 65°C. PCR products were cloned with the TOPO TA Cloning® Kit (Invitrogen) according to the manufacturer's instructions. DNA Sequences obtained from cloned fragments were aligned with ClustalW in Codon-Code Aligner software V3.5.7 (CodonCode Corp.). Hsp70A, Hsp70B, Grp78 and β-actin primers published by Clark et al. (2008a) were checked for matching with the sequences obtained by our cloning process and Hsp70A, Hsp70B and Grp78 primers were used in the subsequent analysis. Actin primers were newly designed using the online program Netprimer (www.premierbiosoft.com). Primers for histone H3 were designed to fit the sequence published for *N. deaurata* and *N. magellanica* by Nakano and Ozawa (2007; GenBank accession nos. AB433688 and AB433689). Primers for HIF-1 (Hypoxia Inducible

Factor 1) subunit α were designed to the sequence from *N. concinna* supplied by Weihe et al. (in progress).

RNA extraction and reverse transcription

For measurements of gene expression, total RNA was extracted from 50 to 100 mg foot tissue samples using TRI Reagent (Sigma) under RNase-free conditions according to the manufacturer's instructions. Tissues were homogenized by vigorous shaking in a three-dimensional motion in a Precellys®24 Dual tissue homogenizer (Bertin Technologies) at 6,500 rpm for 25 s. To prevent possible contamination with genomic DNA, the extracted RNA solutions were digested with RNase-free DNase (1 U per µg RNA, Fermentas) in a 10 mM DTT/100 mM $MgCl_2$ buffer. Subsequently, RNA was reverse transcribed into cDNA with MaximaTM Reverse Transcriptase (Fermentas) using oligo-$(dT)_{18}$ primers, under a protocol adjusted for dissolving secondary structures, at 55°C for 40 min.

Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was conducted using the Type-It™ HRM PCR Master Mix with HotStar- Taq® Plus DNA Polymerase and EvaGreen dye (Qiagen). Cycling was performed in a Rotor-Gene® Q 5-Plex rotary cycler (Qiagen) using the following cycling program: 5 min at 95°C, 40 cycles of 10 s at 95°C and 30 s at 55°C. Each sample was quantified in triplicate, and treatment groups were distributed evenly between runs. To confirm the specificity of the RT-qPCR amplification, melt analysis was performed directly after the cycling, by increasing temperature from 65°C to 90°C in increments of 0.3°C for 2 s each. Replicates showing by-product peaks with a height of more than 10% of the main peak were discarded. Two samples were sequenced for each product peak to confirm identity of the measured fragment. Amplification efficiency and linear range of the assay were tested by relative standard curves. As candidate reference genes for normalization of Hsp70 expression values, β-actin, histon H3, Grp78 and HIF-1α were quantified. The program GeNorm (Vandesompele et al. 2002) suggested the gene combination Grp78 + HIF as most suitable reference genes. The program NormFinder (Andersen et al. 2004) ranked Grp78 as best candidate reference, and HIF was ranked second best in terms of stability of expression over time

of exposure. We therefore decided to use the combination of Grp78 and HIF as reference genes in this study (Table 2).

Tab. 2 Stability ranking of candidate reference gene expression by two different algorithms. GeNorm calculates an average expression stability M based on the standard deviation between all genes and samples, while NormFinder returns a model-based stability value between subpopulation/treatment groups

	GeNorm		NormFinder	
rank	Genes	M	Genes	Stability
1.	Grp78+HIF	1.1	Grp78	0.426
2.	β-actin	1.34	HIF	0.562
3.	Histon	1.53	β-actin	0.567
Best pair			Grp78+Histon	0.412

Enzyme activity measurements of SOD and CAT

The determination of SOD activity was carried out according to Livingstone et al. (1992). Fifty to one hundred milligrammes of frozen tissue were ground in liquid nitrogen and homogenized in a buffer containing 20 mM Tris-HCl and 1 mM EDTA (pH 7.6) as a 5:1 mix. All samples were centrifuged for 3 min at 18,000×g and at 4°C. The supernatant was used to determine SOD activity photometrically at a fixed wavelength of 550 nm for 3 min with an interval time of 10 s in a potassium buffer (43 mM K_2HPO_4, 0.1 mM EDTA, pH 7.68, 100 µM cytochrome *c*, 5 mM xanthine, 0.3 mU/µl xanthine oxidase and 2 M $(NH_4)_2SO_4$). The activity of SOD was determined on the basis of its inhibiting capabilities of the xanthine oxidase/xanthine reaction system, which catalyses the formation of superoxid anions ($O_2^{-\cdot}$). SOD converts the formed $O_2^{-\cdot}$ to hydrogen peroxide (H_2O_2), thus inhibiting the reduction of cytochrome *c*. In the applied assay, 1 U of SOD causes an inhibition of 50% of the XOD reaction.

The CAT was measured using the same extracts as for SOD. Enzyme activities were determined photometrically at 240 nm in a potassium buffer containing 50 mM K_2HPO_4 (pH 7.0) and 1 µM hydrogen peroxide (H_2O_2). CAT activity is determined via the turnover rate of a defined amount of H_2O_2 into water and oxygen, according to Aebi (1984).

Statistics

For quantification of gene expression, replicate CT values were transferred to Excel 2002 (Microsoft Corp.), and linear expression values were obtained using the standard curve equation output by the Rotor-Gene software. The stability of gene expression was tested by running the Excel macros GeNorm (Vandesompele et al. 2002) and Norm-Finder (Andersen et al. 2004) on the expression data. Relative expression values were normalized through division by the geometric mean of the most stable genes Grp78 and HIF-1, and tested for statistical significance of expression changes within subpopulations (independent variable: time of air exposure) by one-way ANOVA with Tukey's post hoc test and among subpopulations by two- way ANOVA (independent variables location and shore height) with Bonferroni post hoc test using Graphpad Prism 5.01 (Graphpad Software Inc.).

Enzyme activity data from all treatments and subgroups were tested for normal distribution using the Kolmogorov-Smirnov test. Statistical analysis of the enzyme activities was performed using Graphpad Prism 5.01. The dependence of SOD and CAT activities on exposure time within a subpopulation was tested by one-way ANOVA with Tukey's post hoc test, and on factors subpopulation site and shore height by two-way ANOVA followed by Bonferroni post hoc test.

An ANCOVA model of the temperature dependence of SOD and CAT activities in gills of *N. magellanica* and *N. deaurata* at PA revealed no significant thermal effects on the activity of either enzyme when assayed at room temperature ($p = 0.076$, $F = 2.201$, $n = 71$). Further, the effective oxygen saturation in the experimental buckets had no modulating effect on the enzyme activities (oxygen level $p = 0.985$, $F = 0.091$, $n = 71$). Therefore, a slight, but statistically significant difference ($p = 0.044$) between CAT activities of the *N. deaurata* initial groups (0 h) between day 1 ($n = 5$) and day 2 ($n = 4$) was attributed to the natural intra-specific variability, and data from both days were pooled into a common 0 h exposure group ($n = 9$).

Results

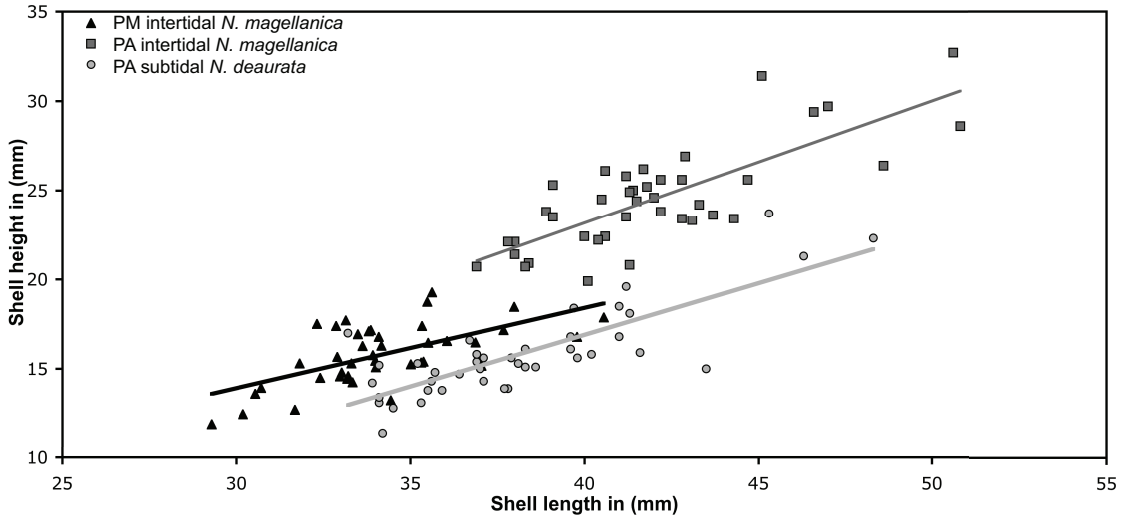

Fig. 3 Shell lengths to shell heights in the three investigated populations from Puerto Montt (black triangles), Punta Arenas intertidal (dark grey squares) and Punta Arenas subtidal (light grey cycles)

Shell morphometry

Shell lengths (SL) and heights (SH) of all experimental individuals are shown in Fig. 3. Mean shell lengths and heights were highest in intertidal *N. magellanica* from PA (SL = 42 ± 3.3 mm, SH = 24.6 ± 2.9 mm). The *N. magellanica* population from PM and the *N. deaurata* population from PA had the same mean SH of 15.8 mm with slightly different height variability (± 1.7 mm and ± 2.5 mm, respectively), but average SL was smaller in *N. magellanica* specimens from PM (34.1 ± 2.4 mm) compared to individuals from the *N. deaurata* population from PA (38.1 ± 3.4 mm).

The strong variations in shell morphometry between the two populations of *N. magellanica* reflect the high phenotypic plasticity in the species complex, with bigger shells in regions where limpets are exposed more frequently to fluctuations caused by the tidal rhythm.

Heat-shock gene expression

Tab. 3 Average gene expression of Hsp70A and Hsp70B in air exposed and submerged individuals from Puerto Montt (PM), Punta Arenas (PA) intertidal and Punta Arenas subtidal, relative to untreated control animals. n=6 for each treatment/ population combination except n = 4 for 6h Punta Arenas subtidal. Values in bold indicate significant up regulation compared to the control animals (One Way ANOVA, Tukey test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)

		PM intertidal *N. magellanica*		PA intertidal *N. magellanica*		PA subtidal *N. deaurata*	
Gene	Incubation	air exposed	submerged	air exposed	submerged	air exposed	submerged
Hsp70A	2h	14.7 ± 9.2	3.9 ± 3.4	23.3 ± 29.9	2.9 ± 2.7	**402.2 ± 285.6*****	4.3 ± 3.3
	6h	**1101.7 ± 513.9*****	229.9 ± 314.8	**99.9 ± 115.9***	**23.8 ± 22.6****	**285.6 ± 172.5***	**20.8 ± 9.7****
	12h	**1113.4 ± 200.3*****	84.9 ± 47.5	2.4 ± 1	4.2 ± 2.1	11.9 ± 12.4	4.6 ± 5.1
Hsp70B	2h	5.3 ± 2.9	1.7 ± 0.7	13.8 ± 16.6	5.0 ± 2.9	**134.9 ± 92.3***	**15.3 ± 8.1****
	6h	**79.5 ± 17.8*****	31.5 ± 44.7	**24 ± 16.5****	**15.5 ± 11.1*****	**149.5 ± 117.3****	**36.5 ± 12.2*****
	12h	**123.3 ± 34.1*****	23.2 ± 8.2	0.9 ± 0.7	0.7 ± 0.5	6.6 ± 5.7	8.0 ± 3.5

The basal, unstressed expression patterns of Hsp70A and B were similar, but up-regulation of Hsp70A was up to ten times higher when comparing the expression of both genes in each subpopulation and treatment (Table 3, Fig. 4). Temperature curves at both sites at the northern PM and the southern PA were markedly different in the course of experimental exposure. Seawater temperatures at the time of collection in PA were 11°C and at PM 15°C, and the

thermal increase during the first 2 h of exposure was 6°C at PA and 3°C at PM. During these first 2 h of air exposure and in spite of 17-18°C of air temperature, no HSR was initiated in either population of *N. magellanica* at the border (PM) or the centre (PA) of the distribution range, indicating these limpets to be well-adapted to short periods of air exposure. Contrary, the subtidal *N. deaurata* from PA responded already after 2 h of air exposure with a significant up-regulation of Hsp70A (~400-fold) and Hsp70B (~150-fold). In the following 4 h until the end of the 6 h treatment, air temperatures at PA dropped by about 3°C, whereas air temperature in PM rose further to 22°C. This prolonged period of air exposure and desiccation elicited up-regulation of Hsp70A and Hsp70B with the response in *N. magellanica* at PM being an order of magnitude higher than in PA *N. magellanica* (PM, ~1,100 fold; PA, ~100-fold) and about three times higher compared to PA *N. deaurata*. Both subpopulations at PA responded to prolonged air exposure (12 h) with down-regulation of the HSR to control level in both Hsp genes. PM *N. magellanica* responded completely differently with a HSR extended until 12 h of air exposure with up-regulation about ~1,000- fold compared to the controls for

Hsp70A and ~120-fold for Hsp70B. It is striking that inter-individual variation in *N. magellanica* and *N. deaurata* from PA is fairly high compared to less inter-individual variation in *N. magellanica* from PM. This further indicates that the severe temperature and desiccation stress at PM force all limpets in the experiment to increase their Hsp70 gene expression whereas temperature stress is less pronounced at PA allowing for greater variation among tested individuals.

Limpets maintained submerged in water during the same experimental period still produced a HSR, but the levels of heat-shock gene induction were much less pronounced than in air-exposed limpets (Fig. 5). For Hsp70A, a significant up-regulation was observed only in individuals from both PA subpopulations and only after 6 h of submersed exposure. Although on average the relative Hsp70A expression after 6 h was much higher in PM (~240-fold) than in both PA limpet subpopulations, statistical testing failed to prove a significant difference due to high inter-individual fluctuations. Expression patterns of Hsp70B were similar to those of Hsp70A, except that in the PA subtidal limpets Hsp70B was already significantly up-regulated after 2 h.

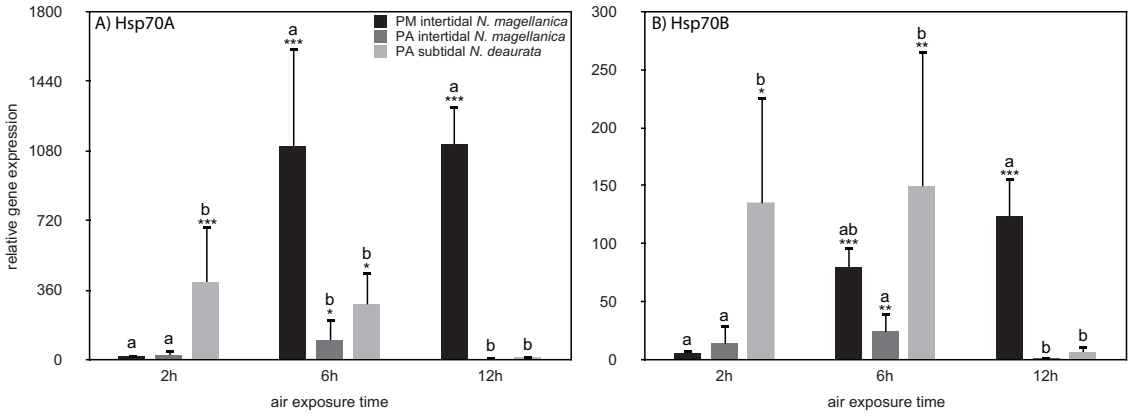

Fig. 4 Average gene expression of A Hsp70A and B Hsp70B in air-exposed individuals from Puerto Montt, Punta Arenas intertidal and Punta Arenas subtidal, relative to untreated control animals (not shown). Asterisks indicate significant up-regulation compared to the control animals (one-way ANOVA, Tukey test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Letters indicate significant differentiation among the populations in each treatment (two-way ANOVA, Bonferroni, $p < 0.05$)

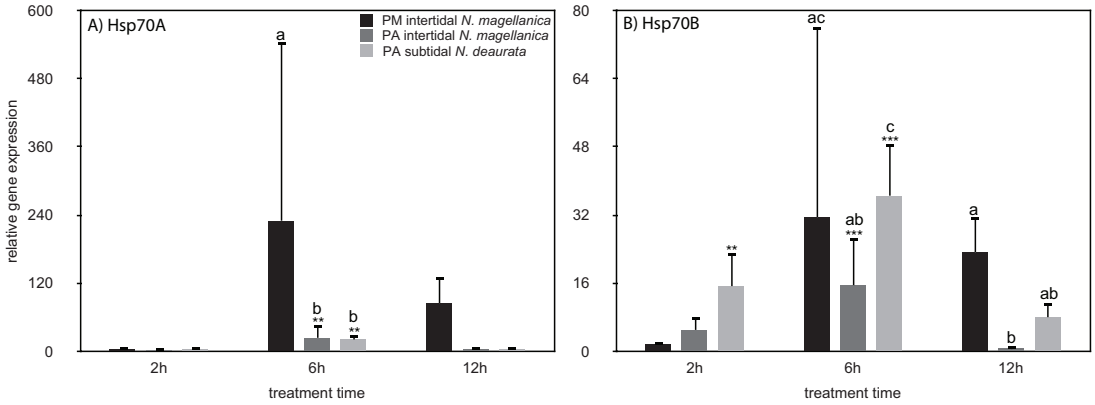

Fig. 5 Average gene expression of A Hsp70A and B Hsp70B in submerged individuals from Puerto Montt, Punta Arenas intertidal and Punta Arenas subtidal, relative to untreated control animals (not shown). Asterisks indicate significant up-regulation compared to the control animals (one-way ANOVA, Tukey test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Letters indicate significant differentiation among the populations in each treatment (two-way ANOVA, Bonferroni, $p < 0.05$)

SOD and CAT enzyme activities in gills of submersed and air-exposed limpets

Tab. 4 Average enzyme activities of SOD and CAT (U/mg_{FW}) in air exposed and submerged individuals from Puerto Montt (PM), Punta Arenas (PA) intertidal and Punta Arenas subtidal. n = 6 for each treatment/population combination, except n = 9 for controls and n = 4 for 6h Punta Arenas intertidal. Significant differences among treatments and populations can be seen in Figs. 6 and 7

		PM intertidal *N. magellanica*		PA intertidal *N. magellanica*		PA subtidal *N. deaurata*	
Enzyme	Incubation	air exposed	submerged	air exposed	submerged	air exposed	submerged
SOD	controls	0.414 ± 0.113	0.414 ± 0.113	0.455 ± 0.066	0.455 ± 0.066	0.485 ± 0.069	0.485 ± 0.069
	2h	0.502 ± 0.166	0.4 ± 0.048	0.713 ± 0.077	0.681 ± 0.017	0.619 ± 0.135	1.111 ± 0.307
	6h	0.449 ± 0.091	0.4 ± 0.083	0.582 ± 0.031	0.447 ± 0.017	0.464 ± 0.028	0.441 ± 0.07
	12h	0.562 ± 0.103	0.437 ± 0.09	0.572 ± 0.095	0.419 ± 0.088	1.371 ± 0.407	1,376 ± 0,442
CAT	controls	0.754 ± 0.211	0.754 ± 0.211	0.883 ± 0.066	0.883 ± 0.066	0.992 ± 0.069	0.992 ± 0.069
	2h	0.819 ± 0.317	0.893 ± 0.317	0.702 ± 0.077	0.867 ± 0.095	1.058 ± 0.135	0,868 ± 0,307
	6h	0.931 ± 0.317	0.653 ± 0.317	1.242 ± 0.031	0.935 ± 0.017	0.731 ± 0.028	1.011 ± 0.07
	12h	0.747 ± 0.317	0.744 ± 0.198	0.647 ± 0.095	0.836 ± 0.017	0.796 ± 0.407	0.578 ± 0.442

Gill CAT activities showed only little variations in air-exposed and submerged individuals of *N. magellanica* and *N. deaurata* at both locations over the time course of the experiments. A significant change in CAT activity could only be detected in gills of the PA intertidal animals after 6 h, where CAT activity was significantly elevated over the activities after 2 and 12 h of air exposure. Contrary, the difference between the 6- and the 0-h control group was not significant (see Table 4 and Fig. 6 for exact values).

Gill SOD activities in *N. magellanica* limpets from the PM intertidal did not change during air exposure with respect to the control groups over time. Contrary, in PA both subpopulations responded with an increase in SOD activities at 2 h of air exposure compared to the submersed controls. While this increase was below significance in subtidal *N. deaurata*, it reached significance level in the intertidal *N. magellanica*. Indeed, SOD activities in intertidal limpets remained elevated over the submersed control groups at 6 and 12 h of air exposure. To the contrary, SOD activity in subtidal *N. deaurata* from PA decreased to control level at 6 h after the initial increase and finally peaked at 12 h of air exposure. Submersed *N. deaurata* in the control group displayed a similar fluctuating SOD pattern over time with highest activities after 2 and 12 h of experimentation (see Table 4 and Fig. 7 for exact values).

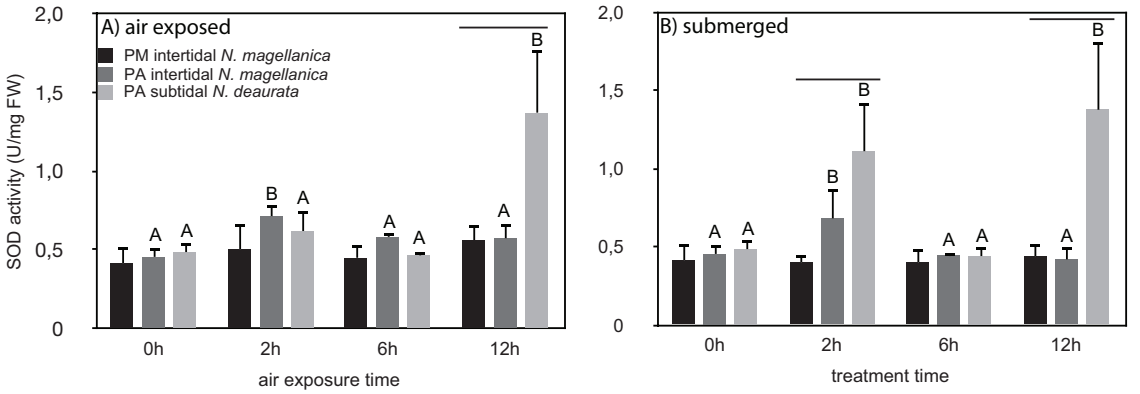

Fig. 6 CAT activities (U/mgFW) in A air-exposed and B submerged individuals from Puerto Montt, Punta Arenas intertidal and Punta Arenas subtidal. Significant changes of enzyme activities among treatments are indicated as capital alphabets (one-way ANOVA, Tukey test, $p<0.05$) among each population

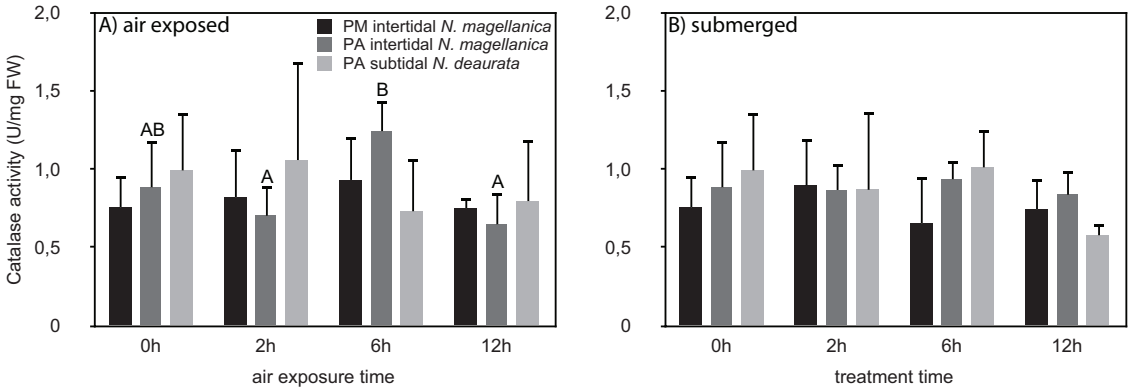

Fig. 7 SOD activities (U/mgFW) in A air-exposed and B submerged individuals from Puerto Montt, Punta Arenas intertidal and Punta Arenas subtidal. Significant changes of enzyme activities among treatments are indicated as capital alphabets (one-way ANOVA, Tukey test, $p < 0.05$). The vertical bars mark significantly higher SOD activities in Punta Arenas subtidal samples compared to the other two populations (two-way ANOVA, Bonferroni, $p < 0.05$)

Discussion

HSR on a biogeographic climate gradient along the Patagonian coast

Populations of marine ectotherms living close to the borders of their biogeographic distribution experience stress to a much greater degree than populations of the same species at the centre of the distribution range (Sorte and Hofmann 2004, Osovitz and Hofmann 2005; Tomanek 2008). This also applies to our study of the HSR of *N. magellanica* along the biogeographical temperature gradient from PM to PA. Short-term air exposure of 2 h did not cause a HSR in *N. magellanica* at either of the two sampling sites, reflecting the general capacity of these limpets to tolerate short periods of tidal uncovering. However, after prolonged air exposure and at a 10°C higher air temperature only at PM, *N. magellanica* at the northern distribution edge expressed Hsp genes in much greater quantities than conspecifics from PA. These results suggest that intertidal animals at PM where environmental factors, especially temperature, fluctuate with wider amplitude and produce a stronger HSR to cope with heating during tidal emersion. The higher air temperature maxima at PM appear to represent the thermal tolerance limit for *N. magellanica*, which would provide one explanation for the absence of limpets in the high intertidal and splash zone of this region. Structuring factors such as predation and competition need to be taking into account during future studies. However, smaller shell and body size of PM compared to PA *N. magellanica* specimens (Fig. 2) are a typical consequence of warmer climate and indicate higher investments into cellular stress compensation which restricts growth in ectotherms (Moore and Folt 1993; Reznick et al. 2001; Gracey et al. 2008; Daufresne et al. 2009). Laboratory acclimation studies indicate differences in Hsp induction and thermo-tolerance among intertidal congeners from thermally differing habitats to be genetically fixed (Tomanek and Somero 1999). This suggests that the physiological capability for a high-stress response is a consequence of evolutionary adaptation, which may prevent successful colonization of stressful habitats and contribute to setting the geographic boundaries for intertidal species habitat expansion.

Generally, two opposing forces determine the adaptive potential of given populations occupying different habitats. Selection, mutation and genetic drift support adaptation to local conditions, whereas gene flow usually reduces the adaptive potential (Slatkin

1987; Lenormand 2002). In the present case, ongoing gene flow between populations from PA and PM was demonstrated using fast evolving microsatellite markers (Chapter 2.1). Therefore, repeated introduction of the Hsp70A and Hsp70B alleles, typical for the populations in the centre of the distribution area, might hamper the fixation of newly evolving Hsp mutants at PM and, consequently, diminish the adaptive potential of *N. magellanica* at its northern edge of distribution.

Interestingly, the study by Clark et al. (2008a) concerning laboratory induction temperatures of the HSR in Antarctic *N. concinna* showed a significant up-regulation (1,000-fold) of Hsp70A only at 18-20°C, a temperature these limpets never experience in their natural environment. Although it is questionable to what degree real-time PCR results from different studies can be compared, there is a striking similarity to the results of this study regarding the HSR in *N. magellanica* from PM also being 1,000-fold at temperatures of ~20°C. In their follow-up study, Clark et al. (2008b) tested the HSR in intertidal *N. concinna* taken from their natural habitat at different time points during low tide and found Hsp gene up-regulation at much lower air temperatures, clearly demonstrating that the HSR is governed by a far more complex parameter scenario, and not only triggered by temperature. The in situ inducibility of Hsp70A in the study of Clark et al. (2008b) was, however, two orders of magnitude lower (5- to 25-fold) than in the laboratory experiments with heat shock at 20°C. The similarity of HSR induction temperature and response in South American and Antarctic congeners indicates that the HSR in *Nacella* is old and preserved, and presumably inherited from the common ancestor of today's extant species. The strong preservation of the HSR limits migration beyond northern habitat boundaries, whereas it might be beneficial for Antarctic intertidal limpets during the ongoing climate change scenario.

Small-scale differences of the HSR in inter- and subtidal populations

At PA, onset and intensity of the HSR to air exposure differed strongly between *Nacella* from the inter- and the subtidal (Figs. 4 and 5). In intertidal *N. magellanica*, up-regulation of Hsp70A and Hsp70B occurred later during air exposure, and the intensity of up-regulation was between 2- and 20-fold lower at all time points compared to air-exposed *N. deaurata* from the subtidal. This again underlines that intertidal *N. magellanica* have developed adaptations that enable them to survive periods of air

exposure during tidal uncovering, which they are prone to experience twice a day, without a necessity of inducing the gene expression of extra Hsps. Comparatively less exposed to tidal emersion than their intertidal relatives, subtidal *N. deaurata* did not develop these adaptations and are forced to activate the HSR earlier and to a higher extent, to survive air exposure, e.g. during spring tides. Aside from this plausible explanation for the different intensities of the HSR in limpets with different shore level distributions, the initial levels of Hsps may already have been higher in intertidal *N. magellanica* prior to air exposure. It is possible that shallow intertidal limpets produce Hsps routinely as a preparative defense and, therefore, showed a less pronounced HSR in the course of the experiment (Dong et al. 2008). However, since real-time qPCR represents a relative approach, we cannot make any statement about the Hsp mRNA ground levels in the control animals. Either way, the presented results clearly demonstrate the adaptations in intertidal limpets to cope with recurring stress following tidal emersion.

Most studies of the HSR in the intertidal have focused on the variation in induction temperature. Higher temperature thresholds have been found in high intertidal molluscan populations than in low- and mid-intertidal zones, even after laboratory acclimation at a common temperature, indicating intrinsic fixation of this physiological characteristic (Sanders et al. 1991; Tomanek and Somero 1999; Dong et al. 2008). Since the main trigger for activation of the HSR is the cellular amount of non-native proteins (Feder and Hofmann 1999), stress levels at defined temperatures can be assessed as temporal stress entities that suffice to produce high enough protein damage to activate a HSR. Adaptation to intertidal conditions implies higher physiological tolerance or behavioral adaptation allowing for a later and all in all weaker HSR compared to subtidal conspecifics from the same location. Similar Hsp70 expression in submersed control and air-exposed animals in the intertidal population support this view. The difference in shell morphometry between the two populations of *N. magellanica* from PM and PA (Fig. 2) reflects phenotypic plasticity in this species complex. Shell height seems adaptive to the habitat rather than genetically fixed. The *N. magellanica* population inhabiting the intertidal zone at PA features much higher SH values, adaptive to tidal air exposure twice per day, than *N. magellanica* from PM and *N. deaurata* from PA, neither of which occurs in the high intertidal. Higher shells provide more space for

shell water storage, which may indeed represent an oxygen reserve during the initial period of emersion and contraction, but moreover isolates the animals thermally and prevents desiccation during air exposure (see Vermeij 1973; Hoffman et al. 2010b).

The antioxidant stress response in Patagonian Nacella *during air exposure*

Using the same experimental set-up and the same individuals as in the heat-shock approach, we measured the oxidative stress response in *N. magellanica* from PM and PA and *N. deaurata* from PA. The basic levels of SOD and CAT activities in gills of unstressed animals were similar in all three subpopulations. These results are in line with a study of gill tissues of *N. magellanica* and *N. deaurata* from Ushuaia, Argentina by Malanga et al. (2005). To the contrary, SOD and CAT activities in digestive gland varied between intertidal and sublitoral limpets at Ushuaia (Malanga et al. 2004) and in the Antarctic (Weihe et al. 2010), and we conclude that antioxidant enzyme activities in South American limpets are a tissue specific phenomenon which, in gills, reflects the oxygenation levels the animals encounter in their respective habitats. The antioxidant activities in gills of the Antarctic congener *N. concinna* (Weihe et al. 2010), however, revealed considerably higher levels of both antioxidants. Especially SOD was higher in intertidal compared to subtidal Antarctic *N. concinna*. The subtidal *N. concinna* had antioxidant levels similar to the South American limpet populations in our study. SOD activities twice as high in Antarctic intertidal *N. concinna* than in South American intertidal *N. magellanica* may represent an adaptation to the harsh environment in the Antarctic high intertidal, where extremely low temperatures, thermal fluctuation and fresh water run-off may call for special metabolic adaptations (see Weihe and Abele 2008).

An oxidative stress response in limpet gills from PM was not detectable throughout the whole experiment. Both SOD and CAT activities in gill tissues of air-exposed animals remained stable. Either it is not necessary to increase SOD levels as respiration is controlled on low levels in stress exposed limpets, or available energy is invested into the HSR under conditions of severe warming, rather than into antioxidant defense. It has been reported that the intensive synthesis of Hsps during severe thermal stress can block synthesis of other non-Hsp stress proteins (Lindquist 1980, 1981; Storti et al. 1980).

By contrast, both subpopulations of PA showed elevated SOD activities after 2 h and the subtidal *N. deaurata* featured their highest SOD activity of all groups at 12 h of air exposure. These patterns of up- and down-regulation of antioxidant enzyme activities can be seen even in the control treatments where animals were kept submerged for the whole experimental period. Strikingly, SOD activity levels seem to correlate with the tidal rhythm in the field, with high SOD activity during low tide and vice versa (Fig. 7). Coupling of gene expression to tidal rhythms could be shown in a large-scale microarray study with the intertidal mussel *Mytilus californianus* where distinct sets of genes link to different tidal periods (Gracey et al. 2008). As submerged and immersed limpets from the Punta Arenas region showed the same response, there might even be an internal trigger, which regulates SOD activity to match the tidal cycle. This mechanism seems superior even for *N. deaurata*, which do not regularly fall dry, but presumably feature higher metabolic activity during low tide periods, when animals are exposed to warmer surface waters. In colder environments, investing into antioxidant defense seems to be important for survival in the intertidal and in the shallow subtidal to minimize the risk of oxidative damage during emersion.

Conclusions

Our study provides new insight into the biogeography, and locally into the time resolution pattern, of the anti-stress response in tidally emerged limpets in Patagonia. The stress response includes the heat-shock protein gene expression and enzymatic antioxidant defense. Patagonian *Nacella* have developed markedly different physiological strategies to survive thermal and air exposure stress upon emersion depending on their climatic and shore level positioning and adaptation. In the Northern Patagonian region at Puerto Montt, we are dealing with a population that, once air exposed on the beach under experimental conditions, is stressed beyond levels normally experienced in their deep intertidal environment. These animals exhibit a pronounced HSR and apparently the heat stress is so high that the animals are unable to increase antioxidant activities for protection. A failure of the antioxidant response has been shown to occur in animals stressed beyond tolerance limits (Abele 2011 in press). In Punta Arenas, thermal stress is very limited and only the subtidal animals show a

pronounced HSR when experimentally exposed to air and to warming. Here, the dynamics of antioxidant defense system correlate to the tidal rhythm, with higher activities during times of tidal air exposure. Apparently, antioxidants are important anti-stress proteins that prepare limpets in their biogeographical optimum range for short periods of regularly occurring tidal emersion. It follows that fluctuations of antioxidants with tidal cycles, together with a mild HSR during emersion indicate optimal adaptation in these intertidal mollusks. Contrary, an extreme HSR and no antioxidant response at all seem to indicate extreme, presumably eventually lethal stress condition at the border of the thermal tolerance range at the northern (warm) edge of geographic distribution.

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Chapter 3

Manuscripts

Chapter 3.1

Was the Antarctic Circumpolar Current always a strict barrier to gene flow? - a molecular study on the Isolation of the Antarctic Ocean

Kevin Pöhlmann, Christoph Held

Manuscript

Abstract

The opening of the Drake Passage marked the beginning of a disintegration of a formerly contiguous faunal assemblage into two separate entities. The isolating effect that the Drake Passage and the creation of the Antarctic Circumpolar Current (ACC) flowing through it about 35 Ma had is largely considered to be the ultimate reason that has caused the high degree of endemic species on the Antarctic shelf. A molecular phylogenetic tree based on mitochondrial and nuclear gene sequences (16S, COI, 18S) of South American and the Antarctic members of the shallow-water limpet *Nacella* confirms the existence of genetically isolated clades on either side of the potential barrier. However, the calculated age of the divergence between these two clades is significantly younger than the age of the forming of the ACC (COI: 6.52 Ma, CI 0.61 - 14.76; even younger estimates in other genes). Extensive genetic exchange must have taken place well into a period of time during which the Drake Passage and the ACC had already been fully established. However, the molecular data also demonstrate that this exchange eventually disappeared in the late Miocene with no exchange happening today. The final divergence into the two clades today occupying Patagonia and the Antarctic Peninsula took place in the Miocene when the strong cooling of the Antarctic Continent around 10 Ma ago increased the selection for physiological and genetic adaptation and specialization on both sides of the Drake Passage.

This unexpected finding forces us to reject the hypothesis that the mere physical presence of the Drake Passage and the ACC sufficed to erect an effective barrier to faunal exchange between Antarctica and South America. Whatever isolates the faunal communities today is more complex than simple physical obstacles such as geographical distance or ocean currents. Our findings are particularly relevant now as the climate change is quickening its pace in Antarctica. It is not going to alter the physical isolation of the Southern Ocean but may change the parameters that still protect the uniqueness of the Southern Ocean fauna very soon.

Keywords Antarctic Circumpolar Current · Allopatric speciation · Geographical barriers · *Nacella*

Introduction

The Antarctic Ocean is one of our planet‘s unique ecosystems with a huge biodiversity of benthic species and high levels of endemism (e.g. Munilla 2001; Clarke and Johnson 2003; Clarke et al. 2005). The explanation for these high endemic rates that do not only encompass the species level but also whole genera and families lies in the evolutionary history of the ecosystem, which is considered to be isolated for more than 20 million years (Clarke and Crame 1992; Arntz et al. 1994; 1997; Clarke and Johnston 2003). Isolation plays a central role in the creation and increase of biodiversity. The process that describes the evolution of new species is called allopatric speciation, where a formerly continuous entity gets separated due to any form of physical barriers and the disjointed populations evolve independently and become new species over the course of time. In the marine realm, however, only a few strict barriers to gene flow are known and many marine species have pelagic larvae with the capability to disperse over long distances, resulting in little genetic differentiation between populations over large geographic scales (Palumbi 1994). The Antarctic Ocean has long been considered as one outstanding example of allopatric speciation in shallow water benthic organisms. Large geographic distances to circumjacent continents and the presence of the Antarctic Circumpolar Current (ACC), representing a biogeographical barrier to biological exchange (Crame 1999; Clarke et al. 2005; Thatje et al. 2005), are widely assumed to be the major reasons for the isolation of Antarctica that led to speciation in allopatry and the high degree of endemism on the Antarctic shelf.

It is commonly believed that the ACC came into existence around the Eocene/Oligocene (E/O) boundary (35 Ma) and triggered the glaciation of the Antarctic continent by decreasing heat transport from temperate regions north of the Southern ocean (Kennett 1977). However, age estimations of the onset of the ACC range from 23 million years (Ma) to around 35 Ma ago (e.g. Barker and Burrel 1977; Lawver and Gahagan 2003; Exon et al. 2004; Livermore et al. 2004, Pfuhl and McCave 2005). The high discrepancy of onset assumptions spanning 12 Ma is a consequence of different applied methods and models and mainly caused by persisting uncertainties in the proper dating of the opening of the Drake Passage that was necessary for the formation of a circumantarctic current system. However, the newest and most comprehensive models developed by Livermore and coworkers (2007) conclude that initial Drake Passage

opening occurred ~50 Ma ago, transformed to a deep pathway at around 34 - 30 Ma allowing for a flow-through of deep currents and fronts and resulted in an ACC comparable to what it is now at 24 - 23 Ma. Controversially, recent molecular studies regarding the biogeography and phylogeny of a variety of different taxa proposed divergence time estimates of South American and Antarctic species to be considerably younger than the proposed dates for the onset of the ACC, raising doubts about the isolating function of the ACC since its existence (Medlin et al. 1994; Patarnello et al. 1996; Bargelloni et al. 2000; Page and Linse 2002; Hunter and Halanych 2008; Thornhill et al. 2008; Wilson et al. 2009; Göbbeler and Klussmann-Kolb 2010). Unfortunately, in nearly all of the above mentioned studies the divergence time estimates were calculated with relatively weak molecular clock calibrations with rates taken from distantly related species. Molecular clocks calculated for related species can have a severe impact on the estimated divergence times because mutation rates are known to vary widely among lineages (Thomas et al. 2006; Bromham et al. 1996). The underlying reasons for those deviations are still not fully understood. It is thought to be mainly influenced by organismal generation times (Laird et al. 1969) and metabolic rates (Martin and Palumbi 1993), but that is still highly debated (Lanfear et al. 2007). However, applying an inappropriate clock can lead to significant under- or overestimations of divergence times of species or groups of interest.

Furthermore, and even more problematic, almost all of the published divergence times of Antarctic and South American relatives cited above consist only of one fixed date as a consequence of rather simple deductions from branch lengths and fixed mutation rates. Confidence intervals are not given in most of the studies, and if, they are a mere product of the uncertainty and variance of the applied calibration points but not of the stochastic variation of the molecular clock itself (Poisson process, Hillis et al. 1996). True confidence intervals that reflect the stochastic effect of the applied clock and describe the variance of the molecular clock estimate are indispensable when we want to accept or reject hypotheses. It is not possible to speak reliably of a discordance of species divergence times and geological events without knowing the confidence intervals of those estimated divergence times. Here, for the first time, we present a profound statistical framework with state-of-the-art bayesian phylogenetic reconstruction methods to exclusively test the hypothesis that the large geographic

distances and the ACC were the ultimate reasons for the isolation of the Antarctic Ocean. The taxon investigated in this study were patellogastropods from the genus *Nacella*: the South American intertidal *N. magellanica* and subtidal *N. deaurata, as well as* the Antarctic *N. concinna,* which inhabits both inter- and subtidal coastal areas along the Antarctic Peninsula. We analyzed three different genes (COI, 16S and 18S) with uncorrelated relaxed molecular clocks where mutation rates are allowed to vary between the different branches of any given tree and also along each lineage (Drummond et al. 2006; Drummond and Rambaut 2007). Thus, we avoid the assumption of constant rates of evolution that can lead to miscalculations of divergence times (Yoder and Yang 2000).

Material and Methods

Sampling sites and preservation

Samples of *N. magellanica* and *N. deaurata* were taken from several locations along the distribution area from Northern Patagonia until Ushuaia (Fig 1). On the Falkland Islands samples of *N. deaurata* and *N. mytilina* were collected. Samples of *N. concinna* were taken from Jubany Station on King George Island and Rothera Station on Adelaide Island. Subtidal animals had to be taken by SCUBA diving, all other animals were collected as a whole during low tide. All samples were immediately preserved in 96% ethanol.

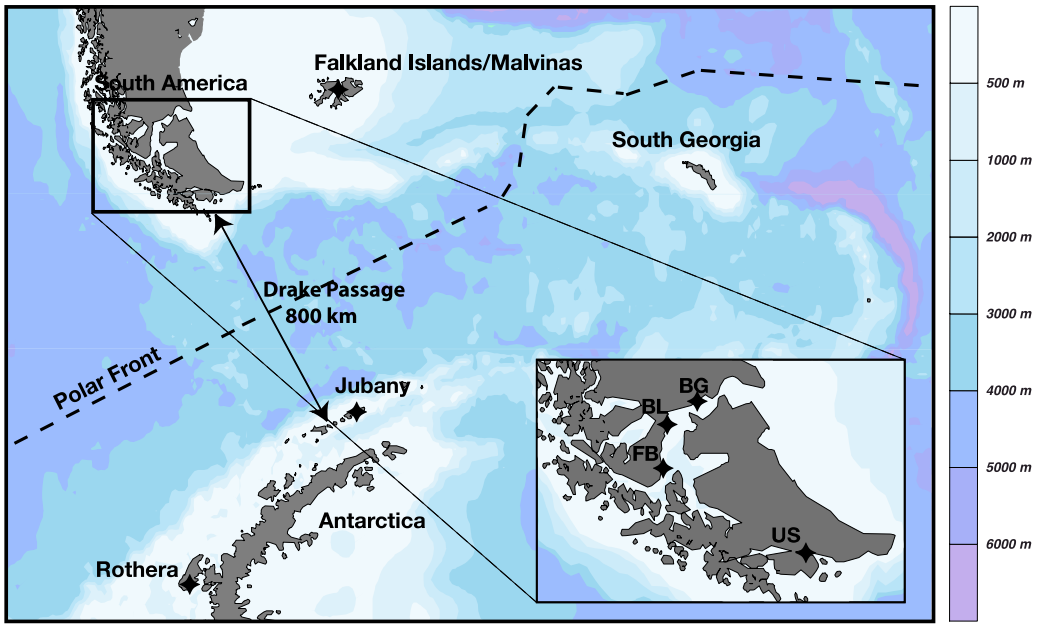

Fig. 1: Map with the sampling stations in South America and the Antarctic Peninsula. BG, Bahia Gregorio; BL, Bahia Laredo; FB, Fuerte Bulnes; US, Ushuaia

DNA extraction, gene amplification and sequencing

DNA was extracted with the QIAamp DNA Mini Kit (Qiagen), according to the manufacturers protocol for standard tissues. A fragment of the 16S gene was amplified using the universal primers 16Sar and 16Sbr (Simon et al. 1994) in a total volume of 25 µl, containing approx. 10-300 ng genomic DNA, 1x HotMaster reaction buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 0.03 U/µl Hotmaster Taq (Eppendorf). Following PCR conditions were applied: 2 min 94°C, 38 cycles of 20 s at 94°C, 20 s at 52°C, 50 s at 65°C and a final extension of 7 min at 65°C. Amplification of a COI gene fragment was

carried out with the universal primers LCO1490 and HCO2198 (Folmer 1994). PCR reaction mix and program specifications were like those described for 16S. Only the annealing temperature was set to 45°C. Successful amplification of a fragment of the nuclear 18S gene was conducted using the primers AGM-18F and AGM-18R published by Harasewych and McArthur (2000). PCR reaction mix and conditions were as described for 16S with annealing temperature changed to 62°C and cycles reduced to 30. All PCR reactions were performed on an epgradient thermocycler (Eppendorf). PCR products were checked on 2% agarose gels and purified with the enzyme mix ExoSAPit (Fermentas) to degrade remaining primers and inactivate remaining dNTPs. Cycle sequencing was carried out using the BigDye Terminator Kit 3.1 (Applied Biosystems) as described in the manufacturer´s instructions. The fragments were purified with the DyeEx Kit (Qiagen) and subsequently sequenced on an ABI 3130xl sequencer.

Phylogeny and molecular dating

Phylogenetic approaches and molecular dating were carried out using the BEAST v1.5 software bundle (Drummond and Rambaut 2007). For all three genes the HKY Model plus invariable sites was applied as suggested by JMODELTEST v0.1.1 (Posada 2008). For all three genes apart of the strict clock (CLOC), also the uncorrelated exponential relaxed clock (UCED) and the uncorrelated lognormal relaxed clock (UCLN) were applied. The former relaxed clock model allows changes of mutation rates among branches only at the nodes with size of change independent of the branch length, whereas the latter allows for mutation rate changes along the branches depending on the branch length. In both cases mutation rates of new branches are uncorrelated to the mutation rates of their ancestral lineage. Tree model for all three genes was the birth death speciation model (Gernhard 2008). Three independent runs for 16S and 18S and six for COI were performed, each comprising of 10 million inferences with sampling frequencies of 1000 and a burn-in of 10%. Pairwise bayes factors were calculated in order to compare the single runs as implemented in TRACER v1.5 (Rambaut and Drummond 2007). Low bayes factor values indicate high compatibility of the single inferences and high convergence of estimates (Drummond et al. 2006). Sequences of the limpet *Cellana*, the closest relative to *Nacella* and the second genus in the family Nacellidae, from the Indo Pacific, Australia and New Zealand served as outgroup. As a

calibration point the divergence time of the *Nacella* and *Cellana* clades was set to 35 Ma or 50 Ma. The former is the proposed date when land connections between Antarctica and Australia and South America, respectively, finally broke apart with the Opening of the Drake Passage and the forming of a deep water gateway between Australia and Antarctica (Sanmartín and Ronquist 2004). However, Woodburne and Case (1996) concluded that faunal exchange between Australia and Antarctica was restricted already 50 Ma ago with the establishment of a shallow marine seaway between Antarctica and Australia. Although it is rather unlikely that this shallow water gap was an effective barrier to migration in animals with pelagic larvae, we calculated divergence times between South American and Antarctic clades according to this more conservative assumption as well. Tree topologies were assessed by first creating a consensus tree out of all sampled trees using TREEANNOTATOR v1.5.3 implemented in the BEAST package and the consensus tree was visualized in FIGTREE v1.2.2 (Rambaut 2009). Additionally, we calculated divergence times based on published mutation rates for closely related taxa for the COI and the 16S dataset.

Results

Phylogenetic reconstructions based on the analyzed COI, 16S and 18S gene fragments revealed a clear divergence between South American and Antarctic limpets of the genus *Nacella* under all applied clock models (see Fig. 2 for COI) with high statistical support (posterior probabilities of 1 for all genes under all clock models) and all geographic groups showed reciprocal monophyly. A consistent genetic separation was not detectable among the two South American species *N. magellanica* and *N. deaurata*. These two groups did not show reciprocal monophyly under all three genes analyzed.

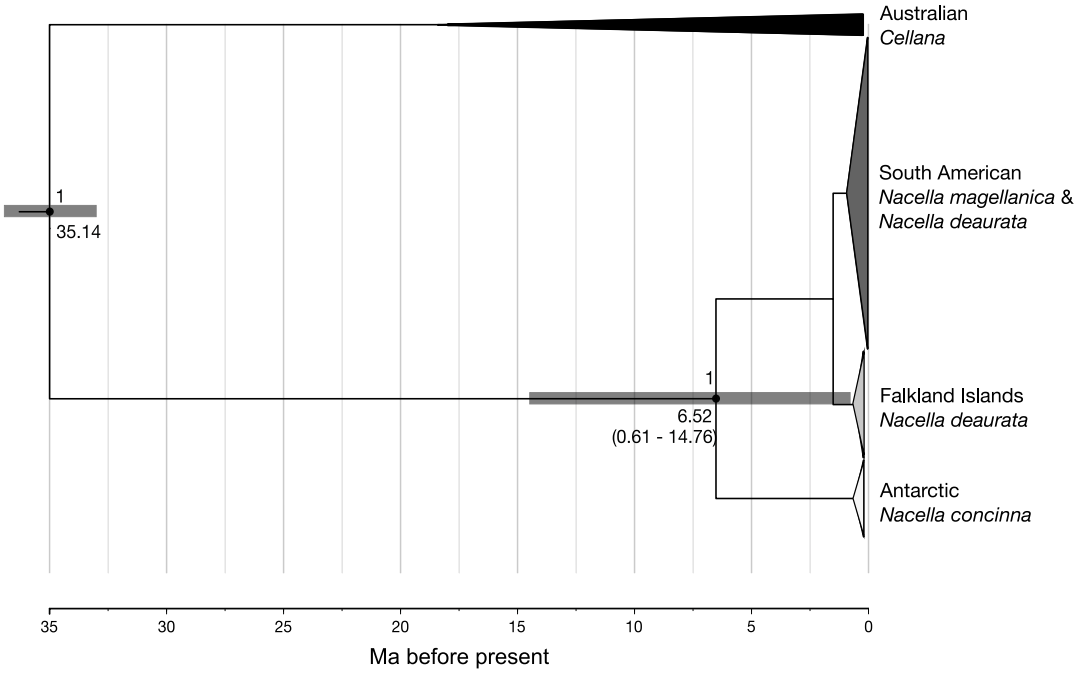

Fig 2: Phylogenetic trees created with Beast based on the COI fragment and the UCED relaxed clock model. A divergence time prior of 35 Ma between the outgroup *Cellana* and the ingroup *Nacella* was used. Dark grey, *N. magellanica* and *N.deaurata* from South America; light grey, *N. deaurata* from the Falklands; white, *N. concinna* from Antarctica

Although the applied strict clock model always produced highest posterior probability values for all three genes investigated, under both relaxed clocks, the uncorrelated lognormal (UCLN) and the uncorrelated exponential relaxed clock (UCED), the substitution rates were consistently higher for the South American lineage than for the Antarctic lineage (UCLN: 3.4%/Ma/site and 1.1%/Ma/site, respectively; UCED: 3.5%/ Ma/site and 1.7%/Ma/site, respectively; Tab. 1). For this reason, we applied both strict and relaxed clocks to completely rule out the possibility of rejecting the null hypothesis as a mere consequence of wrong model assumptions.

Tab. 1: Comparison of the strict clock (CLOC) and the two relaxed clock models, the uncorrelated exponential (UCED) and the uncorrelated lognormal (UCLN) model. Dates and rates were calculated with a divergence prior of 35 Ma between *Nacella* and *Cellana*. Divergence times with a 50 Ma prior were calculated additionally under the UCED clock model. PP, posterior probability; r_a, overall mutation rate; r_{SA}, mutation rate of the South American clade; r_{Ant}, mutation rate of the Antarctic clade

Gene	Clock Mode	PP (35 Ma)	rate heterogeneity (35 Ma)	Divergence SA-Ant (35 M	Divergence SA-Ant (50 M
COI	CLOC	-1859	$r_a = 0.016$	5.44 (2.96 - 8.01)	-
	UCED	-1903	$r_a = 0.015$, $r_{SA} = 0.035$, $r_{Ant} = 0.017$	6.702 (0.74 - 14.23)	10.18 (1.403 - 21.37)
	UCLN	-1931	$r_a = 0.015$, $r_{SA} = 0.034$, $r_{Ant} = 0.011$	6.56 (0.88 - 14.86)	-
18S	CLOC	-1278	$r_a = 0.0046$	3.85 (2.02 - 5.89)	-
	UCED	-1303	$= 0.0041$, $r_{SA} = 0.0046$, $r_{Ant} = 0.005$	5.32 (0.67 - 12.02)	7.29 (1.08 - 16.2)
16S	CLOC	-1162	$r_a = 0.0034$	2.02 (0.78 - 3.48)	-
	UCED	-1414	$_a = 0.0049$, $r_{SA} = 0.0055$, $r_{Ant} = 0.00$	2.49 (0.31 - 5.89)	3.37 (0.38 - 8.04

We calculated the divergence times of South American and Antarctic Nacellids using two different strategies. First, we calculated a limpet-specific rate based on the divergence of *Cellana* species today inhabiting Australia‘s coast and being the closest relative to *Nacella* including our *Nacella* species that today are restricted to Antarctica and South America. No overlaps of the distribution areas of both genera exist. The underlying assumption for this approach is that the well dated deep water opening of the Tasman seagate 35 Ma ago (Stickley et al. 2004), that separated Australia and Antarctica, led to the divergence of *Nacella* and *Cellana*. Due to the fact that the divergence time of two species is a function of the phylogenetic distance and the mutation rate, we could use the limpet-specific rate to calculate the divergence time of South American and Antarctic congeners of the genus *Nacella*. Woodburne and Case (1996) proposed that the divergence between Antarctic and Australian land mammal species have already occurred around 50 Ma ago with the formation of a shallow water marine seaway between both continents. Although it is rather unlikely that such shallow water barrier that was effective for terrestrial organisms had the same effect to marine shallow-water animals with pelagic larval stages, we also applied these 50 Ma as a clock calibration point to test the hypothesis.

When applying 35 Ma as the calibration point for our molecular clock the oldest divergence time of South American and Antarctic Nacellids was calculated to have taken place 6.7 Ma ago (for COI and under the UCED relaxed clock, see Tab. 1 and Fig. 3). Confidence intervals reached a maximum of 14.2 Ma. Applying 50 Ma as clock calibration point, the oldest time of divergence between *Nacella* from both sides of the Drake Passage calculated with our datasets was 10.2 Ma ago with confidence intervals

stretching to a maximum of 21.4 Ma (COI and UCED). The confidence intervals do of course also extend to younger estimates but since these are of interest in our approach,they are not mentioned here. Under relaxed and strict clocks and under both applied molecular clock calibration prior assumptions (35 Ma and 50 Ma) our calculated divergence times for South American and Antarctic limpets of the genus *Nacella* are far younger than the youngest dates published for the onset of the ACC being 23 Ma ago (Barker and Burrell 1977, Pfuhl and McCave 2005).

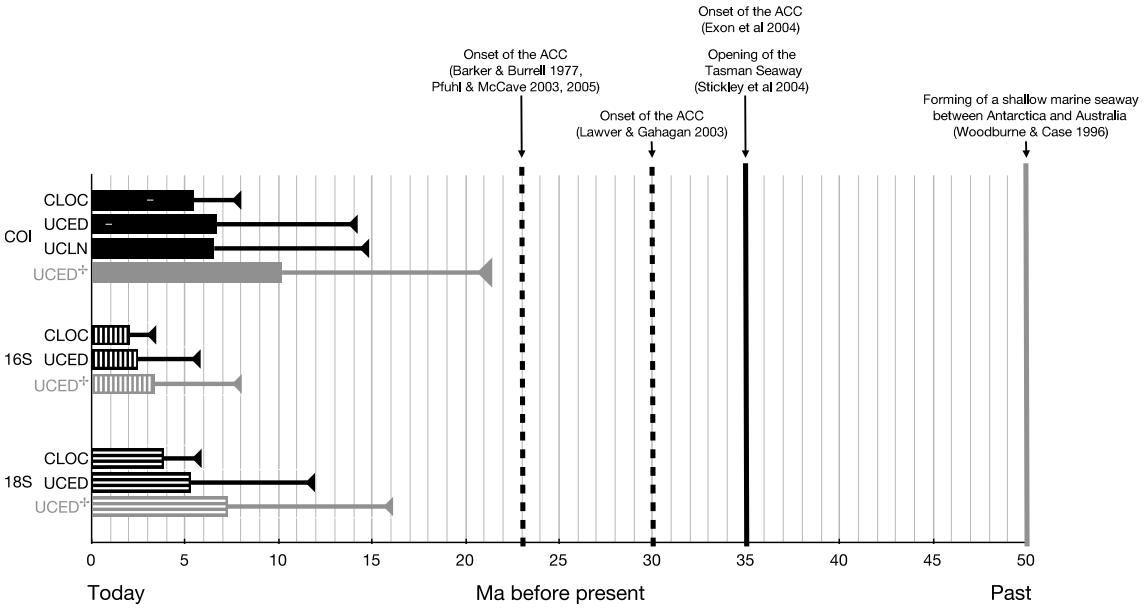

Fig. 3: Estimated divergence times between South American and Antarctic limpets of the genus *Nacella*. The three different genes COI, 16S and 18S were investigated. Three different clock models were used, a strict clock (CLOC), a relaxed exponential clock (UCED) and a relaxed lognormal clock (UCLN). Divergence time estimates indicated as black bars were calculated using a divergence prior of 35 Ma between *Nacella* and *Cellana*. Grey bars were calculated using a 50 Ma prior as divergence time between both groups

However, we are aware of the fact that our two chosen priors of 35 Ma and 50 Ma as divergence times of the genera *Nacella* and *Cellana* are not ultimately the only possibilities because processes not considered in our prior design might have caused a divergence of both genera before the Antarctic and the Australian continents broke apart. Therefore, in a second approach, we applied a range of different plausible mutation rates published for closely related taxa and also widely used mutation rates found in the literature (Tab. 2). All estimations were made under the the UCED relaxed clock model. With the slowest applied rate of 0.7%/Ma/site for COI, published by Marko (2002), we calculated a divergence time estimate for South American and Antarctic limpets to be 11.91 Ma ago. The slowest rate for 16S that we applied was 0.084%/Ma/site (Koufopanou et al. 1999) and resulted in divergence time estimates of

10.45 Ma for South American and Antarctic *Nacella* species. The confidence intervals for COI and 16S extended to 24.09 Ma and 22.45 Ma, respectively. Even with the slowest available mutation rates taken from the literature for related species we still don‘t find overlaps of the divergence time of South American and Antarctic *Nacellids* and the proposed onset of the ACC. We would have to assume extremely low rates of evolution, never calculated in any phylogenetic work so far, to accept our null hypothesis that the onset of the ACC initiated the divergence of Antarctic and South American *Nacella*.

Tab. 2: Divergence times estimates of South American (Sa) and Antarctic (Ant) limpets from the genus *Nacella* and of *Nacella* (Na) and *Cellana* (Ce) calculated with published mutation rates for COI and 16S. All runs were performed under the relaxed exponential clock (UCED) model. Mutations rate per site per million years for COI: 0.7% (Arcidae, Marko 2002), 1.2% (Arcidae, Marko 2002), 2.3% (*Alpheus*, Knowlton et al. 1993; *Tegula,* Hellberg and Vacquier 1999). Mutation rates for 16S: 0.084% (*Littorina*, Koufopanou et al. 1999), 0.14% (Patellids, Koufopanou et al. 1999), 0.23% (*Cellana/Nacella*, Goldstien et al. 2006)

	COI			16S		
assumed mutation rate	0.7 %	1.2 %	2.3 %	0.084 %	0.14 %	0.23 %
Divergence time SA-Ant (M	11.91	5.92	3.44	10.45	6.31	3.87
CI (Ma)	3.69 - 24.09	1.59 - 12.96	0.96 - 7.68	2.69 - 22.45	1.39 - 12.28	1.05 - 8.53
Divergence time Na-Ce (Ma	56.48	30.39	13.96	134.78	73.83	46.27
CI (Ma)	24.41 - 95.34	12.32 - 55.7	6.43 - 23.09	52.91 - 248. 21	30.21 - 137.64	18.47 - 86.17

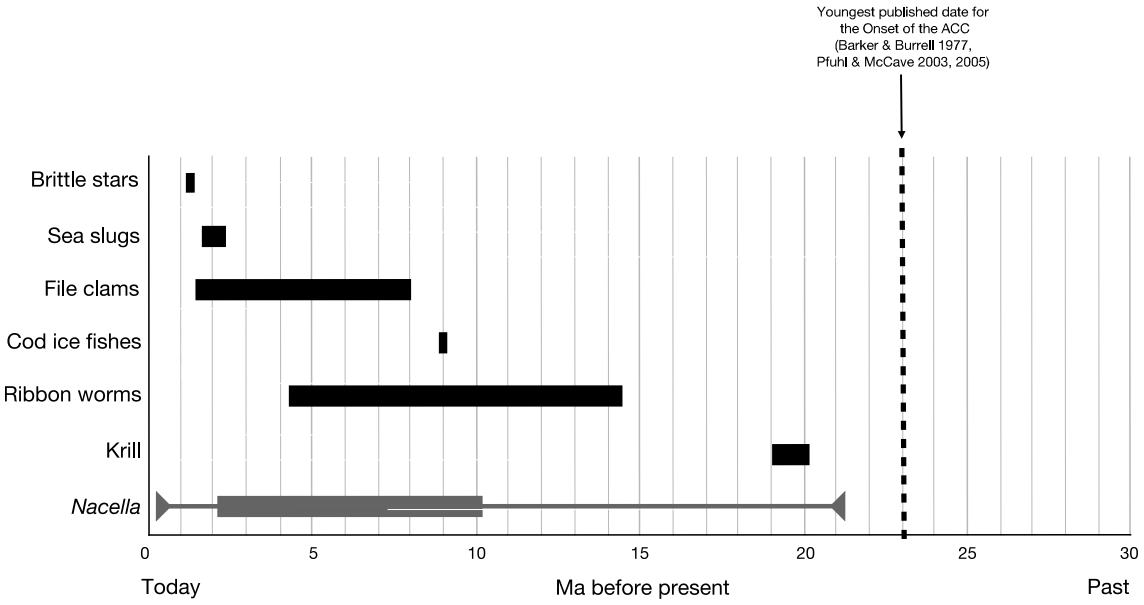

Fig. 4: Published divergence times of various taxa from both sides of the Drake Passage. The first three, labelled with an a, represent taxa without larval stages, including the brooding brittle star *Astrotoma agassizii* (Hunter and Halanych 2008), the direct developing sea slug *Doris kerguelenensis* (Wilson et al. 2009) and the brooding file clams from the genus *Limatula* (Page and Linse 2002). The lower four taxa, labelled with a b, represent species with a planktonic larval life stage, encompassing Cod ice fishes (Bargelloni et al. 2000), the ribbon worm *Parbolasia corrugatus* (Thornhill et al. 2008), Krill (Patarnello et al. 1996) and *Nacella* from the present study

Discussion

Our results presented here, support the assumption that the Antarctic Ocean is presently isolated from South America without any gene flow across the Drake Passage. The investigated South American and Antarctic limpet species show a striking degree of genetic divergence and reciprocal monophyly of all lineages in all investigated genes with no shared haplotypes (Fig. 2). Our calculated divergence time estimates, however, conflict with the common hypothesis of vicariant speciation in the Southern Ocean caused by the opening of the Drake Passage and the subsequent onset of the ACC 23 - 35 Ma ago. Regardless which method we applied, our estimated divergence times were never older than 10 Ma. Under the assumption that the final breakup of Antarctica and Australia initiated the disintegration of Australian *Cellana* and South American and Antarctic *Nacella* 35 Ma ago (Kennett 1977; Stickley et al. 2004), our calculations of divergence times of South American and Antarctic Nacellids resulted in ages between 2.02 - 6.7 Ma ago with confidence intervals extending to 14.9 Ma, depending on the investigated gene and the applied clock model. Under the assumption that the forming of a shallow water gap 50 Ma ago between Antarctica and Australia was already sufficient to prevent gene flow between both continents (as proposed for land mammals by Woodburne and Case 1996), our estimated divergence times of South American and Antarctic limpets were dated to be 3.4 - 10.2 Ma ago with a maximum confidence interval of 21.4 Ma. Similar results were obtained by applying published mutation rates to our datasets. The range of divergence times between *Nacella* from South America and Antarctica was then 3.4 - 11.91 Ma. All these calculated dates are much younger than the youngest published date of the forming of the ACC at 23 Ma (Barker and Burrell 1977; Pfuhl and McCave 2005) and strikingly younger than the ACC onset date of >30 Ma (Fig.3) which is today most accepted (Livermore et al. 2007; Lagabrielle et al. 2009).

On the basis of these results we propose that the mere presence of the geographical barriers have not exclusively been the major reasons leading to the isolation of the Antarctic Ocean. Gene flow over a deep water passage and an already established ACC must have been possible much after the occurrence of both geographical barriers.

Our findings are in line with other recent publications that found surprisingly young estimates for divergence times of South American and Antarctic species (Medlin et al.

1994; Patarnello et al. 1996; Bargelloni et al. 2000; Page and Linse 2002; Hunter and Halanych 2008; Thornhill et al. 2008; Wilson et al. 2009; Göbbeler and Klussmann-Kolb 2010). This congruence of divergence in a variety of investigated taxa rules out the possibility that *Nacella* represents one exceptional genus that by chance surmounted the Drake Passage after the forming of the ACC. Additionally, it also excludes the possibility that younger divergence times are a cause of one particular life cycle. Divergence time estimates younger than 23 Ma were found not only in species with pelagic larvae and consequently with the potential of long distance dispersal like *Nacella* or the ribbon worm *Parbolasia corrugatus* (Thornhill et al. 2008) but also in brooders like South American and Antarctic bivalves from the genus *Limatula* (Page and Linse 2002) or the brittle star *Astrotoma agassizii* (Hunter and Halanych 2008) and the direct developing sea slug *Doris kerguelenensis* (Wilson et al. 2009, see Fig. 4). Furthermore, also the pelagic Euphausiacea *Euphausia superba* from south of the Polar Front and *Euphausia vallentini* from north of it, as well as the benthopelagic and moderately active Notothenioid fishes *Lepidonotothen nudifrons* from Antarctica and *Patagonotothen tessellata* from South America were calculated to have diverged much later than the forming of the ACC (Patarnello et al. 1996; Bargelloni et al. 2000).

If it was not a vicariant event caused by the opening of the Drake Passage and the forming of the ACC that had caused speciation of South American and Antarctic species, the question still persist: what was the reason then? Taking a closer look into the historical climate oscillations of Antarctica reveals an initial cooling and partial glaciation of Antarctica at the Eocene/Oligocene boundary (~35 Ma), followed by a warm phase from the late Oligocene to middle Miocene (~ 26 to 15 Ma; Miller et al. 1991, Wright et al. 1992) with maximum temperatures in the late middle Miocene climatic optimum at 17 to 15 Ma (Zachos et al. 2001, 2008). This period was followed by a gradual cooling and reestablishment of the major Antarctic ice-sheet by 10 Ma and the glaciation of the Antarctic Peninsula (Flower and Kennett 1995). A profound explanation for the warming phase between 26 and 15 Ma could yet not be made but newest publications link this warming anomaly to a constriction of the Drake Passage having started at 29 Ma as a cause of an interplay of several major tectonic events (see Lagabrielle 2009 for further details). As a consequence, the narrowing of the Drake Passage reduced water flow through the passage and led to a weakening of the ACC

(Lagabrielle 2009). A constriction of the Drake Passage plus a reduced strength of the ACC could have promoted migration and gene flow between South American and Antarctic populations. The widening of the Drake Passage and the strong cooling of water temperatures after 14 Ma could have driven evolution of cold adaptation in Antarctica and prevented gene flow between moderate sub polar and cold Antarctic regions through a combination of re-emerging geographical barriers and disruptive selection. However, the insights provided by Lagabrielle and co-workers demonstrate that the ACC might not have been a constant current ever since its forming but fluctuated in its strength.

According to Livermore et al. (2007) the ACC did not only fluctuate in its strength but also in its main flow routes through the Drake Passage over the course of time. They state that there might have been times where the ACC‘s main jets flowed south of South Georgia instead of north of it as today. It is therefore imaginable that meandering fronts and jets allowed step wise and delayed gene flow between South America and Antarctica until 16 - 17 Ma, when the ACC current system finally settled to its present course (Livermore et al. 2007). Hoffman et al. (2010a) examined the population genetic structure of the Antarctic limpet *N. concinna* along the Antarctic Peninsula and the islands of the Scotia Arc. They found little to no genetic differentiation among the different populations from the Peninsula and Signy Island indicating strong gene flow among those populations but a strong differentiation compared to the population on South Georgia. This could be a consequence of the large geographic distance as suggested by the authors. It could furthermore corroborate the hypothesis of a meandering ACC that once flowed south of South Georgia and thereby restricted gene flow between populations on this island and those from the other islands of the Scotia Arc and the Antarctic Peninsula leading to a certain degree of genetic differentiation that is still detectable today.

Given the large uncertainties especially in dating the existence of a deep water passage between South America and Antarctica that was necessary for building up the ACC (see Barker et al. 2007), we want to highlight one other explanation. As already listed above, divergence times between South American and Antarctic congeners are younger than 14 Ma in a variety of taxa, spanning immobile and mobile species, broadcast spawners and also brooding species. One more answer might therefore be taken into consideration,

precisely that there was no ACC before the mid-Miocene. The common knowledge that the ACC started at the E/O boundary and was the reason for the glaciation of the Antarctic continent by decreasing heat transport from temperate regions north of the Southern Ocean (Kennett 1977) has been doubted by several authors. They conclude that a fall in atmospheric greenhouse gas concentrations had caused the global cooling and the Antarctic glaciation (De Conto and Pollard 2003; Coxhall et al. 2005; Tripati et al. 2005). According to these results the glaciation of Antarctica is not necessarily linked to the onset of the ACC allowing for much younger ACC formation dates than the widely accepted date for the glaciation of the Antarctic at the E/O boundary. Therefore, our estimated divergence of South American and Antarctic limpets of the genus *Nacella* could still reflect the isolating effect of the ACC when this current system is indeed much younger than previously thought.

In summary, we suggest two scenarios that might have driven the evolution of the Antarctic fauna which is so unique today:

- fluctuations in the strength of the ACC due to periods of constriction during the Oligocene to mid-Miocene (29 - 15 Ma) fluctuations in main flow routes enabling stepwise and delayed gene flow between South America and Antarctica through the islands of the Scotia Arc. An effective isolation of the Antarctic ocean did not start before the second major cooling event of Antarctica at 15 Ma that led to the evolution of cold adaptation and resulted in restricted gene flow between moderate sub polar and cold Antarctic regions through disruptive selection
- the ACC did not exist prior to the mid Miocene cooling and could therefore not act as an effective barrier to gene flow across the Drake Passage

In both scenarios the opening of the Drake Passage and the forming of the ACC play a crucial part. However, contrary to the common assumption that the mere presence of these geographical barriers led to the isolation of Antarctica the likely interplay of the proposed scenarios here make the evolution of Antarctic life far more complex than previously thought.

Chapter 3.2

Analyzing multiple neutral and selected genetic markers to overcome biases in population genetic studies: the recent evolutionary history of two South American shallow-water limpets of the Genus *Nacella*

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Manuscript

Abstract

The importance of natural selection and local adaptation is still not well understood in the marine realm. Population genetic studies mostly only apply neutral genetic markers that are not affected by natural selection. In the present thesis we used a gene fragment of a heat-shock protein, Hsp70, as a candidate locus to investigate adaptive divergence in two South American patellogastropods from the genus *Nacella*, the intertidal *N. magellanica* and the subtidal *N. deaurata*, that are different in morphology and vertical zonation. Additionally, we tested seven microsatellites and the mitochondrial gene COI to perform a baseline study on population structure and gene flow patterns with presumably neutral markers.

The results from the neutral marker approach suggest Southern South America to be a high gene flow environment with only little genetic differences among the single populations of both nominal species. Furthermore, no genetic divergence was found between the two species describing them as two morphotypes of one species with high phenotypic plasticity. Analysis of allelic distribution of the Hsp70 candidate gene showed a strong genetic divergence between the two morphotypes *N. magellanica* and *N. deaurata*. The results highlight that despite ongoing gene flow, local selection along a vertical stress gradient can drive adaptive divergence. Two major allele groups of Hsp70 were found, one dominant in the Pacific and the other in the Atlantic. The initial divergence in Hsp70 therefore likely happened during allopatric separation of Atlantic and Pacific populations during the Pleistocene glaciation. At secondary contact after the end of the Last Glacial Maximum (approx. 10,000 years before present) a partial reproductive barrier allowed gene flow in certain genomic regions as indicated by the absence of genetic divergence in microsatellites and COI. In other regions, presumably under selection as Hsp70, gene flow was restricted to ensure adaptation to the intertidal and subtidal, respectively.

The study suggests adaptive divergence between *N. magellanica* and *N. deaurata* and highlights that local adaptation is possible in the background of high gene flow in South American limpets. Investigations applying Hsp70 provide a useful means to study the role of local selection in marine invertebrates.

Keywords *Nacella* · Patagonia · Natural selection · Adaptive divergence · Gene flow

Introduction

Understanding the evolutionary processes that lead to the high biodiversity in the marine realm are a challenging task for evolutionary biologists. The absence of strict barriers to gene flow over large geographic scales, and many marine species exhibiting developmental stages with pelagic larvae allowing for dispersal over long distances, contradict the common belief that speciation needs some kind of geographical barriers that restrict gene flow (Mayr 1963). A growing body of marine case studies is, however, becoming available that challenges the paradigm of allopatric speciation. Instead, these studies demonstrate speciation in sympatry in the ocean (Rocha et al. 2005; Forslund and Kautsky 2009; Bongaerts et al. 2010; Crow et al. 2010; Johannesson et al. 2010; Bird et al. 2011). The emerging examples of sympatric speciation in the marine environment have furthermore challenged the assumption that natural selection and adaptive divergence can be neglected as drivers of population structure and speciation in marine systems owing to high gene flow overriding local effects of selection (Palumbi 1994; Conover et al. 2006). Recent molecular studies with genetic markers under selection encourage a re-evaluation of the common belief that adaptive divergence plays only a minor role in marine evolution. A study on the flounder *Platichthys flesus* by Hemmer-Hansen and co-workers (2007) showed strong population differentiation among geographically close populations with high gene flow when analyzing heat-shock protein genes under selection (the heat-shock cognate Hsc70). Larmuseau and colleagues (2010) showed adaptive divergence in rhodopsin genes among populations of the sand goby *Pomatoschistus minutus* inhabiting waters of different water turbidity, again on the background of high gene flow conditions. These are only two examples of an increasing body of literature describing the impact of adaptive divergence on scales that are much finer than previously believed, thus highlighting the potential of natural selection to vanquish even high gene flow (see Conover et al. 2006 and references therein).

In order to unravel the evolutionary history of a species or a group of closely related species a profound genetic and analytical framework is indispensable as evolutionary studies rest on mathematical reconstructions based on present day genetic information and are therefore highly dependent on the molecular markers of choice. Each of the many different genetic markers today available comes with certain advantages, but to

date not a single one exists that is free from any caveats and limitations (Avise 1989; Zhang and Hewitt 2003; Schlötterer 2004). Mitochondrial genes are susceptible to pseudogenes (Bensasson et al. 2001), forces like selective sweeps and hitchhiking (Ballard and Kreitman 1995; Hurst and Jiggins 2005), and biases stemming from their uniparental inheritance. Multilocus markers like RAPDs, AFLPs or ISSRs are fundamentally restricted by their dominant mode of inheritance (Müller and Wolfenbarger 1999) and microsatellite studies can be biased by homoplasious mutations (Estoup et al. 2002; Selkoe and Toonen 2006). Therefore, population genetic studies focusing on one marker system are prone to reconstruct the genealogy of the marker of choice but not the species genealogy (Avise 1989). Additionally, the vast majority of population genetic studies apply neutral markers which should per definition not be subject to selective forces and therefore allow only for speculations about the potential roles of selection and adaptive divergence for speciation (Hemmer-Hansen et al. 2007).

Here we present a comprehensive analytical and statistical framework for a population genetic study with multiple genetic markers. We employed one mtDNA marker (COI), seven fast evolving nuclear microsatellites (Pöhlmann and Held 2011) and a fragment of a heat-shock protein (Hsp) of the Hsp70 class highly inducible and, therefore, likely to be under selection (Pöhlmann et al. 2011). With the present study we are aiming at demonstrating how powerful combined genetic analyses can be to reveal the evolutionary histories and the processes that shape the population structure of species of interest.

We studied an abundant taxon inhabiting the Southern South American shallow waters of Chilean and Argentinian Patagonia and Tierra del Fuego, as well as on the Falklands/ Malvinas, the patellogastropod genus *Nacella.* The two most conspicuous species, the intertidal *N. magellanica* and the subtidal *N. deaurata* have been subject to several scientific studies attempting to disentangle their genetic status. Despite clear morphological distinctiveness in shell shape (Valdovinos and Rüth 2005) and profound differences in physiological adaptation (Malanga et al. 2004, Malanga et al. 2005, González et al. 2008, Pöhlmann et al. 2011), it is controversial whether the classification of the two different morphotypes truly reflects reproductively isolated species. Several genetic analyses using mitochondrial and nuclear genes failed to demonstrate reciprocal monophyly of specimens assigned to the two species on the

grounds of their morphology (de Aranzamendi et al. 2009; González-Wevar et al. 2010). Interestingly, fast evolving ISSR markers (Inter simple sequence repeats) revealed significant amounts of genetic differentiation among the species *N. magellanica* and *N. deaurata* (de Aranzamendi et al. 2009) and microsatellite amplification success and failure of a subset of the applied loci were consistent with the nominal species boundaries (Pöhlmann and Held 2011; but see Bailie et al. 2010).

Our superordinate aim of this study was to understand the prevalent forces that drove the evolution of Patagonian *Nacella* by addressing the following questions: Do we find evidence for genetic differentiation between both morphotypes when analyzing multiple independent genetic markers or are morphological and physiological differences merely caused by phenotypic plasticity? What role did the glacial periods which repeatedly affected Patagonian coasts during the Pleistocene (Clapperton et al. 1995; McCulloch et al. 2000; Hulton et al. 2002) play for the population structure of Patagonian limpets? Is there evidence that the steep vertical gradients in abiotic conditions such as temperature shifts, desiccation and osmotic stress characterizing intertidal habitat stress scenarios have promoted disruptive ecological selection between intertidal *N. magellanica* and subtidal *N. deaurata*? This could be demonstrated for the galician intertidal snail *Littorina saxatilis*, which is supposed to be in an incipient state of sympatric speciation (Quesada et al. 2007; Rolán-Alvarez 2006; Johannesson et al. 2010). Our strategy was to perform a baseline study with the neutral markers COI and seven microsatellites to assess population differentiation and gene flow between populations of *N. magellanica* and *N. deaurata* from all over the distribution area. In a second attempt, we investigated the allele distribution of a heat-shock protein gene from the Hsp70 family that is most likely under selection (see Hemmer-Hansen et al. 2007) to test whether selective forces drive local adaptation and speciation in the South American *Nacella* complex.

Material and Methods

Sampling

Samples of *Nacella magellanica* and *N. deaurata* were collected in Patagonia and Tierra del Fuego during an expedition from January till March 2009. Samples of *N. deaurata* and *N. mytilina* from the Falkland Islands were provided by Judith Brown (Paul Brickle) and colleagues at the Falkland Islands Fisheries Department. Individuals were collected by hand during low tide and directly preserved in 96% ethanol. Sampling stations and number of sampled specimen can be seen in figure 1 and table 1.

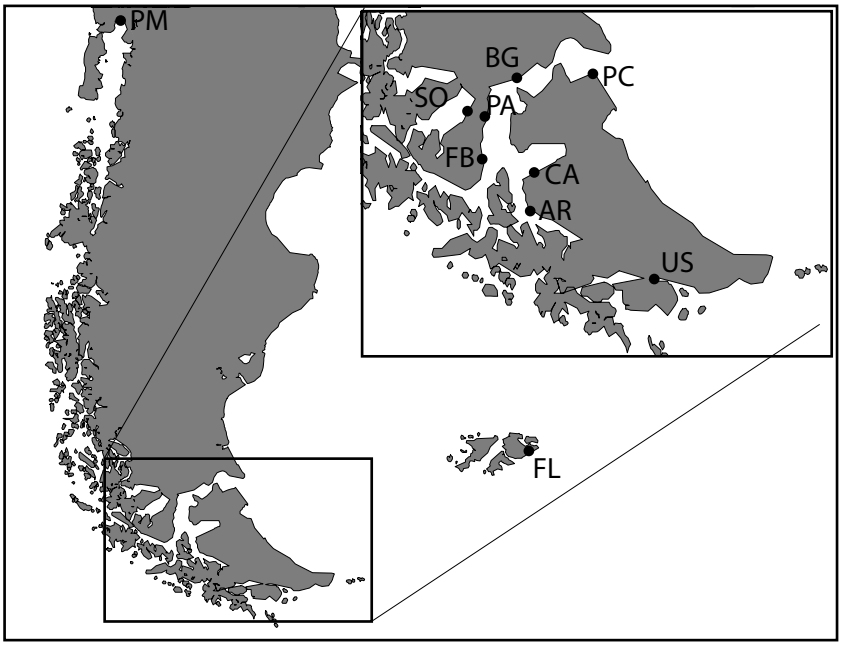

Fig. 1: Map with sampling sites. PM, Puerto Montt; SO, Seno Otway; FB, Fuerte Bulnes; AR, Puerto Arturo; CA, Camaron; PA, Punta Arenas; BG, Bahia Gregorio; PC, Punta Catalina; US, Ushuaia; FL, Falkland Islands/Malvinas

DNA extraction, PCR and sequencing

The QIAamp DNA Mini Kit (Qiagen) was used for DNA extractions of a small piece of foot tissue.

Amplification of a COI gene fragment Amplification of a COI gene fragment was carried out with the universal primers LCO1490 and HCO2198 (Folmer 1994) in a total volume of 25 µl, containing approx. 10-300 ng genomic DNA, 1x HotMaster reaction buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 0.03 U/µl Hotmaster Taq (Eppendorf). The following PCR conditions were applied: 2 min 94°C, 38 cycles of 20 sec at 94°C,

20 sec at 45°C, 50 sec at 65°C and a final extension of 7 min at 65°C. A 693bp fragment of the HspA gene was amplified using the forward primer HSP70A-1F AAGTTATGTGGCGTTCACAGAC and the degenerated reverse primer HSP70-1R GCNACAGCYTCRTCNGGRTT in total volumes of 25 µl, containing ~10 ng genomic DNA, 1x HotMaster reaction buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 0.03 U/µl Hotmaster Taq (Eppendorf) under the following conditions: 2 min 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 50°C, 2.5 min 65°C and a final extension of 5 min 65°C. All PCR reactions were performed on an Ep Gradient thermocycler (Eppendorf). Gel electrophoresis with 2% agarose gels was applied to check for successful PCR reactions. Purification of PCR product was conducted using the enzyme mix ExoSAPit (Fermentas). Cycle sequencing was carried out using the BigDye Terminator Kit 3.1 (Applied Biosystems) in 10 µl reactions containing 1 µl template DNA and 1 µM of each forward and reverse primer, respectively. Cycle sequencing conditions were as described in the manufacturer´s instructions. The fragments were purified with the DyeEx Kit (Qiagen) and subsequently sequenced on an ABI 3130xl sequencer.

The sequences were aligned using the ClustalW algorithm as implemented in the program Codon Code Aligner v3.5.7 (CodonCode Corporation, Dedham, MA, USA). In about one fourth of the 158 sequenced individuals a consistent pattern of base ambiguities occurred, characterized by two peaks of the same height in the chromatograms coding for two different bases.

In order to resolve these base ambiguities a specific reverse primer Hsp70A-3R 5'-AACCCCCAACCAGAACAAC-3' was designed based on the sequences that were obtained with the degenerate primers and the PCR was repeated with all samples showing the mentioned ambiguities. The PCR conditions were similar to that mentioned above with increased annealing temperature of 55°C and reduced annealing and extension times of 20 sec and 70 sec, respectively. Additionally 0.5 M Betaine betaine was added to each PCR reaction. PCR products were cloned with the TOPO TA Cloning® Kit (Invitrogen) according to the manufacturer's instructions.

Amplification of the microsatellite loci Nma3, Nma4, Nma6, Nma12, Nde1, Nde2, Nde3 and Nde8 (Pöhlmann and Held 2011) was conducted in total volume of 25 µl, containing ~10 ng genomic DNA, 0.2 mM dNTPs, 0.5 µm primer, 0.5 M Betaine, 2.5 mM MgCl, 0.03 U/µl Hotmaster Taq (Eppendorf). One primer of each pair was labeled

with either HEX or FAM, and PCR reactions were performed as multiplex PCRs amplifying to loci simultaneously (Kraemer et al. 2009). The following PCR conditions were applied: 2 min 94°C, 32 cycles of 20 sec at 94°C, 10 sec at annealing temperatures, 60 sec at 65°C and a final extension of 45 min at 65°C. PCR products were purified using ExoSAPit (Fermentas) adding 5 µl of PCR products to a mix of 0.25 µl Exo I (20 U/µl) and 1 µl SAP (1 U/µl) and incubating at 37°C for 30 min and 15 min at 80°C. The purified PCR products were denatured and analysed on an ABI 3130xl sequencer using ROX GS500 size standard (ABI). Genotyping was performed using the software GENEMAPPER 4.0 (Applied Biosystems). To minimize genotyping errors chosen samples were run twice to ensure reproducibility and additionally scoring was cross checked by experienced co-workers.

Data evaluation

All population genetic analyses were conducted assuming both morphotypes to represent two different species and assuming them to be one species. The reason for performing the study with both groups as separate species was because we detected signs of microsatellite amplification failure in certain loci to be consistent with species membership (Pöhlmann and Held 2011) and because of the results from the Hsp70 allele distribution that indicates a strong genetic differentiation between the two nominal species *N. magellanica* and *N. deaurata.*

Presence of genotyping errors and null alleles in the microsatellite datasets was tested with the program MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004). GENETIX 4.05 (Belkhir et al. 2004) was used to check whether all populations and loci are in the Hardy-Weinberg-Equilibrium (HWE) and to ensure that no loci are linked to others (linkage disequilibrium LD). Allelic richness and the inbreeding coefficient F_{IS} were calculated with FSTAT v2.9.3 (Goudet 2001). The pairwise population differentiation measures F_{ST} and R_{ST} were calculated using ARLEQUIN v3.5 (Excoffier et al. 2005) with significance testing by 10,000 bootstrap replicates. Additionally, F_{ST} estimates were standardized according to the approach by Hedrick (2005). Maximum possible F_{ST} values were calculated by the program RECODEDATA (Meirmans 2006) and F_{ST} values obtained in GENEPOP were subsequently divided by this maximum F_{ST} value to get the standardized F'_{ST}. Sequential Bonferroni corrections (Rice 1989) were performed for

both F_{ST} and R_{ST} to correct for multiple testing.

A mantel test was performed using the program GENALEX 6 (Peakall and Smouse 2006) in which we tested for correlations of genetic and geographical distances between populations to detect a possible isolation by distance signal in the *N. magellanica* and the *N. deaurata* datasets. Geographical distances were determined as shortest sea distances between populations. The test was run with 9999 inferences.

In order to estimate any genetic clustering in our data without *a priori* information on the number of sampled populations we used the bayesian cluster analysis as implemented in STRUCTURE v.2.3.2 (Pritchart et al. 2000). This approach provides an estimation of the population structure and assigns individuals to corresponding clusters without being biased by sampling locations. Population numbers were inferred under several model assumptions, such as independent or correlated allele frequency distributions and population admixture or no admixture. Calculated log likelihood values (Ln P(D)) for tested models can subsequently be used to choose the appropriate prior informations for any given dataset. We performed tests with each model combination using 50.000 inferences and a burn-in of 10.000. Every simulation was run as five replicates and number of clusters K=1-9 for *N. magellanica* and K=1-5 for *N. deaurata*, where K=1 represents no population structure and K=9 (*N. magellanica*) and K=5 (*N. deaurata*) assume that all sampled locations represent genetically distinct populations. In addition to Ln P(D) we calculated ΔK as in many studies the obtained Ln P(D) values tend to reach a plateau with increasing K and, therefore, render it difficult to choose the correct number of clusters (Evanno et al. 2005). ΔK is described as the second order of change in the Ln P(D) between successive K values. The program CLUMPP v.1.1.1 (Jakobsson and Rosenberg 2007) was used to combine the data from the five replicates of each simulation and the program DISTRUCT v.1.1 (Rosenberg 2004) was used to visualize the results.

The microsatellite loci were tested for neutrality applying the FDIST F_{ST} outlier method by Beaumont and Nichols (1996) as implemented in LOSITAN (Antao et al. 2008). On the basis of the observed levels of population differentiation, the program calculates the area of heterozygosity each locus has to fall into to be regarded selectively neutral. We ran 10,000 inferences under the IAM and the SMM models. In order to increase

reliability of the overall mean F_{ST} all runs were performed with activated neutral mean F_{ST} and force mean F_{ST} options.

Data Analysis

Sequence assembly and editing of the COI sequences were conducted using the program CODON CODE ALIGNER v3.5.7 (CodonCode Corporation, Dedham, MA, USA). The implemented ClustalW algorithm was applied to perform aligning of sequences. Collapsing of sequences into haplotypes was carried out online with the web based program FABOX (http://gump.auburn.edu/srsantos/fabox).

A statistical parsimony network was calculated using TCS 1.21 (Clement et al. 2000). The connection limit was set to 40 steps to create one single network.

The Hsp70A clone sequences were used to create a median-joining network analysis with the program NETWORK (www.fluxus-engineering.com).

Results

Microsatellites

Genetic diversity

Allelic richness was always higher for *N. magellanica* than for *N. deaurata* (mean = 8.64 and 5.34, respectively; see Tab. 1 and 2). Genetic diversity in terms of expected heterozygosity (H_E) was relatively uniform across all tested populations of both *N. magellanica* (mean = 0.77) and *N. deaurata* (mean = 0.79). No population deviated from Hardy-Weinberg equilibrium. Intra-population genetic diversity for *N. deaurata* was evenly distributed both in terms of allelic richness and expected heterozygosity. Only the population at US showed elevated levels of genetic diversity. However, this might rather be a consequence the extremely low number of sampled individual (n = 5) and should not be interpreted as biologically significant information. For *N. magellanica* the northernmost population at PM and the central Magellan population at PA exhibited highest values/indices for H_E and allelic richness for *N. magellanica.*

Tab 1: Number of samples individuals (N_S), scored alleles (N_A), observed (H_0) and expected heterozygosity (H_E), the inbreeding coefficient (F_{IS}) and allelic richness (A_R) per population for *N. magellanica* based on six microsatellites.

	N_S	N_A	H_0	H_E	F_{IS}	A_R
PM	49	15	0.69	0.79	0.114	9.62
SO	32	11	0.73	0.75	0.034	8.53
FB	16	9	0.68	0.75	0.098	7.84
AR	13	9	0.62	0.76	0.196	8.3
CA	16	10	0.72	0.78	0.085	8.65
PA	24	12	0.71	0.79	0.101	9.28
BG	22	11	0.71	0.76	0.065	8.63
PC	41	12	0.7	0.76	0.069	8.32
US	40	12	0.66	0.77	0.15	8.63
All		11	0.69	0.77	0.1	8.64

Tab. 2: Number of samples individuals (N_S), scored alleles (N_A), observed (H_0) and expected heterozygosity (H_E), the inbreeding coefficient (F_{IS}) and allelic richness (A_R) per population for *N. deaurata*, based on five microsatellites.

	N_S	N_A	H_0	H_E	F_{IS}	A_R
US	5	6	0.85	0.91	0.068	5.6
AR	15	8	0.67	0.85	0.214	4.94
FB	46	11	0.61	0.73	0.168	5.39
PA	42	12	0.63	0.73	0.14	5.46
FL	52	12	0.68	0.72	0.053	5.31
All		10	0.69	0.79	0.13	5.34

Population structuring

Assignment of microsatellite allele distribution to the geographic distribution of investigated populations resulted in very little genetic structuring both for *N. magellanica* and *N. deaurata.* For *N. magellanica* pairwise F_{ST} values were significant after Bonferroni correction only among the three populations at the far sites of the sampled locations between the northernmost population at PM and the easternmost population at PC and also between PM and the southernmost population at US. When calculating R_{ST} values, that take the magnitude of allele length difference into account, PM is significantly different to almost all other populations of *N. magellanica* except the population at AR, located at the Pacific opening of the Magellan Strait and geographically closest to PM.

The results for *N. deaurata* are very similar to those obtained for *N. magellanica.* Among the populations from the Magellan region no population structuring was found. The three populations from the Magellan region AR, FB and PA are all significantly different both in F_{ST} and R_{ST} to the population from the Falkland Islands/Malvinas (Tab.

4). Only the population at US shows no significant genetic divergence to all other populations, but as mentioned above this may be attributable to the small sample size at this site.

Tab. 3: Genetic differentiation among populations of *N. magellanica*, based on six microsatellite loci. F_{ST} values are given below diagonal and R_{ST} above. PM, Puerto Montt; SO, Seno Otway; FB, Fuerte Bulnes; AR, Puerto Arturo; CA, Camaron; PA, Punta Arenas; BG, Bahia Gregorio; PC, Punta Catalina; US, Ushuaia. Bold numbers indicate significance (Bonferroni corrected $p < 0.01$).

	PM	SO	FB	AR	CA	PA	BG	PC	US
PM		**0.0827**	**0.1438**	0.0585	**0.1302**	**0.0947**	**0.1013**	**0.108**	**0.1063**
SO	0.0049		0.0019	-0.0223	-0.0088	-0.0133	-0.016	-0.0073	-0.0067
FB	0.0086	-0.0022		-0.0129	0.0147	-0.0122	-0.0156	-0.0093	-0.0081
AR	0.0046	0.0018	-0.0063		-0.0115	-0.0263	-0.0279	-0.0251	-0.0165
CA	0.0097	0.0012	0.0021	-0.0018		-0.013	-0.0116	-0.0086	0.0119
PA	0.0065	-0.0028	-0.0016	0.0026	-0.0008		-0.0221	-0.0065	-0.0108
BG	0.0056	-0.0059	-0.0033	-0.0022	-0.0079	-0.0086		-0.0073	-0.0151
PC	**0.0133**	0.005	0.0023	0.0138	-0.0044	0.0026	-0.0037		0.0029
US	**0.0088**	0.0029	0.0011	0.004	-0.0007	-0.0019	-0.0007	0.0055	

Tab. 4: Genetic differentiation among populations of *N. deaurata*, based on five microsatellite loci. F_{ST} values are given below diagonal and R_{ST} above. US, Ushuaia; FB, Fuerte Bulnes; AR, Puerto Arturo; CA, Camaron; PA, Punta Arenas; BG, Bahia Gregorio; PC, Punta Catalina. Bold numbers indicate significance (Bonferroni corrected $p < 0.01$).

	US	AR	FB	PA	FL
US		-0.0349	-0.0352	-0.0155	-0.054
AR	0.0048		-0.0207	-0.0105	**0.0363**
FB	-0.016	0.0001		0.006	**0.0416**
PA	-0.0085	0.0024	-0.0008		**0.0624**
FL	0.0184	**0.049**	**0.0317**	**0.0331**	

Regarding the question whether or not *N. magellanica* and *N. deaurata* represent two species, two possible conclusions can be drawn from the microsatellite data. On one hand, calculations of F_{ST} and R_{ST} values between population of *N. magellanica* and *N. deaurata* with the four loci that worked for both of them revealed no significant genetic differentiation, thus refuting the assumption that both morphotypes truly reflect reproductively isolated species (Tab. 5). Only R_{ST} between both subgroups at AR revealed a significant difference, but the level of differentiation was still low (R_{ST} = 0.0665). On the other hand, the three loci that amplified only in the species they were developed for lend credibility to the idea that *N. magellanica* and *N. deaurata* indeed represent differentiated taxonomic units.

Tab. 5: Genetic differentiation between populations of *N. magellanica* and *N. deaurata*, based on four microsatellite markers. Compared were only those populations occurring at the same sampling site to rule out any distance effects. FB, Fuerte Bulnes; AR, Puerto Arturo; PA, Punta Arenas; US, Ushuaia. Bold numbers indicate significance (Bonferroni corrected $p < 0.01$).

	F_{ST}	R_{ST}
FB	0.0017	**0.0665**
AR	0.0165	0.0676
PA	-0.0018	0.0251
US	-0.0051	0.1291

Testing for selective neutrality revealed the two loci Nma3 and Nma12 that only amplified in *N. magellanica* to be slightly above the upper 95% confidence interval indicating elevated levels of diversifying selection (Fig. 2a). All other loci applied to *N. magellanica* genetic material were selectively neutral. Surprisingly, of the 5 different loci used in *N. deaurata* all except one were outside the 95% confidence interval, which in the case of Nde8 and Nde4 indicated diversifying selection and in the case of Nde1 and Nde2 balancing selection (Fig. 2b). A microsatellite locus that is linked to a locus under selection might lead to inflated levels of population structure because of diversifying selection or to reduced levels due to balancing selection. In the present case, diversifying selection in some loci could lead to failure of amplification although in loci that worked for both species no population differentiation signal could be detected (Bailie et al. 2010).

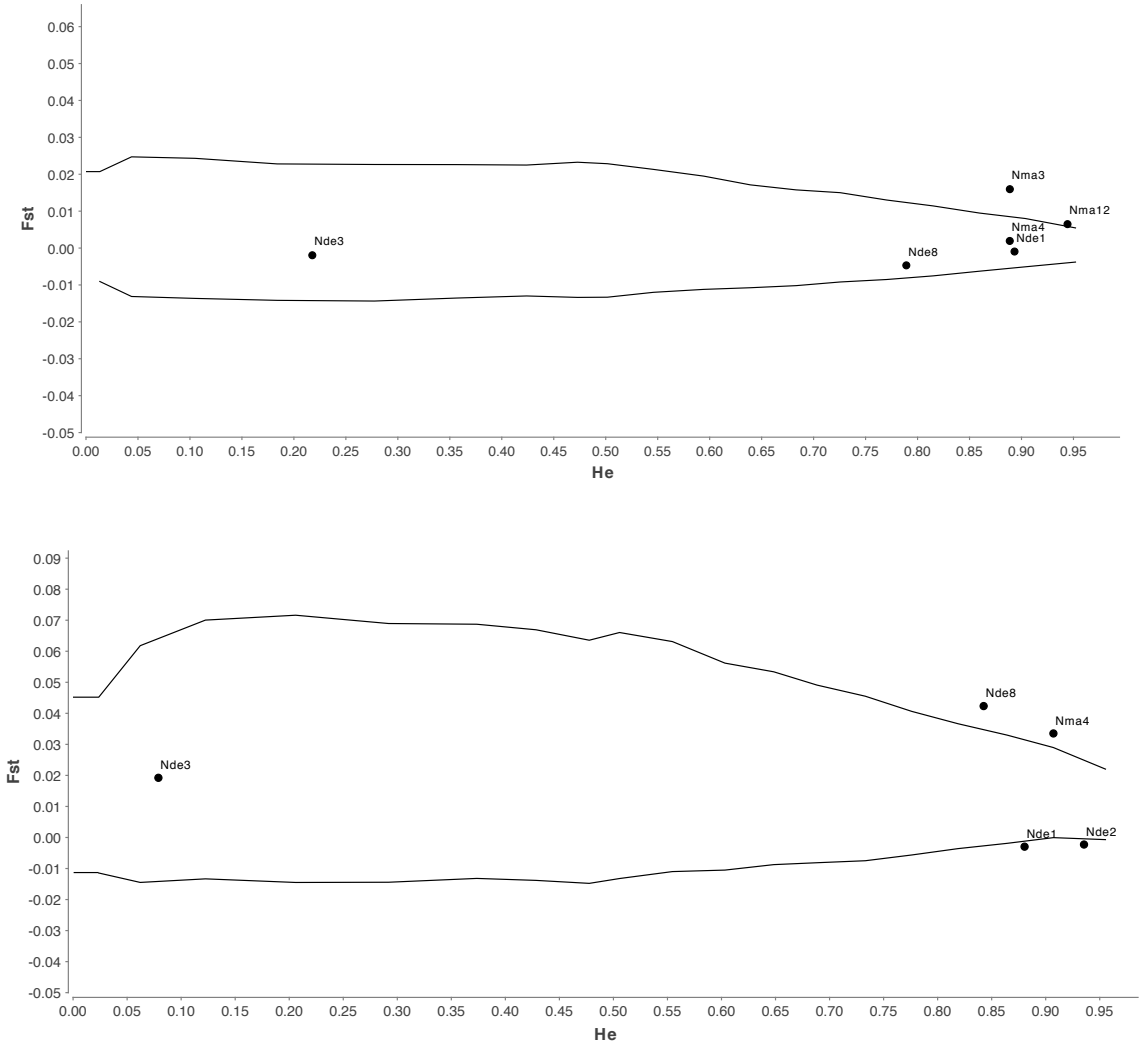

Fig. 2: Microsatellite results from FDIST F_{ST} outlier method under the stepwise mutation model (IAM model results similar and therefor not shown). The solid lines represent the 95% confidence interval of the distribution of F_{ST} values from 500000 simulated loci as well as the estimated values of individual microsatellite loci. Loci outside the lines show signs of diversifying (above) and balancing (below) selection. Upper graph shows results for *N. magellanica*, low graph for *N. deaurata*

In the STRUCTURE analysis highest Ln P(D) values were obtained when K = 3 clusters and no admixture were assumed for both *N. magellanica* and *N. deaurata.* In Fig. 4 the likelihood curves for the different applied K values are shown, and a sharp increase can be seen starting from K = 1 to K = 3, followed by a less pronounced tailing-off for *N. magellanica* and a plateau for *N. deaurata* with higher K. This phenomenon is described by Evanno et al. (2005) who state that once the true K is reached it can further slightly increase or reach a plateau phase which complicates the correct choice for K. The calculation of ΔK, the second order rate of change, is proposed by the authors to help effectively in assigning the correct number of K. In our case for both subgroups the ΔK method resulted in K = 2. Fig. 3 shows graphically the assignment of each individual,

represented by a vertical bar, to the inferred clusters. Each population is defined by high admixture levels and large numbers of individuals with shared genotype proportions. Under the prior assumption of K = 3, admixture is still high but the two geographically farthest populations, PM for *N. magellanica* and FL for *N. deaurata,* exhibit high proportions of individuals assigned to a third genotype, indicating signs of isolation by distance.

In order to test whether the STRUCTURE results indicate isolation by distance effects (IBD), we performed a Mantel test and indeed highly significant correlations between genetic and geographic distance were found for *N. magellanica* (R = 0.71, p = 0.02) and *N. deaurata* (r = 0.86, p = 0.02) when all populations were included. However, it seems that the IBD effects detectable for both subgroups are a consequence of the large geographic distances between the Magellan region and PM on the one side and FL on the other side. When the northernmost population PM from the *N. magellanica* dataset and the easternmost population FL from the *N. deaurata* dataset were excluded, the Mantel tests for both groups were insignificant (*N. magellanica*: R = 0.28, p = 0.17; *N. deaurata*: R = 0.2, p = 0.33) clearly rejecting isolation by distance effects.

COI

The statistical parsimony network resulted in two major subnetworks (Fig. 5), a shallow one represented by the Falklands/Malvinas population (10 haplotypes, H70-H79) and a more diverse one comprising all samples from South American mainland populations (65 haplotypes, H5-H69). There is no obvious structure in the South American subnetwork, but H5 to H16 form a small subclade only consisting of samples of nominal *N. magellanica* and H58 to H69 represent a subclade mainly comprising *N. deaurata.*

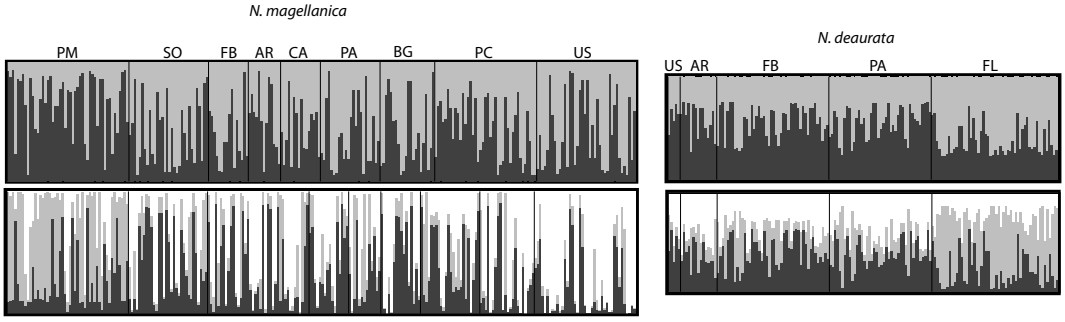

Fig. 3: Structure results based on microsatellite data showing the group membership coefficients of *N. magellanica* (left) and *N. deaurata* (right). The upper graphs show results under the assumption of k = 2, and the lower assuming K = 3. Each vertical bar represents one individual. The color and partitioning of each bar describes the membership probability of the individual fractioned in K clusters.

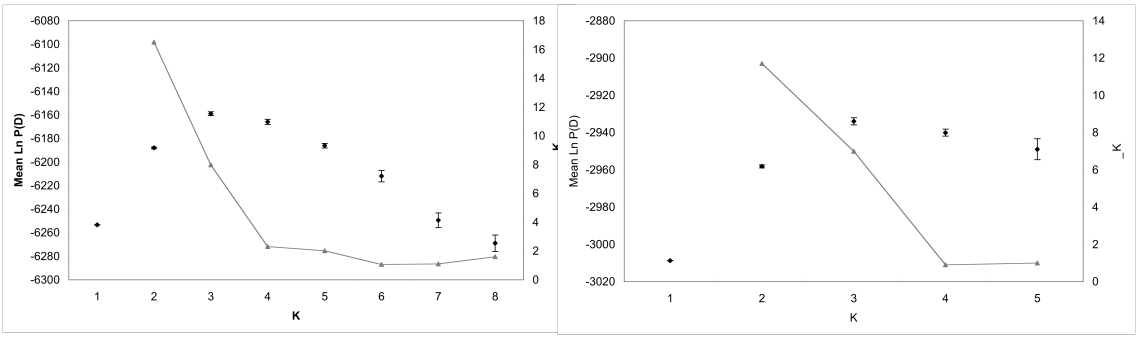

Fig. 4: Ln P(D) (black, witch standard deviations) and ΔK (grey) values as calculated in Structure for a) *N. magellanica* and b) *N. deaurata.*

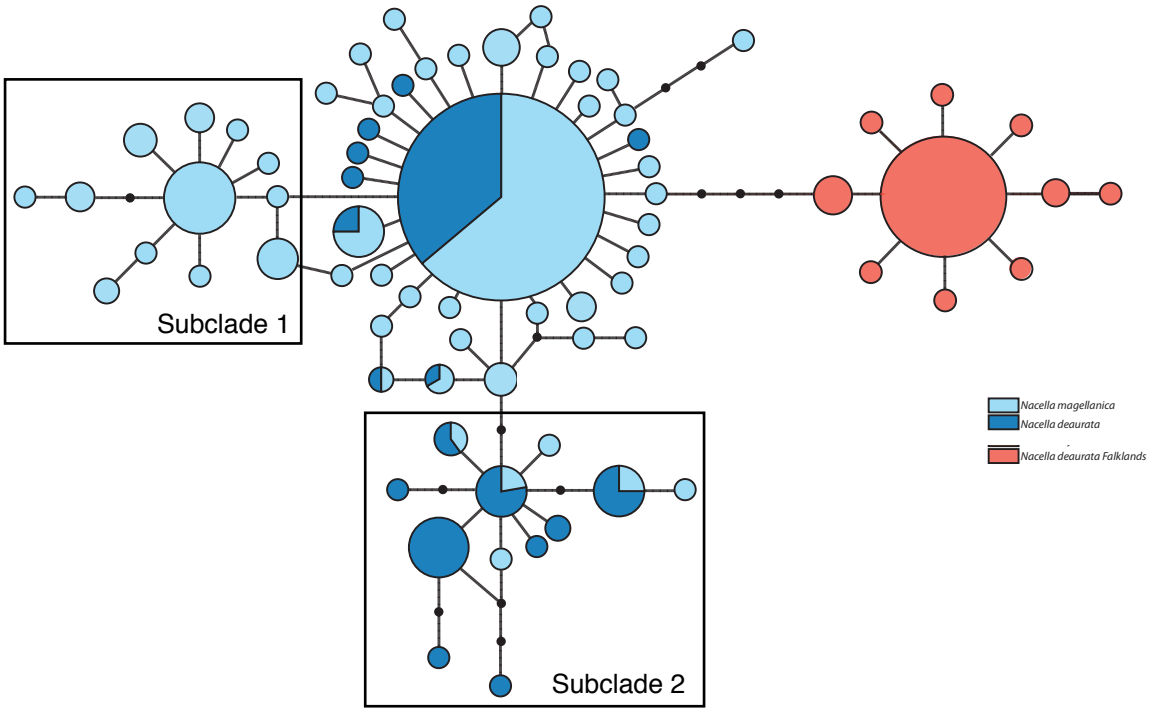

Fig. 5: Statistical parsimony network of COI haplotypes. Color code according to nominal species. Black dots indicate missing haplotypes.

Hsp Alleles

Tab. 5: Consistent polymorphic sites in the sequences fragment of the analyzed Hsp70 gene. Three different main groups can be identified, Hsp1, largely comprising the *N. magellanica* morphotype and Hsp2, mainly consisting of *N. deaurata.* The group Hsp3 represents individuals that are heterozygous carrying one allele of each Hsp1 and 2.

Group	n	87	93	111	114	123	129	252	255	312	319	321	330	433	531	561	576	609	649
Hsp1	81	G	T (59) K (22)	T	A	A	T	C	M (37) A (44	T	A	G (78) K (3	T	C	A	C	G	T	T
Hsp2	40	C	G	C	G	G	A	C	T	C	T	T	G	T	C	T	A	G	C
Hsp3	22	S	K	Y	R	R	W	M	W	Y	W	K	K	Y	M	Y	R	K	Y

The sequencing of a 693 bp fragment of the Hsp70 gene in 158 individuals of *N. magellanica* and *N. deaurata* resulted in four different major allele clusters. The two main clusters Hsp1 and Hsp2 are distinguished by 15 consistent base pair mismatches (97.8% sequence similarity, Tab. 5). Three more sites (nucleotide positions 93, 255 and 321) were polymorphic between both clusters but a proportion of individuals assigned to cluster Hsp1 had nucleotide ambiguities. Additionally, we found a cluster Hsp3 standing out by the high degree of ambiguous sites. In all 18 sites that separate the allele clusters Hsp1 and Hsp2, the cluster Hsp3 has peaks in the sequence chromatograms for both bases. These patterns indicate that individuals belonging to the latter cluster are heterozygotes carrying one allele of the Hsp1 and one of the Hsp2 allele group. Figure 6 shows the geographical distribution of the different Hsp allele clusters. The most intriguing information that can be extracted from the figure is the striking correlation of allele distribution and the two nominal species *N. magellanica* and *N. deaurata.* The allele group Hsp1 mainly occurs in *N. magellanica.* The Nma population in the Pacific ocean at PM, SO, FB and US exclusively carried the Hsp1 alleles. In the Magellan Strait, however, the more the populations are located at the Atlantic opening the higher become the proportions of heterozygote individuals with the easternmost *N. magellanica* population at PC having even one individual with Hsp2 alleles. Contrary, all four populations of *N. deaurata* are dominated by individuals carrying alleles of the Hsp2 type but in each of these populations a minor fraction was heterozygote and very few individuals were homozygote for the Hsp1 cluster. It can be concluded the the two major allele groups Hsp1 and Hsp2 can largely be assigned to the two nominal species *N. magellanica* and *N. deaurata.* The distribution pattern of our investigated Hsp70 gene resembles that of a bimodal hybrid zone (Jiggins and Mallet 2000) in which two genetically incompletely isolated groups of individuals overlap in certain parts of their

distribution areas and between which to a low extent gene exchange is still facilitated due to incomplete reproductive isolation.

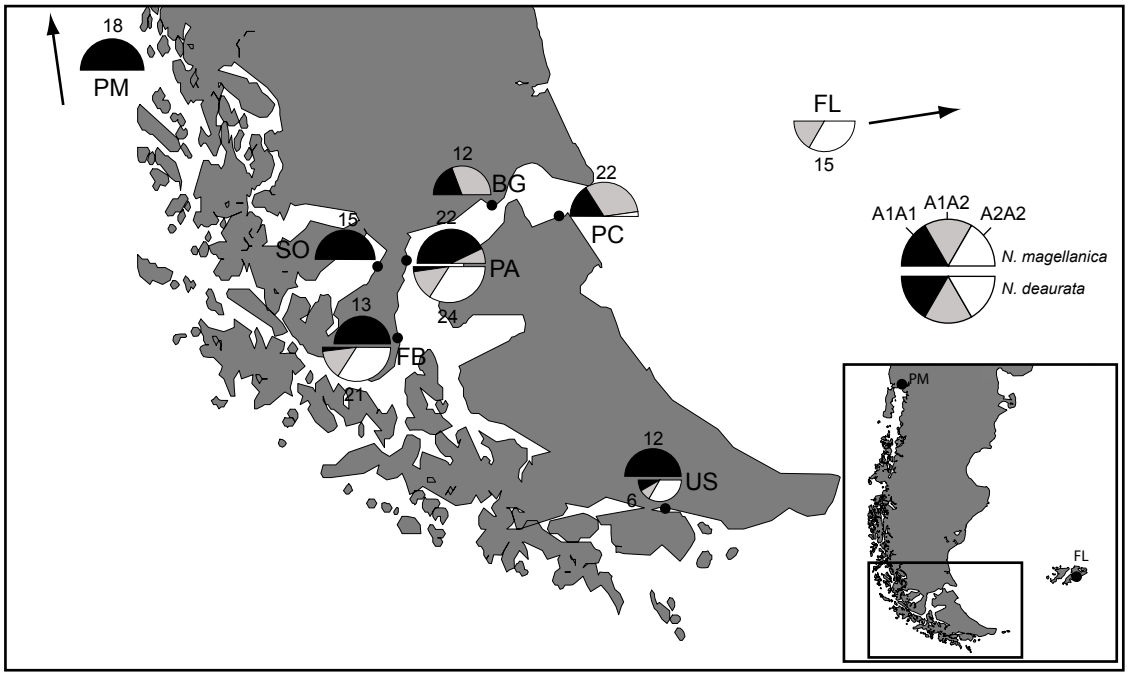

Fig. 6: Allelic distribution of the two different Hsp70 allele groups Hsp1 (black) and Hsp2 (white). Proportions of individuals being heterozygote carrying one allele of each Hsp group 1 and be are displayed in gray. Upper semi circles give distribution for *N. magellanica*, lower semi circles for *N. deaurata.* Numbers indicate sequenced individuals for each populations. For sampling location abbreviations see Fig.1

In order to verify if the cluster Hsp3 truly reflects heterozygote individuals carrying one allele of each Hsp1 and Hsp2 we cloned twelve individuals of each nominal species belonging to that cluster. A 700 bp long fragment sequenced from 160 clones stemming from the twelve heterozygote individuals and each five sequences from the homozygote clusters Hsp1 (yellow) and Hsp2 (light blue) were used to create a minimum spanning network (Fig. 7). The allele distribution supports our hypothesis as the two main allele clusters are easily identifiable in the graphic. Additionally, alleles from the heterozygote individuals (blue = heterozygous *N. magellanica*, orange = heterozygous *N. deaurata*) group with both the main clusters verifying that indeed those individuals assigned to heterozygous Hsp3 carried one allele from either of the two major allele groups. However, the cloning of 24 individuals in total resulted in 69 different alleles which would be impossible if we were dealing with a single copy gene and therefore expecting a maximum of 48 different alleles. Apparently, we are investigating a multi copy Hsp70 gene but more studies are necessary to understand the state of the investigated Hsp70

gene fragment. This does, however, not change the interpretation of the results as such because of the clear genetic break between *N. magellanica* and *N. deaurata*, regardless whether we are looking at different alleles of one gene or several gene copies.

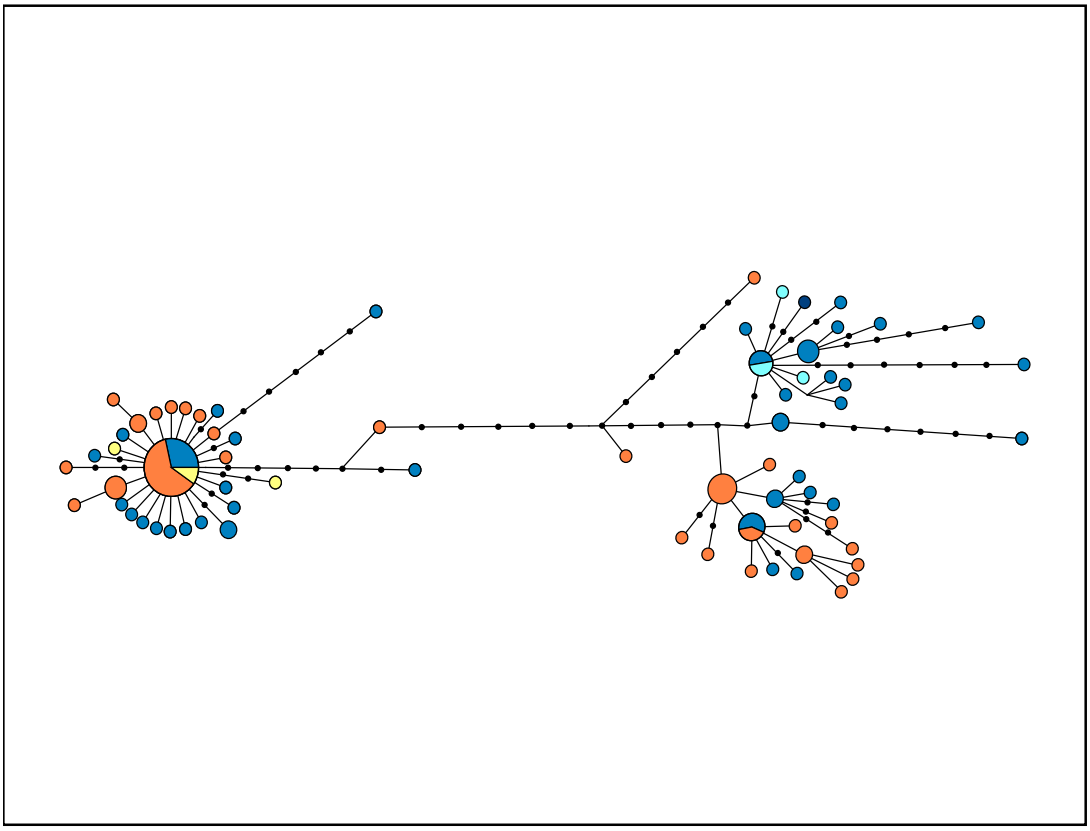

Fig. 7: Minimum spanning network showing the allele distribution of heterozygote individuals of *N. magellanica* (blue) and *N. deaurata* (orange) according to the sequences obtained by cloning of ambiguous individuals of the Hsp3 cluster (Tab. 5). Two major clusters can be identified on both ends of the network. On the right side is the cluster Hsp1, identified by light blue circles that represent homozygous *N. magellanica* Hsp1 individuals and yellow cycles representing homozygous *N. deaurata* on the left side identify this cluster as Hsp2

Discussion

The present study highlights the importance and the power of multimarker approaches in comprehensive population genetic studies to get deeper insights into the various evolutionary and ecological factors that contribute to the evolutionary history of a species. Neutral markers alone, even if they are fast evolving and highly informative like microsatellites, might not always provide the resolution to completely unravel recent and contemporary processes that explain present day population structure. The inclusion of markers under selection contributed significantly to track the history of *Nacella* evolution in our work by confirming profound genetic divergence between nominal *N. magellanica* and *N. deaurata*, not detectable when applying only neutrally evolving markers. This work is a good example how natural selection maintains a genetic divergence and can be an important driver of increasing biodiversity in marine and coastal high gene flow environments.

Neutral genetic baseline study COI and microsatellites

Based on the neutral markers we applied population structuring is largely absent for both *N. magellanica* and *N. deaurata* revealing Patagonia to be a high gene flow environment for limpets. F-statistics revealed very little genetic differentiation between populations of either morphotype. Exceptions are the northernmost population in PM and the easternmost population in FL as inferred by small albeit significant F_{ST} and R_{ST} values (Tabs. 3 and 4) and from bayesian cluster analysis (Fig. 4). Statistical tests suggest that these observed genetic differences are most likely attributable to an isolation by distance effects. A comparable microsatellite study by Leese et al. (2008) with the serolid isopod *Serolis paradoxa* revealed genetic divergence between populations from the Magellan region and the Falklands/Malvinas to be in the same order of magnitude generally accepted for different species. Although in the present study the pairwise F_{ST} values between limpet populations from the Falklands/Malvinas and from southern South America are significant, and a clear haplotype divergence exists based on COI (Fig. 3), the bayesian cluster analyses showed considerable amounts of genetic admixture. This lesser degree in spatial genetic partitioning in *Nacella* compared to *Serolis*, as well as the generally marginal population structure of investigated limpets is best explained by the reproductive modes. Serolid isopods are

benthic brooders with rather low dispersal capacities (Leese et al. 2008) whereas *Nacella* have pelagic larval stages that stay in the water column for several weeks enabling them to disperse large distances by drifting with ocean currents (Picken 1980; Morriconi 1999; Hofmann et al. 2010).

On the basis of the four different neutral microsatellite markers that amplified both in *N. magellanica* and *N. deaurata* no consistent differentiation between the two nominal species on the basis of F_{ST}, R_{ST} and bayesian cluster analyses could be determined (Tab. 3 and 4, Fig. 3). The investigated microsatellites do not support a separation on the species level indicating genetic exchange between both morphotypes. Similar results were obtained when displaying the haplotype distribution of the analyzed COI fragment in a parsimony network (Fig. 3).

This pattern of homogeneity and absence of genetic differentiation between *N. magellanica* and *N. deaurata* is comprehensible when considering the facts that both formal species have mildly overlapping vertical distributions, external fertilization with free swimming gametes, mate choice mediated by gamete protein recognition and pelagic larvae with high dispersal potential. All these points would make a diversification rather unlikely (Bird et al. 2011). This theoretical high potential to overcome small scale geographic barriers have also been recognized in other marine invertebrates, however, it has been widely shown that the potential of high dispersal does not preclude absence of genetic differentiation. Several studies have been addressing similar situations with closely related sympatric species or ecotypes with high theoretical dispersal capacities showing non-allopatric modes of speciation in intertidal habitats. One intriguing example for sympatric speciation mediated by pre-zygotic selection is given by the galician marine snail *Littorina saxatilis* that occurs in two ecotypes that are largely sympatric but show slightly different vertical intertidal zonation pattern. Hybridization is possible but restricted due to assortative mating, which is believed to be the driver that establishes a genetic discordance (Rolán-Alvarez 2006). Small-scale habitat stratification in three sympatric broadcast-spawning Hawaiian limpets of the genus *Cellana* further underlines the importance of ecological factors along vertical intertidal gradients in non-allopatric speciation processes where steep shore gradients can be a basis for sympatric speciation, presumably with the objective of habitat expansion (Bird et al. 2011).

Allopatric divergence of N. magellanica *and* N. deaurata *mediated by glacial maxima*

Contrary to the results obtained with the neutral marker approach, the analysis of Hsp allele patterns shows strong genetic structuring across the species boundaries (Fig. 6). We found a clear bimodal distribution of the two main Hsp70 allele clusters with one being dominant in *N. magellanica* (Hsp1) and the other in *N. deaurata* (Hsp2). Populations of *N. magellanica* along the Pacific coast consisted entirely of the Hsp1 group and the gradual increase of *N. magellanica* individuals carrying either one or even both alleles of the Hsp2 group to the eastern Atlantic opening of the Magellan Strait suggest that this allele group originated in the Pacific Ocean. The Atlantic population on FL consisted entirely of *N. deaurata* dominated by the Hsp2 allele group. The bimodal distribution of the two Hsp allele groups underlines a genetic divergence between *N. magellanica* and *N. deaurata*, and we believe that the creation of the two distinct Hsp allele groups originated in times of glacial maxima when formerly contiguous gene pools were separated and evolved independently over time. During the LGM, that ended 9,000-14,000 years BP, populations on either side of the South American continent were isolated. We hypothesize that the Falklands/Malvinas and areas around Puerto Montt served as refuge areas during the LGM. The Falklands/ Malvinas were only little affected by the LGM (Clapperton 1990; Leese et al. 2008) and Puerto Montt represents the northern boundary of the Patagonian glaciation (Rostami et al. 2000; Hulton et al. 2002). The gradual increase of hybrid *N. magellanica* individuals to the eastern Atlantic opening of the Magellan Strait suggest that this allele group originated in the Pacific ocean and dispersed eastward after the LGM ended and surviving Pacific limpet populations re-colonized the Magellan Strait. The Hsp2 group, dominant in *N. deaurata*, shows an opposite distribution pattern suggesting its Atlantic origin. It is still in the range of possibility that dispersal of individuals was maintained around Cape Horn but it remains questionable whether larvae could travel such long distances and whether ice free areas in Tierra del Fuego existed that could have served as stepping stones. Present-day reconstructions of the extent of ice during the LGM do not speak for the existence of larger ice-free areas at the southern tip of South America (Clapperton et al. 1995; McCulloch et al. 2000; Rostami et al. 2000; Hulton et al. 2002). We therefore conclude that Atlantic and Pacific populations have indeed been isolated during the LGM. Different ecological selection pressures on both sides of South

America and larger impacts of genetic drift on small populations surviving in refuge areas might have driven the evolution of the two different Hsp allele clusters detectable today. It is also likely that the amount of genetic divergence stems not only from the LGM but from previous glacial maxima. However, at the end of the LGM, when the Magellan Strait finally became inhabitable again, limpets from both sides of South America re-colonized that area and formed a secondary hybrid zone, as can be nicely seen in the gradual increase of hybrids in *N. magellanica* across the Magellan Strait from Pacific to Atlantic populations. The fact that the Pacific coast north of the Magellan Strait is only colonized by *N. magellanica* and on the Falklands/Malvinas all sampled individuals where genotyped as *N. deaurata* might further suggest the origin of the two nominal species. However, this study lacks samples from the Atlantic coast north of the Magellan Strait and we need to analyze populations from that area to reinforce our statements.

Based on their studies on the genetic diversity of the serolid isopod *Serolis paradoxa,* Leese and co-workers (2008) concluded that extraordinarily high genetic diversities of populations in the center of the Magellan Strait support the theory of two-sided re-colonization of the Magellan Strait. Normally, populations in newly available habitats would be characterized by low genetic diversities due to founder effects (Mayr 1963), but a bilateral colonization of populations from different refuge areas that merge in the central Magellan Strait would increase the genetic diversity as polymorphisms that originated in either of the refuge populations get mixed (Leese et al. 2008).

Natural selection maintains disrupted gene pools of N. magellanica *and* N. deaurata *in the Magellan Strait*

One important question that arises when studying hybrid zones is whether observed habitat preferences and niche specializations have already emerged in allopatry or whether they are exclusively the outcome of local adaptations and reinforcement (Riginos and Cunningham 2005). One fundamental difference between the situations of *Littorina* and *Cellana*, which are believed to be two examples for true sympatric speciation (Rolán-Alvarez 2006; Bird et al. 2011), compared to that of *Nacella* is that in Southern South America allopatric isolation mediated by glacial maxima has likely played a major role in the extant diverging Nacella genotypes as discussed above.

Coyne and Orr (2004) listed four criteria for correct identification of sympatric speciation of which the present *Nacella* case violates the fourth that states „the biogeographic and evolutionary history of the groups must make the existence of an allopatric phase very unlikely“.

However, as seen in the microsatellite data, gene flow is not totally restricted between *N. magellanica* and *N. deaurata* after secondary contact in the Magellan Strait as indicated by the absence of any genetic structure between both morphotypes based on F_{ST}, R_{ST} and bayesian cluster analyses of two different neutral markers: microsatellites and mitochondrial COI (Tab. 3 and 4, Fig. 3 and 4). This means that a reproductive barrier is not fully established but selective forces exist that restrict reproduction. If mating between *N. magellanica* and *N. deaurata* , which is principally possible, would be favored, a unimodal distribution of Hsp alleles would be expected with an excess of hybrids in zones of overlapping distribution. As this is not the case in nearly all investigated populations, we assume natural selective environmental conditions to favor reproduction among individuals of the same morphotype over hybridizing mating. The importance of selection establishing a reproductive barrier between both nominal species is supported by the fact that two microsatellite loci developed for *N. magellanica* did not cross-amplify in *N. deaurata* and one locus vice versa indicates that a certain degree of genetic distinction is present, caused by mutations in microsatellite flanking regions (Bailie et al. 2010). These loci are likely linked to areas of the genome under selection and indicate a certain degree of genetic distinction.

The divergent habitat use of both morphotypes in the Magellan Strait can lead to sexual selection by reduced chances of intraspecific fertilization. The probability that male and female gametes of the same morphotype meet would be higher than melting with a gamete of the other morphotype. Furthermore, asynchronous times of gamete release can reduce the chance of hybridization as it has been shown in sympatric Caribbean corals (Fukami et al. 2004; Levitan et al. 2004). It is also imaginable that selection acts on hybridizing reproduction by decreased gamete recognition. Finally, the selective pressure may be on the hybrids themselves when hybridizing reproduction produces unfit or sterile offspring. This process is called reinforcement and is thought to be especially important in speciation, where initial assortative mating is already high because otherwise high gene flow would possibly break down associations of loci

responsible for hybrid fitness (Dobzhansky 1951; Felsenstein 1981; Liou and Price 1994).

Additional evidence for reinforcing selection is provided by morphometric analyses done with samples from Puerto Montt (northern distribution edge of *N. magellanica*, Punta Arenas (central Magellan Strait, zone of sympatric occurrence of both morphotypes) and the Falklands/Malvinas (Fig. 7). On the Falklands/Malvinas *N. deaurata* was found occupying a broader vertical shallow-water range than in the Magellan Strait, where it is clearly restricted to the shallow subtidal. Morphological analysis of shell heights and lengths shows that *N. deaurata* reaches much greater shell sizes on the Falklands/Malvinas compared to Punta Arenas. *N. magellanica*, on the other side, has considerable smaller shells at Puerto Montt than at Punta Arenas. It is conspicuous that in Punta Arenas, an area where both morphotypes, large *N. magellanica* and small *N. deaurata*, occur in sympatry, body sizes of each morphotype reach their extremes, opposite to regions where only one of the morphotypes occurs. In locations where sympatry is possible, survival chances of extreme morphotypes might guarantee best chances for survival as a consequence of habitat specialization (intertidal vs subtidal, see Pöhlmann et al. 2011), highlighting the importance of ecological factors for speciation in sympatry. Gene flow among limpets from different shore levels does not cease completely as seen in the microsatellite data, but ecological selection can possibly drive the incipient speciation in *Nacella* to completeness (Nosil 2008; Nosil et al. 2009). A study by Pöhlmann et al. (2011) demonstrated that *N. deaurata* is not adapted to prolonged phases of air exposure during low tides compared to well-adapted *N. magellanica.* Hybrids might therefore be under negative selection as the result of maladaptation to the tidal gradients. On the Atlantic side, where the tidal change is least pronounced in the whole Magellan Strait, we sampled two populations, BG and PC, which were dominated by hybrid *N. magellanica*, carrying one allele of either Hsp allele cluster. In this area ecological selection is probably so weak that hybrids can survive and reproduce. However, this is only a vague assumption and more research is required to explain the high hybrid incidence in the eastern Magellan Strait.

However, whether the enhanced reproductive barriers are because of intrinsic genomic and genotypic incompatibilities leading to reduced hybrid viability and reproducibility or determined by extrinsic factors such as differences in spawning time or decreased cross-fertilization success cannot be determined on the basis of the available data. The likelihood of reduced hybrid fitness to be the cause of separation is somewhat weakened by the fact that hybrids dominate in the two *N. magellanica* populations located close to the Atlantic opening of the Magellan Strait. Regardless what the dominant mode of diversifying selection is in the *Nacella* case, our results suggest the Magellan Strait to be a secondary hybrid zone of limpets with distinct genotypes of Atlantic and Pacific origins between which reproductive isolation is not complete but restricted.

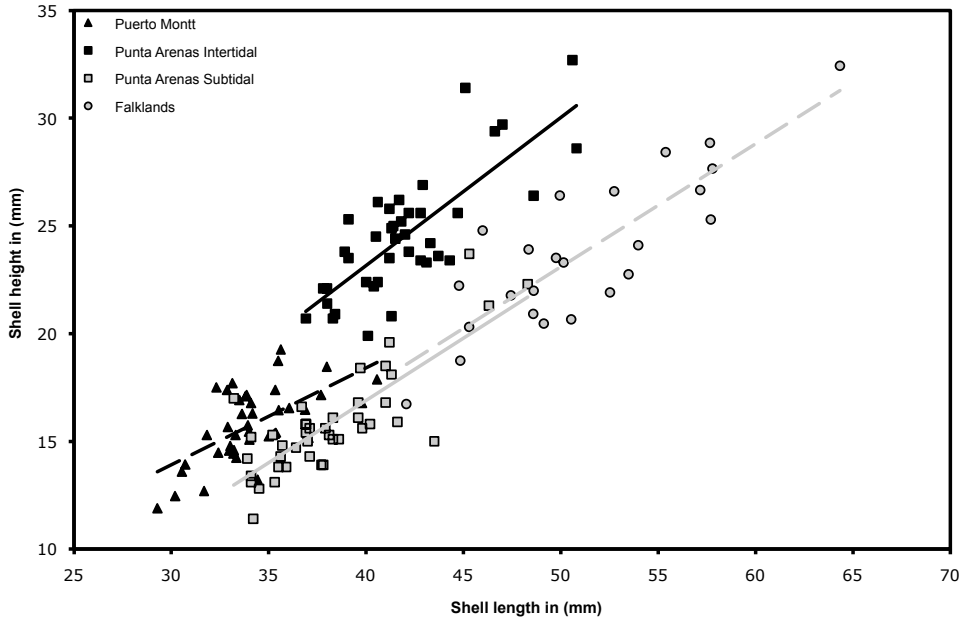

Fig. 7: Shell lenghts to shell heights in four investigated populations from *N. magellanica* from Puerto Montt (black triangles) and Punta Arenas intertidal (black squares) and from *N. deaurata* from Punta Arenas subtidal (grey triangles) and from the Falklands/Malvinas (grey cycles)

Conclusions

The combined application of neutral and selected markers as shown in this study allows much deeper insights into the evolutionary history of Patagonian *Nacella* and highlights the important role of natural selection in driving speciation processes that might possibly have started in phases of allopatry but were not completed. A study based on the neutral markers microsatellites and COI alone, representing two different genomes (nuclear and mitochondrial), would have led to the conclusion that *N. magellanica* and

N. deaurata are not genetically diverged and represent two morphotypes caused by mere phenotypic plasticity. The addition of selected markers provided us much deeper insights into the evolutionary history of South American *Nacella,* where geographical isolations summoned by glacial maxima resulted in a deep divergence of a Hsp70 gene. Neutral microsatellites demonstrate that gene flow among both morphotypes was not restricted upon secondary contact after the LGM had ended, but the bimodal distribution of Hsp70 alleles displays how divergent selection maintains diverged gene pools and highlights the impact of ecological selection in high gene flow environments. The *Nacella* evolution is an outstanding example how allopatric isolation and sympatric divergence, acting on different time scales, both shaped the evolution of Patagonian limpets. A study with flies of the genus *Rhagoletis* could show how chromosomal inversions arisen in times of allopatry have promoted sympatric disruptive selection of genes involved in diapause (Feder et al. 2003).

Whether or not *N. magellanica* and *N. deaurata* should yet be considered as different species is a matter of perspective and depends on which species definition one prefers. According to the biological species concept (Mayr 1942) that basically defines species as reproductively isolated entities, our results would reject the assumption that *N. magellanica* and *N. deaurata* represent two different species. According do the Darwinian concept of species (see Mallet 2008 for review) speciation is driven by divergent selection where intermediates between species might occur but diversifying selection ensures that the parental forms persistent in the longer run would lead to extinction of intermediates. Such species definition would actually allow to call *N. magellanica* and *N. deaurata* two different incipient species.

Chapter 4

Additional results

Chapter 4.1

Declining genetic richness across the Drake Passage: A demographic analysis of the impact of ice on the population structure of intertidal limpets from the genus *Nacella*

Kevin Pöhlmann and Christoph Held

Abstract

This section comprises additional data on the genetic diversity and demography of limpet populations from both sides of the Drake Passage based on COI sequence data. Investigated populations of the Antarctic shallow-water limpet *Nacella concinna* showed very low genetic diversities compared to South American *N. magellanica* and *N. deaurata* indicating the severe influence of annual sea ice extensions on Antarctic intertidal coastal communities. The results raise concern about the genetic adaptability of *N. concinna* under further rapidly ongoing climate warming in the Southern Ocean as low genetic variability can be understood as low potential for adaptation to changing environmental conditions. Additionally, the results presented in chapter 3.1 question the isolating capability of the Antarctic Circumpolar Current. Thus, an ongoing climate change that further warms the Southern Ocean could lead to invasion events of South American species into Antarctica.

Keywords Glacial cycles · Genetic variability · Adaptive potential · Global warming · *Nacella*

Introducion

Glacial cycles and the accompanying extent and retreat of sea ice have dramatic effects on organisms in high latitudes causing shifts in species‘ distributions and even leading to extinction of populations (Hewitt 1996). The formation of sea ice during glacial maxima especially affects those species that inhabit coastal areas. Whole benthic communities can be eliminated due to ice scouring and large regions in the high intertidal can become inhabitable due to icefoot (Gutt 2001; Barnes and Conlan 2007; Fraser et al. 2009). In times of glacial maxima species move towards lower latitudes surviving in refuge areas or survive in ice free areas on the shelf (Clarke and Crame 1992; Thatje et al. 2008). At the end of those glacial maxima when ice retreats a typical pattern of genetic homogeneity in recolonizing populations could be shown in a variety of northern hemisphere taxa. This phenomenon of genetic homogeneity is called the founder effect (Hewitt 1996, 2000). The explanation for such loss of genetic diversity is due to reduced population sizes during survival in refuge areas and accordingly stronger impacts of genetic drift and inbreeding, both leading to the reduction of genetic

variation. Additionally, only a fraction of a surviving population, equivalent to a fraction of the gene pool, forms the leading edge of migration into new habitats further decreasing the genetic diversity (Hewitt 2000). Less intensive research has been done in the Antarctic ocean but the few works published could support this general pattern of genetic homogeneity in areas affected by ice during glacial maxima (Holderegger et al. 2003; Fraser et al. 2009; Hunter and Halanych 2008; Thornhill et al. 2008). Here, we aim at analyzing and comparing the impact of glacial advances and retreat during interglacial and glacial periods on the genetic diversity of closely related shallow-water benthic organisms from two different areas. In southern South America large areas of the Chilean coasts of Patagonia and Tierra del Fuego were covered with ice during the Last Glacial Maximum (LGM), thus inhabitable. The Magellan Strait for example did not exist during the LGM and only became inhabitable after glacial retreat that started around 9,000-14,000 years BP (Clapperton et al. 1995; McCulloch et al. 2000; Rostami et al. 2000; Hulton et al. 2002). Today no ice affects these coasts anymore. Along the Antarctic Peninsula, on the other side of the Drake Passage, the shallow-water coasts are still affected by ice even in this interglacial period. Especially in winter shallow-water communities are still heavily affected by ice foots and scouring ice bergs which should have drastic effects on intertidal organisms (Gutt 2001).

Limpets of the genus *Nacella* inhabit the rocky shores on both sides of the Drake Passage in Patagonia and the Antarctic Peninsula. The Antarctic limpet *Nacella concinna* inhabits the highly disturbed intertidal rocky shores along the Antarctic Peninsula and on the island of the Scotia Arc (Davenport 1988). In Southern South America mainly two nominal species of the genus *Nacella* can be found, the intertidal *N.magellanica* and the subtidal *N. deaurata.* These two nominal species are characterized by morphological differences like Apex position and shell heights (Pöhlmann et al. 2011). The genetic status of the South American species is not fully understood yet. Competing works exists stating that profound genetic divergence can be found (de Aranzamendi et al. 2009) or that there is no genetic differentiation between them (González-Wevar et al. 2010).

The focus of the present chapter is to understand the impact of glacial advances and retreat during interglacial and glacial periods on southern hemisphere shallow-water benthic organisms. We compared the genetic structure of populations that now occupy

habitats not affected by ice anymore (South America) and populations inhabiting coasts that are still heavily influenced on an annual cycle by sea ice formation and glacial advances (Antarctic Peninsula). We therefore sequenced a COI gene fragment of more than 400 samples and performed several statistical test to assess the genetic diversity and population structure of South American and Antarctic limpets of the genus *Nacella.*

Material and Methods

Sampling sites and preservation

Samples of *Nacella magellanica* and *N. deaurata* were taken from several locations along the distribution area from Northern Patagonia until Ushuaia (Fig. 1). On the Falkland Islands samples of *N. deaurata* were collected. Samples of *N. concinna* were taken from Jubany Station on King George Island and Rothera Station on Adelaide Island. Subtidal animals had to be taken by SCUBA diving, all other animals were collected as a whole during low tide. All samples were immediately preserved in 96% ethanol and the position of every sampled individual relative to the water level (intertidal or subtidal) was recorded. Species identification was conducted later on in the lab based on morphological and morphometric criteria (chapter 3.2) as well as based on microsatellite data (Pöhlmann and Held 2011).

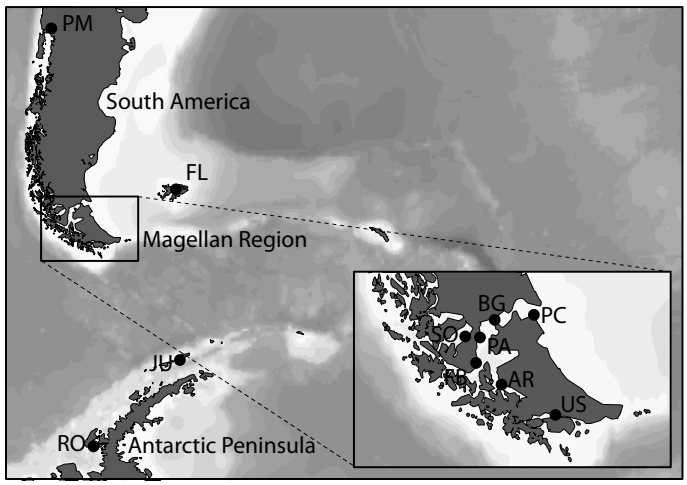

Fig. 1: Map with sampling sites

DNA extraction, gene amplification and sequencing

DNA was extracted with the QIAamp DNA Mini Kit (Qiagen), according to the manufacturers protocol for standard tissues. A small piece of foot tissue was dissected, digested overnight with Proteinase K and DNA was extracted subsequently via spin columns. A fragment of the 16S gene was amplified using the universal primers 16Sar and 16Sbr (Simon et al. 1994) in a total volume of 25 µl, containing approx. 10-300 ng genomic DNA, 1x HotMaster reaction buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 0.03 U/µl Hotmaster Taq (Eppendorf). Following PCR conditions were applied: 2 min 94°C, 38 cycles of 20 sec at 94°C, 20 sec at 52°C, 50 sec at 65°C and a final extension of 7 min at 65°C. Amplification of a COI gene fragment was carried out with the universal primers LCO1490 and HCO2198 (Folmer 1994). PCR reaction mix and program specifications were like those described for 16S. Only the annealing temperature was set to 45°C. Successful amplification of a fragment of the nuclear 18S gene was conducted using the primers AGM-18F and AGM-18R published by Harasewych and McArthur (2000). PCR reaction mix and conditions were as described for 16S with annealing temperature changed to 62°C and cycles reduced to 30. All PCR reactions were performed on an epgradient thermocycler (Eppendorf). PCR products were checked on 2% agarose gels and purified with the enzyme mix ExoSAPit (Fermentas) to degrade remaining primers and inactivate remaining dNTPs.

Cycle sequencing was carried out using the BigDye Terminator Kit 3.1 (Applied Biosystems) in 10 µl reactions containing 1 µl template DNA and 1 µM of each forward and reverse primer, respectively. Cycle sequencing conditions were as described in the manufacturer´s instructions. The fragments were purified with the DyeEx Kit (Qiagen) and subsequently sequenced on an ABI 3130xl sequencer.

Data Analysis

Sequence assembly and editing were conducted using the program Codon Code Aligner v3.5.7 (CodonCode Corporation, Dedham, MA, USA). The implemented ClustalW algorithm was applied to perform aligning of sequences. Collapsing of sequences into haplotypes was carried out online with the web based program fabox (http://gump.auburn.edu/srsantos/fabox).

A statistical parsimony network was calculated using TCS 1.21 (Clement et al. 2000). The connection limit was set to 40 steps to create one single network.

In order to analyze differences in genetic diversity in populations on both sides of the Drake Passage several indices were calculated with Arlequin v.3.5 (Excouffier et al. 2005), the haplotype diversity (h), the nucleotide diversity (π), and the population genetic estimators Theta (S) and Theta (π). Arlequin was further used to calculate pairwise F_{ST} values in order to investigate the presence of population structuring in populations of each continent. The setup was 100,000 MCMC (Markov Chain Monte Carlo) inferences and an initial burn in of 10,000. Significance between pairwise comparisons was tested by a permutation test as implemented in the program.

Demographic expansion was investigated to reveal whether glacial and interglacial periods had different impacts on population expansion and contradiction in South American, Falklands and in Antarctic limpets populations. Therefore we applied Tajima's D-Test (Tajima 1989) and Fu's F_s-Test (Fu 1997) of selective neutrality, all tests implemented in Arlequin v.3.5. Tajima's D-Test and Fu's F_s analyse whether all mutations are selectively neutral. Departures from neutrality which are indicated by an excess of low frequency polymorphisms result in significantly negative values of Tajima's D and Fu's F_s, indicating population size expansion and/or positive selection. The tests were conducted using parametric bootstrapping with 10,000 replicates.

Changes in effective population sizes in populations from South America, the Falkland Islands and Antarctica were analyzed using the Bayesian Skyline Plot implementation provided in Beast v1.5. A subset of data was used for the South American group comprising populations from the Magellan Province (SO, FB, AR, PA, BG) with low and insignificant pairwise F_{ST} values to exclude effects of population structuring on the demographic history reconstructions. Three independent runs with 20 millions MCMC steps each and a burn-in of 200,000 steps were conducted and combined. The uncorrelated exponential molecular clock was applied. Mutation rates for the South American and Falkland groups were 3.5%/Ma and 1.5%/Ma for the Antarctic group (Chapter 3.1). Plots were generated in Tracer v1.5 (Rambaut and Drummond 2007).

Results

Analyses of genetic diversity in populations from both sides of the Drake Passage revealed strong differences among the regions (Tab. 1). Genetic richness was much higher in the South American population (h = 0.69 and π = 0.0042) than in the population from the Falkland Islands (h = 0.5 and π = 0.0029) and the Antarctic Peninsula that showed the lowest genetic diversity (h = 0.54 and π = 0.0012). The two genetic diversity estimators of Theta resulted in the same pattern with highest diversity in South America (θ_s = 8.08 and θ_π = 2.05) and lowest in Antarctica (θ_s = 0.72 and θ_π = 0.6). A closer look at the genetic diversities of the different populations sampled in South America shows that the diversity is rather low in the population situated at the margins of the distribution area of the investigated South American *Nacella* species like Puerto Montt (PM) at the northern distribution limit, Ushuaia (US) at the southern end of Terra del Fuego and Punta Catalina (PC) at the Atlantic opening of the Magellan Strait (Tab. 1, Fig. 2). In the Magellan Province, which lies in the center of the limpets distribution area the genetic diversity was in general higher with the two populations of Seno Otway (SO) and Bahia Gregorio (BG) showing the highest genetic richness. The MDS plot based on the pairwise genetic distances (Fig. 4) showed a central clustering in among the populations of BG, PA and SO. The populations located at the ends of the investigated distribution area (PM, PC and US) exhibited the greatest distances to the central cluster.

Tab. 1: Estimated genetic diversity indices and the demographic expansion parameters Tajima's D and Fu's F_s. Significant values for D and F_s are indicated by * ($p < 0.05$) and ** ($p < 0.01$). N, number of sampled individuals; R, number of haplotypes; h, haplotype diversity; π, nucleotide diversity; AR, Puerto Arturo, BG, Bahia Gregorio; FB, Fuerte Bulnes; PA, Punta Arenas; PC, Punta Catalina; PM, Puerto Montt, SO, Seno Otway; US, Ushuaia; FL, Falklands; Ju, Jubany; RO, Rothera

Population	N	R	h	π	Theta (S)	Theta (π)	D	F_s
PM	41	10	0.565 ± 0.091	0.0018 ± 0.0015	2.104 ± 0.901	0.885 ± 0.702	-1.684*	-6.682**
SO	31	16	0.882 ± 0.047	0.0051 ± 0.0032	4.756 ± 1.777	2.473 ± 1.528	-1.653*	-9.624**
US	44	15	0.667 ± 0.078	0.0034 ± 0.0023	4.598 ± 1.626	1.629 ± 1.089	-2.102**	-9.566**
AR	25	10	0.69 ± 0.102	0.0045 ± 0.0029	3.178 ± 1.333	2.153 ± 1.378	-1.096	-3.386*
FB	57	16	0.64 ± 0.073	0.0041 ± 0.0026	3.903 ± 1.367	1.966 ± 1.253	-1.53*	-7.964**
PA	71	15	0.66 ± 0.163	0.0048 ± 0.0029	3.931 ± 1.33	2.336 ± 1.431	-1.221	-4.557*
BG	27	13	0.801 ± 0.077	0.0062 ± 0.0037	5.448 ± 2.043	2.974 ± 1.787	-1.623*	-4.822*
PC	37	12	0.691 ± 0.078	0.0032 ± 0.0022	3.114 ± 1.227	1.532 ± 1.044	-1.609*	-6.382**
FL	54	12	0.503 ± 0.083	0.0029 ± 0.0021	4.398 ± 1.514	1.396 ± 0.966	-2.149**	-5.703**
JU	18	4	0.543 ± 0.123	0.0013 ± 0.0012	0.872 ± 0.56	0.614 ± 0.569	-0.819	-1.209
RO	19	3	0.55 ± 0.099	0.0013 ± 0.0012	0.572 ± 0.427	0.608 ± 0.563	0.15	0.123
SA	348	69	0.695 ± 0.028	0.0042 ± 0.0026	8.089 ±1.927	2.045 ± 1.273	-2.131**	-26.956**
FL	54	12	0.503 ± 0.083	0.0029 ± 0.0021	4.398 ± 1.514	1.396 ± 0.966	-2.149**	-5.703**
ANT	37	4	0.535 ± 0.078	0.0012 ±0.0011	0.719 ± 0.449	0.601 ± 0.544	-0.365	-0.626

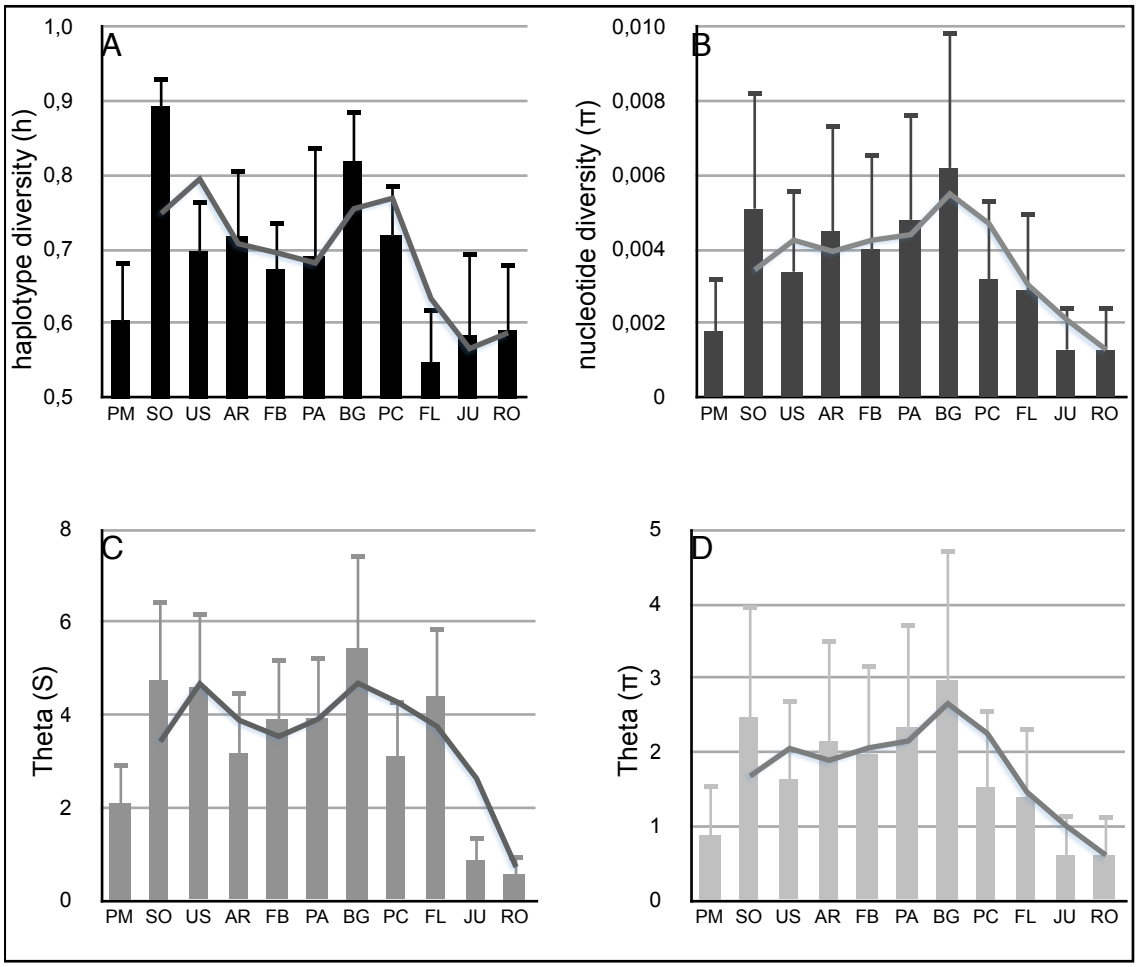

Fig. 2: Bar graphs of the four estimated genetic diversity indices h, π, θ_s and θ_π. The lines represent gliding mean trend lines. AR, Puerto Arturo, BG, Bahia Gregorio; FB, Fuerte Bulnes; PA, Punta Arenas; PC, Punta Catalina; PM, Puerto Montt, SO, Seno Otway; US, Ushuaia; FL, Falklands; Ju, Jubany; RO, Rothera

Tab. 2: Pairwise F_{ST} values. Significant values are bold ($p < 0.05$). AR, Puerto Arturo, BG, Bahia Gregorio; FB, Fuerte Bulnes; PA, Punta Arenas; PC, Punta Catalina; PM, Puerto Montt, SO, Seno Otway; US, Ushuaia; FL, Falklands; Ju, Jubany; RO, Rothera

Population	PM	SO	US	AR	FB	PA	BG	PC	FL	JU	RO
PM	⁕										
SO	0.033	⁕									
US	0.006	-0.004	⁕								
AR	**0.142**	0.068	**0.095**	⁕							
FB	0.071	0.05	0.054	-0.012	⁕						
PA	**0.098**	0.059	**0.075**	0.018	-0.006	⁕					
BG	0.037	-0.007	0.007	0.031	0.021	0.029	⁕				
PC	0.039	0.032	0.025	**0.133**	**0.079**	**0.105**	0.037	⁕			
FL	**0.814**	**0.745**	**0.777**	**0.749**	**0.743**	**0.715**	**0.727**	**0.783**	⁕		
JU	**0.982**	**0.959**	**0.969**	**0.966**	**0.963**	**0.956**	**0.954**	**0.972**	**0.973**	⁕	
RO	**0.982**	**0.96**	**0.97**	**0.967**	**0.964**	**0.956**	**0.955**	**0.972**	**0.973**	-0.035	⁕

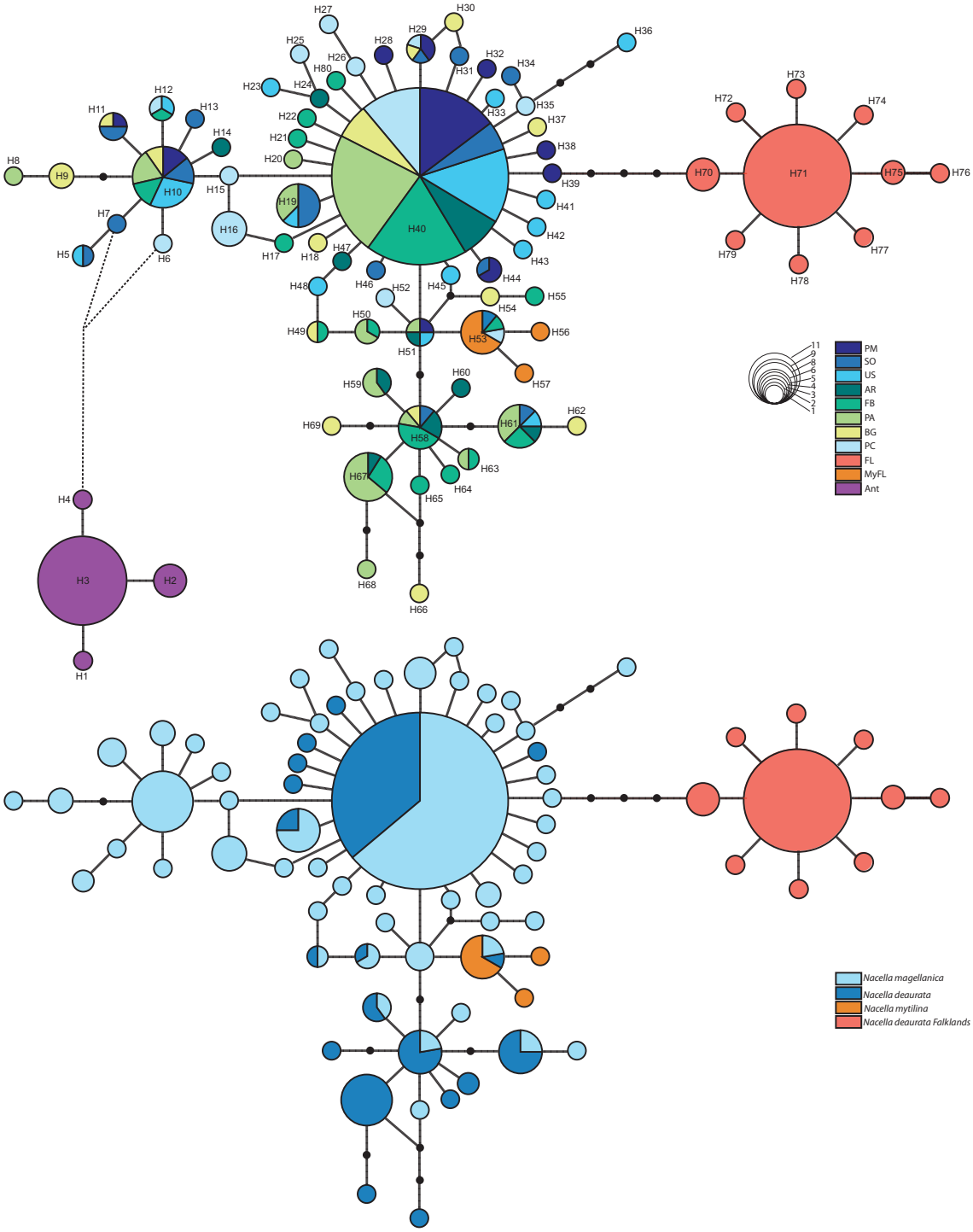

Fig. 3: Statistical parsimony network of COI haplotypes. A) Color code according to sampled populations. B) Color code according to nominal species. Black dots indicate missing haplotypes

The statistical parsimony network resulted in three major subnetworks. Two shallow ones were represented by the Antarctic populations (4 haplotypes, H1-H4) and the Falklands/Malvinas population (10 haplotypes, H70-H79). In between those two networks a more diverse one is located comprising all samples from South American mainland populations (65 haplotypes, H5-H69). There is no obvious structure in the

South American subnetwork, but H5 to H16 form a small subclade only consisting of samples of nominal *N. magellanica* and H58 to H69 represent a subclade mainly comprising *N. deaurata.* The apparent domination of *N. magellanica* haplotypes is a mere effect of a sampling bias towards *N. magellanica* samples.

The two investigated neutrality indices D and F_S were significantly negative ($p < 0.01$) for South American (D = -2.13, F_S = -26.96) and Falkland populations (D = -2.15; F_S = -5.7) indicating an excess of low frequency polymorphisms which can be interpreted as an indication of recent population expansion and/or positive selection. No such signs of population expansion could be found in Antarctic *Nacella* populations. Both D and F_S did not express a significant departure from neutrality. The results of Bayesian Skyline Plot analysis show a sharp increase in population size in South American Nacellids starting at around 14,000 years BP (Fig. 5). An increasing population size was also found in the Falkland population but started later at around 8,000 years resulting in present population sizes one order of magnitude smaller than that in South America. No pronounced increase in the effective population size could be detected in the Antarctic species *N. concinna.*

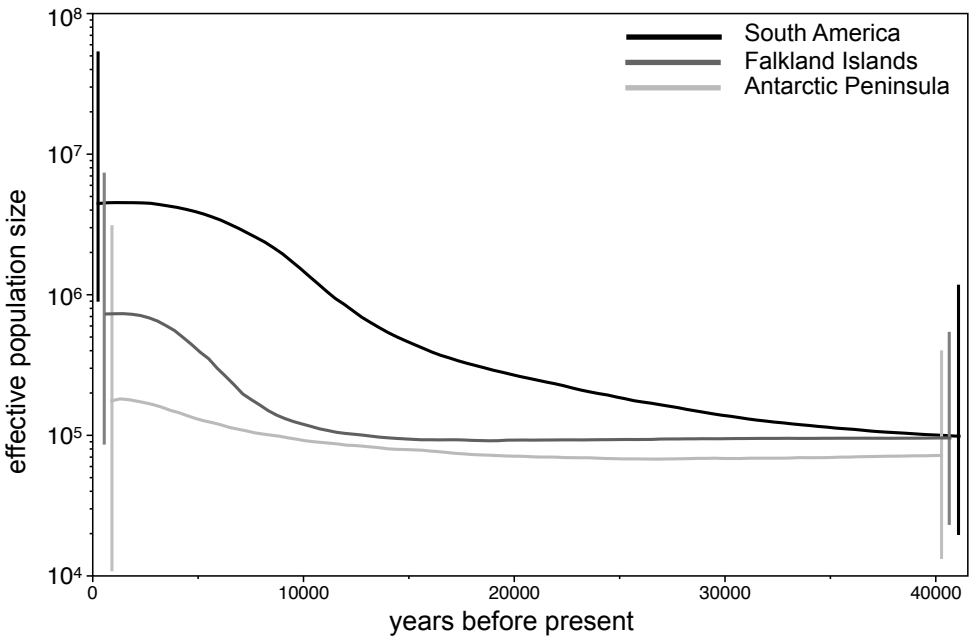

Fig. 4: Bayesian skyline plots demonstrating the effective population sizes (N_e) of *N. magellanica* and *N. deaurata* from South America (black line), *N. deaurata* from the Falkland Islands (dark grey line) and *N. concinna* from the Antarctic Peninsula (light grey line) from present to their most recent common ancestor. Bars at both ends of each timeline indicate the 95% confidence intervals (CI). Mutation rate was set to 1.55% per million years, according to Pöhlmann and Held, Chapter 3.1. All lines end at zero years before present. For a better visualization of the CI, they are shifted a little bit to avoid overlapping

Discussion

Here we present for the first time a comparative analysis of genetic diversity in organisms inhabiting the high intertidal zone of highly disturbed Antarctic coasts and undisturbed sub-Antarctic Patagonian coasts, with respect to sea ice. The results of our analysis of COI variation clearly demonstrate a severe decrease of genetic richness in shallow-water limpets across the Drake Passage. Populations of *N. concinna* on the Antarctic Peninsula exhibit a striking degree of genetic homogeneity and show no signs of post-glacial population expansions compared to South American Subantarctic populations of *N. magellanica* and *N. deaurata.* These findings suggest that the annual extent and retreat of glaciers and sea ice have a strong negative influence on the genetic structure of Antarctic shallow-water organisms.

Low genetic diversity in Antarctic limpets

In a variety of studies on the genetic structure of high latitude species that occupy areas affected by the LGM, reduced genetic diversities could be found (e.g. Hunter and Halanych 2008; Thornhill et al. 2008; Dahlgren et al. 2000; Larmuseau et al. 2009; Hewitt 1996, 2000). Typical for those species is a flat and star-like haplotype network pattern with one dominating haplotype surrounded by several to many low frequency haplotypes, indicating recent mutations. Such patterns are explained by the loss of genetic variability during survival in refuge areas where populations sizes are small and genetic drift and inbreeding are high, which together reduce the genetic diversity (Hewitt 2000). The excess of low frequency polymorphisms together with negative Tajima‘s D and Fu‘s F_S values (Tajima 1989; Fu 1997) are generally caused by population expansion. Such expansion events are most likely directly connected to the availability of habitat with retreat of ice at the end of glacial maxima (Hewitt 2000, Thatje et al. 2005). Population expansion events linked to glacial retreat were found in a variety of taxa on both hemispheres (e.g. European sand goby, Larmuseau et al. 2009; North American Nearctic and Palearctic fishes, Bernatchez and Wilson 1998; Antarctic Nemerteans, Thornhill et al. 2008).

The extremely low genetic diversities and the absence of typical signs for post-glacial population expansion in Antarctic *N. concinna* can be explained two-fold (Tab. 1, Fig.

4). Grounding sea ice during winter and elevated ice scouring in spring and autumn have strong effects on the genetic population structure of *N. concinna* by reducing effective population sizes. That prevents new mutations from getting fixed due to larger impacts of genetic drift and higher inbreeding due to habitat fragmentation (Fraser et al. 2009). The absence of any genetic differentiation between populations from Jubany, King George Island, and Rothera, Adelaide Island, which are more than 700 km apart furthermore suggests a second explanation for the low genetic diversity in Antarctic limpets. The broadcast-spawning life history equips *N. concinna* with high dispersal capacities that might prevent local adaptations and override local mutations by strong gene flow (Hoffman et al. 2010a). The authors concluded on the basis of the results of their AFLP analysis that *N. concinna* along the Antarctic Peninsula belong to one single panmictic population, where strong north- and southward currents facilitate larval dispersal across such large distances (Moffat et al. 2008; Savidge and Amft 2009). It has been stated that in other taxa with brooding behavior the survival in different refuge areas and the subsequent independent evolution of unique polymorphisms is maintained even after glacial maxima end, due to low dispersal capacities and therefore low chances of gene pool mixing (Wilson et al. 2009). Consequently, our data might furthermore indicate that limpet populations from King George Island and Adelaide Island originated from the same glacial refuge area during the LGM. High gene flow prevents population differentiation among populations along the Antarctic Peninsula.
Another possibility for low genetic richness is that polar climate slows mutation rates due to lower metabolic rates in the cold. However, Held (2001) could not find any correlation between environmental temperature and mutation rates so that we neglect this possibility.

High genetic diversity in Patagonian limpets

Calculations of genetic richness show rather high diversities in South American limpet populations compared to the two populations along the Antarctic Peninsula and demographic analyses reveal strong population expansion correlating with the end of the LGM 10,000 years BP (Tab. 1, Fig. 4). These results suggest a typical pattern of post-glacial recolonization and expansion, as seen in a variety of northern hemisphere species (see above). Surprisingly, the populations with the highest genetic richness are

those located in the Magellan Strait. Such distribution of genetic variability is rather counterintuitive because the central Magellan region was not inhabitable during the LGM and was the last habitat to become available again after the LGM ended. Therefore, we would expect signs of founder effects in *Nacella* populations located in Magellan Strait. Leese and co-workers (2008) suggest an elegant solution for the understanding of this phenomenon, as they got similar results in a study of benthic isopods of the genus *Serolis* displaying a distribution comparable to *Nacella.* The existence of different refuge areas on both the Pacific and the Atlantic Patagonian coasts with subsequent recolonization of the Magellan Strait after the LGM might have resulted in the mixture of genetic polymorphisms evolved in each of these refuge areas causing inflation of genetic variability of populations at the center of the Magellan Strait. A possible support for this theory might lay in the COI haplotype network (Fig. 3). Although the major haplotypes are shared by both *N. magellanica* and *N. deaurata* there are two smaller subclades mainly consisting of either one or the other species. This could indicate that certain haplotypes originated during phases of allopatric speciation and show a slight degree of differentiation between both nominal species. The explanation fits the results of the single genetic diversity estimates for each investigated population, that are lowest in the populations at putative refuge locations in northern PM and eastern FL (Chapter 4.1), areas that were not much affected by ice during the LGM (Clapperton 1990; Rostami et al. 2000; Hulton et al. 2002).

Conclusion and future perspectives

One important question that has been asked in Antarctic research is, what will happen to Antarctic marine species under an ongoing global warming scenario leading to further retreat of ice and the permanent availability of high intertidal habitats throughout the year. Taking South America as a model scenario for what could happen in Antarctica under ongoing global warming, *N. concinna* could be considered a winner of climate change. In South America the retreat of ice and the subsequent availability of new habitats led to population expansion and increasing genetic diversity in investigated *N. magellanica* and *N. deaurata* where populations from different refugia meet. Ice free coastal areas in the whole Antarctic Peninsula throughout the year could therefore also lead to such increases of population sizes and genetic diversities as seen in South American limpets. Presently, no such expansion can be seen over the last 20,000 years in the Antarctic *Nacella*, likely as a consequence of annual glacial advances and sea ice scouring in winter leading to reduction of genetic diversity by loss of rare alleles through genetic drift and higher inbreeding by habitat fragmentation (Fraser et al. 2009). However, the observed low genetic diversities in *N. concinna* leave room for speculation about how reduced species fitness and low potential for adaptation could significantly decrease the survivability of *N. concinna* in a fast changing environment like the Antarctic Peninsula (Booy et al. 2000, Reed and Frankham 2002). Bearing in mind the results of Chapter 3.1 that the ACC might not be such a strict barrier to gene flow across the Drake Passage, invasion of genetically more diverse South American limpets into Antarctica could become a threat for *N. concinna* when Antarctica climate further warms up. Definitely, more genetic research comprising more markers with different evolutionary rates will be needed to make profound assumptions on the future of *N. concinna.*

Chapter 5

Concluding discussion

5. Concluding discussion

In this concluding chapter the major advances of this thesis‘ results will be highlighted divided in three major aspects. First, the great advantages and additional insights of multimarker approaches in evolutionary research will be discussed. Then, I will dedicate the second and third part to the impacts of geographical isolation and local adaptation in speciation processes in marine organisms with a comprehensive reconstruction of the evolutionary history of *Nacella*.

5.1 The power of combined molecular markers in evolutionary studies

One of the major keys to understanding evolution is the process of speciation (Turelli et al. 2001; Coyne and Orr 2004). The importance of natural selection in speciation was introduced 150 years ago in the book of Charles Darwin (1859) and by now its concept is widely accepted (Coyne and Orr 2004). The basic understanding of how speciation is mediated by natural selection is, however, still not very well-resolved (Schluter 2001, 2009; Via and West 2008). On the one hand classical advances in speciation studies have been accomplished at *species-level* (Mayr 1942) that show how gene flow barriers can have promoted speciation in the past. On the other hand, investigations of well-defined species as the final ‘product‘ of historical speciation processes render assumptions on the importance of natural selection difficult. When a speciation process is complete, it is hard to infer which genetic traits contributed to the reproductive isolation and which arose after the speciation process had reached completeness (Via 2009).

An alternative approach to understand how selection promotes speciation is to study diverging populations to identify the impacts of ecology and genetics that cause barriers to gene flow. In this *population-level* analysis the focus is on identifying the genes that contribute to final reproductive barriers (Via 2001; Coyne and Orr 2004; Via 2009). Most of the molecular approaches that reveal patterns of genetic diversity in population-level studies are exclusively based on neutral molecular markers which do not allow predictions on natural selection (Reed and Frankham 2001). Thus, a comparison between neutral loci and those loci that are under potential selection is the prime method to reveal existing selective pressure and patterns of adaptive divergence among

populations and relate them to baseline neutral gene flow (Ward et al. 1994; Canino et al. 2005; Hemmer-Hansen et al. 2007).

One aim of my thesis was to demonstrate the benefits from combined neutral markers and markers under selection with studies of physiological adaptation. As an example for additional insight that can be gained by multimarker approaches, I will address the question whether or not the two South American limpets *N. magellanica* and *N. deaurata* are two different, reproductively isolated species making the premise assumption that only one marker type is applied.

Genetic markers applied in this thesis such as COI, 16S, 18S and microsatellites that are assumed to be neutral do all reveal genetic homogeneity without a break according to the proposed species limits. Although microsatellites and the three marker genes differ in their mutation rates by several orders of magnitude, the effects of gene flow and random genetic drift normally show similar patterns across neutral loci (Buonaccorsi et al. 2001; Canino et al. 2005). As long as gene flow is not restricted, a genetic divergence among populations is usually not detected with neutral markers (Hemmer-Hansen et al. 2007).

Tab. 1: Overview of different markers applied in the course of this thesis to understand the evolutionary processes in the South American *Nacella* species. The neutral genetic markers failed to reveal any genetic differentiation between *N. magellanica* and *N. deaurata* defining them as one interbreeding population. In contrast, genotypic and phenotypic markers under selection showed considerable divergence between both nominal species

selective neutrality	marker type	marker	*N. magellanica*	*N. deaurata*
neutral	genetic, mtDNA	COI	No genetic differentiation	
	genetic nuclear DNA	Microsatellites		
under selection	genetic, nuclear DNA	Hsp70	Allele group Hsp1	Allele group Hsp2
	phenotypic, morphological	Shell morphology	Shells thick and big	Shells thin and flat
	phenotypic, enzyme activitiy	SOD, CAT	low stress response	high stress response
	phenotypic, gene expression	Hsp70	low stress response	high stress response

Phenotypic markers under selection like shell morphology and the stress response measured as enzyme activities of SOD and CAT or Hsp70 gene regulation show strong divergence not only between *N. magellanica* and *N. deaurata* but also among populations of the same species. The response to air exposure in *N. magellanica* and *N. deaurata* (chapter 2.2) confirmed that environmental temperature and desiccation stress upon tidal uncovering are two important factors that determine the vertical distribution of the investigated species. Where *N. magellanica* can inhabit intertidal coasts in the sub-polar Magellan Strait, at the northern distribution boundary in Puerto Montt temperature stress is too high during periods of low tides, as shown by the vast increase of Hsp70 genes in chapter 2.2. In the Magellan Strait *N. magellanica* furthermore displays a much better adaptation to tidal air exposure than *N. deaurata*, which is only found in the shallow-subtidal. Phenotypic traits can help to identify the stressors acting on populations but do not allow to distinguish genetic divergence from phenotypic plasticity. With respect to *Nacella*, the strong differences in shell morphology as well as physiological parameters seem suggestive but are no proof for restricted gene flow between the nominal species. Instead the strong differences might as well be caused by a selective advantage of higher shells in the shallow intertidal. Moreover, phenotypic plasticity can allow for increased gene flow between selective regimes, by enabling individuals to phenotypically adapt to alternate environments (Crispo and Chapman 2008).

When reproduction between divergent populations is not restricted, establishment of certain regions on the genome that resist gene flow and maintain differentiation is likely only when ecological divergent selection is strong enough (Via 2001; Hendry et al. 2007). Rice and Hostert (1993) stated that speciation with gene flow only needs the involvement of a handful of genes to promote adaptive divergence. That implies that genomes of incipient species are largely homogenized by continued gene flow and remain genetically similar. In ecologically specialized populations, divergent selection on traits associated with the use of habitat is strong enough to maintain divergence in the respective parts of the genome, while gene flow continues in other genomic sections. It is the challenge to identify those key loci involved in divergent selection in a given species to make profound statements on the evolutionary development of its diverging populations. The third marker type applied in this thesis, a fragment of a

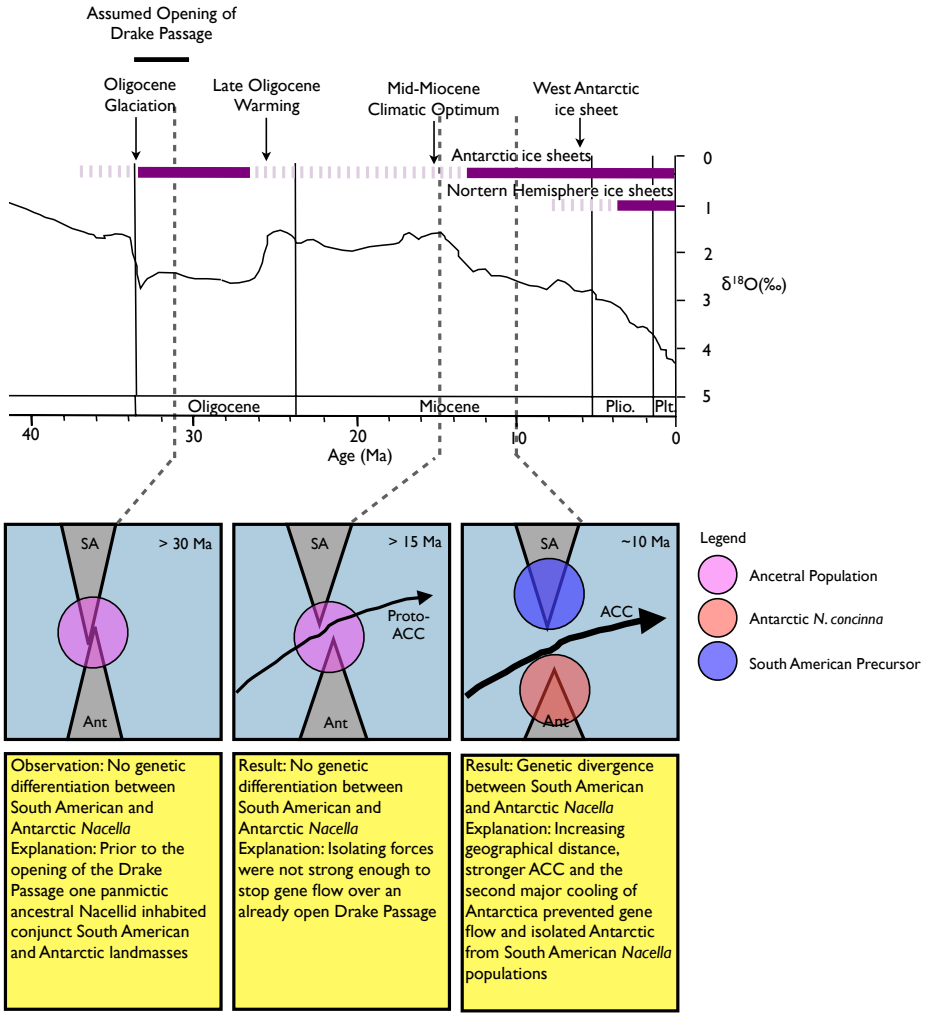

Fig. 1: Reconstruction of the divergence of South American and Antarctic *Nacella*. ACC, Antarctic Circumpolar Current; Ant, Antarctic Peninsula; SA, South America. Climate curve showing the change during the last 40 million years, inferred from oxygen isotopes, was adapted from Poulin et al. (2002)

Hsp70 gene, represents such a genotypic locus that is very likely under strong selection (Hemmer-Hansen et al. 2007). The evaluation of the results regarding the population structure of *N. magellanica* and *N. deaurata* obtained by the Hsp70 gene shows a complete opposite picture to the one obtained with the neutral markers. The allele distribution of Hsp70 displays a clear divergence between the two nominal species supporting the assumption of restricted gene flow, if no other markers would have been tested. The application of marker genes under selection could identify a genetic discordance between *N. magellanica* and *N. deaurata* most likely summoned and maintained by divergent selection that was undetected by the neutral markers.

When comparing the results obtained by the different markers applied, the question arises whether or not a gene flow barrier exists between populations or ecotypes and which factors decide over the dispersal of some loci and restrict it for others in the limpets. A deeper evaluation of these ambiguous result with interpretations regarding

the evolutionary history of South American and Antarctic limpets of the genus *Nacella* will be given in the following chapters.

5.2 Geographical barriers to gene flow - an obvious case?

Allopatric speciation is considered the most plausible and frequent mode of speciation (Coyne and Orr 2004). The concept of evolution in allopatry requires some sort of geographical barriers that separate populations of a formerly interbreeding species, where disjunct populations evolved independently over time of adaptation in diverse habitats and random processes of genetic drift. Although the physical barriers that prevent gene flow are indispensable for allopatric speciation, mostly almost nothing is known about the particular barriers that are involved in a given speciation process (Turelli et al. 2001). Therefore, I tested the actual importance of gene flow barriers in the evolution of South American and Antarctic limpets from the genus *Nacella*, today separated by one of the most prominent physical dispersal barriers in the ocean, the Antarctic Circumpolar Current (ACC). The ACC is believed to represent a geographical barrier to biological exchange between the Antarctic and surrounding continents (Crame 1999; Clarke et al. 2005; Thatje et al. 2005) and is, together with large geographical distances to circumjacent continents, assumed to be the major reason for the isolation of Antarctica that led to speciation in allopatry and the high degree of endemism on the Antarctic shelf.

The results presented in chapter 3.1 support the assumption that the Southern Ocean is presently isolated from South America without any gene flow across the Drake Passage rendering the ACC a functioning contemporary barrier to genetic exchange between South American and Antarctic Nacellids (Fig. 1). This scenario resembles the classical *species-level* investigations on speciation events and suggests that the appearance of the gene flow barrier is the cause of speciation. Therefore, the time of divergence between South American and Antarctic *Nacella* should concur with the formation time of the barrier. Our calculated divergence time estimates of South American and Antarctic limpets, however, conflict with the common hypothesis of vicariant speciation in the Southern Ocean caused by the opening of the Drake Passage and the subsequent onset of the ACC 23 - 35 Ma BP (Barker and Burrell 1977; Pfuhl and McCave 2005;

Livermore et al. 2007; Lagabrielle et al. 2009). The Antarctic Circumpolar Current is amongst the strongest currents on this planet but its supposed role to be the major reason for the isolation of the Antarctic continent must be questioned. Regardless which method we applied, never were our estimate of divergence times between South American and Antarctic *Nacella* were never older than 10 Ma (chapter 3.1).

One might conjecture that *Nacella* describes a special case where high dispersal capacities could have allowed to surmount the ACC long after its formation. However, divergence times of other South American and Antarctic sibling species were found to be in the range of that of *Nacella* in a variety of different taxa spanning all kinds of reproductive modes. Divergence time estimates younger than 23 Ma were found not only in species with pelagic larvae and consequently with the potential of long distance dispersal like *Nacella* or the ribbon worm *Parbolasia corrugatus* (Thornhill et al. 2008) but also in brooding bivalves from the genus *Limatula* (Page and Linse 2002) or the brittle star *Astrotoma agassizii* (Hunter and Halanych 2008, see chapter 3.1). Furthermore, also the pelagic Euphausiacea *Euphausia superba* from south of the Polar Front and *Euphausia vallentini* from north of it and the benthopelagic and moderately active Notothenioid fishes *Lepidonotothen nudifrons* from Antarctica and *Patagonotothen tessellata* from South America are estimated to have diverged much later than the onset of the ACC (Patarnello et al. 1996; Bargelloni et al. 2000). In fact, of all studies that calculated species divergence times between South America and Antarctica none came up with divergence dates older than 15 Ma (see chapter 3.1 for details). To the contrary, the more modern and sophisticated geological techniques tend to redate the onset of the ACC to later dates as investigation progresses. The most recent publications by Livermore et al. (2007) and Lagabrielle et al. (2009) both date the onset of the ACC to around 30 Ma BP.

It cannot be ruled out that the ACC was in general isolating Antarctica since its formation and that there was a single migration event from South America to Antarctica around 10 Ma BP. This scenario is, to my conception, somewhat weakened by the fact that similar divergence times were found in a variety of investigated South American and Antarctic sister species with different life styles and reproductive modes. A mass migration event of South American biota into Antarctica is hard to imagine given the

fact that it was at a time of the second major cooling of Antarctica, where strongly different selective pressures must have been imposed on organisms on both sides of the Drake Passage.

Among several, feasible explanations two stick out: I) Fluctuations in the strength of the ACC due to periods of constriction during the Oligocene to mid-Miocene (29 - 15 Ma, Lagabrielle et al. 2009) allowed for migration over the ACC, and II) fluctuations in main flow routes enabled stepwise and delayed gene flow between South America and Antarctica through the islands of the Scotia Arc (Livermore et al. 2007). As an effective isolation of the Antarctic ocean did not start before the second major cooling event of Antarctica at 15 Ma (Flower and Kennett 1995), I conjecture that gene flow between both continents was diminished due to growing climate discrepancies that forced divergent selection and led to the evolution of cold adaptation and prevented warm adapted species to colonize the Antarctic.

This influence of temperature to set the biogeographical border of a distribution area can also be seen at the northern distribution edge Southern American *N. magellanica.* Although the potential habitat continues to the north, the distribution only extends into Puerto Montt at 42°S. The physiological stress experiments presented in chapter 2.2 demonstrate that *N. magellanica* lives at their border of the thermal stress tolerance range at the northern edge of the distribution area. When exposed to arial conditions, Puerto Montt animals react with a heat-shock response to endure phases of thermal stress that is around 10 times higher than that of *N. magellanica* at the center of the distribution area in the Magellan Strait, where the thermal stress is rather limited. The energy expenses to cope with thermal stress at the northern distribution boundary are at the limits of what these animals can sustain and therefore temperature most likely represents the main limiting factor for migration further northwards. Therefore, temperature could also have been among the important factors that restricted gene flow into Antarctica after the second global cooling event around 15 Ma BP.

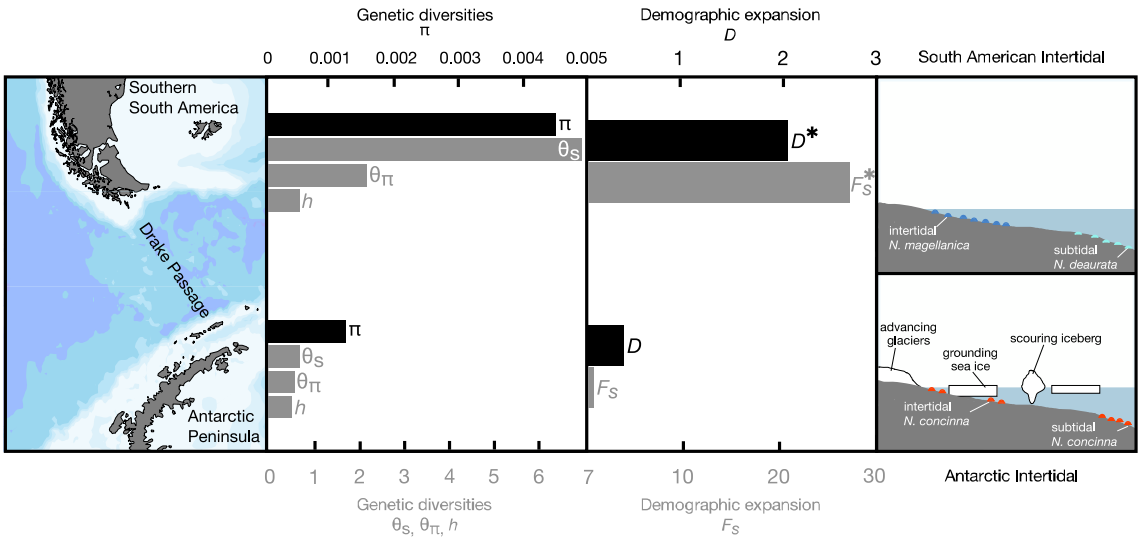

Fig. 2: Comparison of several genetic diversity and demographic expansion indices estimated for South American and Antarctic limpets of the genus *Nacella*. On the right side is the different impact on sea and glacial ice on the intertidal zones in South America and the Antarctic Peninsula depicted. Black bars refer to the upper axis, grey bars to the lower axis. π, nucleotide diversity; *h*, haplotype diversity; θ_s, and θ_π, population genetic estimator Theta based on segregating sites and pairwise nucleotide polymorphisms, respectively; *D,* Tajima's *D*; F_s, Fu's F_s

Testing the efficiency of gene flow barriers is especially important on the background of global warming. It has been demonstrated that species move towards lower latitudes in times of glacial periods (Hewitt 2000), which raises the question how species distributions will alter under global change. The results of my thesis raise concern about the isolating capabilities of the ACC in protecting the unique Antarctic Ocean fauna from the invasion of South American species (Clarke et al. 2005), especially as climate change is accelerating in Antarctica. Antarctic intertidal species such as *N. concinna* are heavily influenced by grounding sea ice and elevated ice scouring in spring and autumn (Fig. 2). Sea ice can have strong effects on the genetic population structure by reducing effective population sizes, preventing new mutations from getting fixed due to larger impacts of genetic drift and higher inbreeding due to habitat fragmentation (Fraser et al. 2009). The observed low genetic diversities in Antarctic *N. concinna* (Fig. 2 and chapter 4.1 for more details) leave room for speculation as to how reduced species fitness and low potential for adaptation could significantly decrease the survivability of *N. concinna* in a quickly changing environment such as the Antarctic Peninsula (Booy et al. 2000; Reed and Frankham 2002). Invasion of genetically more diverse South American limpets into Antarctica could become a threat for *N. concinna* when Antarctica climate further warms up (Fig. 2).

5.3 The recent evolutionary history of the Patagonian limpets: local adaptation and gene flow

The South American nominal species *N. magellanica* and *N. deaurata* have repeatedly been subject to the question whether they represent different, reproductively isolated species or not (Valdovinos and Rüth 2005; de Aranzamendi et al. 2009; González-Wevar et al. 2010). Both morphotypes occur in the shallow-water zone of the Patagonian rocky shores with overlapping vertical zonation. The most intriguing question is: if the two morphotypes truly are two different species how has a reproductive barrier established in the first place in species that stand out by high dispersal capabilities living in an area without clear gene flow barriers?

The scarcity of strict geographical gene flow barriers in the marine system and the uncertainty regarding the isolation capability of identified barriers, such as the ACC described above, have fueled the controversial discussion that alternative ways of speciation must be possible in the ocean. A growing body of literature in the last decade has highlighted sympatric speciation as the alternative mode of speciation in which geographically overlapping populations diverge because of selection on microhabitat scales (Schluter 2001; Rolán-Alvarez 2007; Fitzpatrick et al. 2008, 2009; Mallet et al. 2009). So far, however, only few examples actually confirm such a way of speciation (Rolán-Alvarez 2007). Coyne and Orr (2004) described four criteria that must be satisfied to identify sympatric speciation: (1) species must have largely overlapping geographical ranges, (2) the speciation process must be complete, (3) clades thought to arise via sympatric speciation must be sister species or monophyletic groups and (4) evolutionary history must make existence of an allopatric phase very unlikely. In empirical studies dedicated to prove the existence of sympatric speciation especially criteria 2 and 4 are difficult to be completely ruled out.

Fitzpatrick and colleagues (2009) demonstrated that also among the most prominent examples of sympatric speciation such as the fruit flies *Rhagoletis pomonella* (Feder et al. 2003), the cychlid fishes *Amphilophus zaliosus* and *A. citrinellus* (Barluenga et al. 2006), or the walking-stick insects *Timema cristinae* (Nosil 2009) some uncertainties exist regarding their classification as sympatric speciation according to the Coyne and Orr criteria. Therefore, Fitzpatrick et al. (2009) point out that the whole discussion on allopatric versus sympatric speciation can mislead the understanding of evolution when

the true question should be about the influence of natural selection on speciation processes. The assumption that sympatric speciation is rare in nature because it can rarely be found in empirical studies does not at all imply that natural selection driving local adaptation is a negligible force in speciation processes (Schluter 2001; Fitzpatrick 2002; Fitzpatrick et al. 2009). Studies on speciation processes are not of minor importance, when sympatry cannot be proven as the understanding of "historical biogeography, contemporary spatial structure, natural selection and gene flow helps to understand the origin and maintenance of biological diversity" (Fitzpatrick et al. 2009).

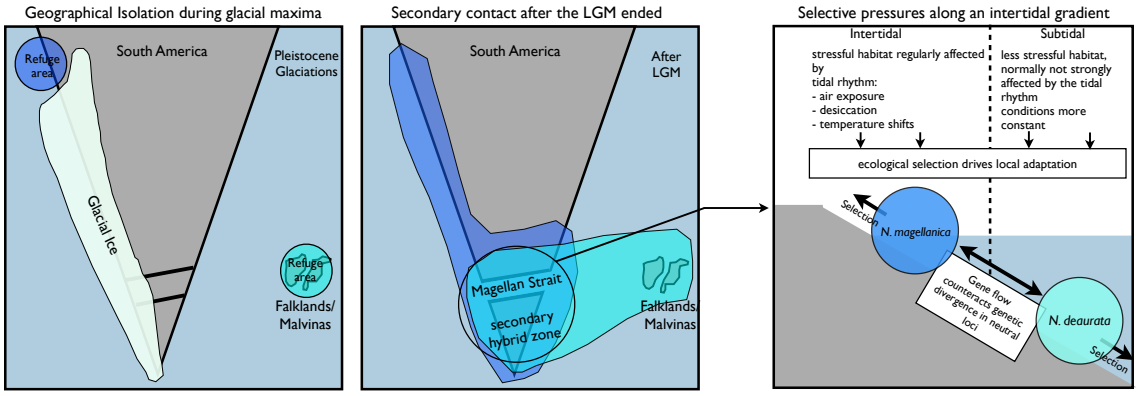

Fig. 3: Graphical demonstration of recent and contemporary events shaping the evolution of South American *Nacella magellanica* and *N. deaurata*. Ice extent during Pleistocene glaciations separated populations on Pacific and Atlantic sides of South America (left). After the Last Glacial Maximum (LGM) ended populations from either side of the continent migrated into the Magellan Strait and formed a secondary hybrid zone (middle). Ecological selection between shallow-intertidal *N. magellanica* and subtidal *N. deaurata* populations promotes local adaptation (right) as seen in strong divergence in investigated markers under selection. A reproductive barrier is not fully established because neutral markers indicate ongoing gene flow between both morphotypes

The *Nacella* species complex exemplifies how an interplay of historical phases of allopatry and contemporary selective divergence in sympatry both shaped the population structure of South American limpets on different time scales (Fig. 3). During the Pleistocene glaciations (2 Ma - 10 kyears BP) gene pools on either side of the South American continent in the Pacific and the Atlantic ocean were repeatedly geographically separated in phases of glacial maxima (Fig 3, left panel). After the LGM ended around 10 kyears ago (Clapperton et al. 1995; McCulloch et al. 2000; Rostami et al. 2000; Hulton et al. 2002), the Magellan Strait that serves as a direct connection between the Atlantic and the Pacific Ocean became inhabitable again and was recolonized by *Nacella* from both sides (Fig. 3, middle panel). Thus, we are looking at two important

time points. One is the separation of a once contiguous gene pool during glacial maxima. This separation affected the whole genomes of Atlantic and Pacific surviving populations. The second is the secondary contact after the LGM ended and here the investigated genetic markers show two completely different pictures depending on whether they are neutral or under selection.

The neutral markers COI and microsatellites showed no hint for gene flow restrictions between *N. magellanica* and *N. deaurata* characterizing Southern South America as a high gene flow environment inhabited by one panmictic *Nacella* population (chapter 3.2).

The results obtained from the analysis of the genetic marker under selection Hsp70 (Fig. 6 in chapter 3.2), however, revealed a clear bimodal distribution of the two main Hsp70 allele clusters with one being dominant in *N. magellanica* (Hsp1) and the other in *N. deaurata* (Hsp2). Populations of *N. magellanica* along the Pacific coast consisted entirely of the Hsp1 group and the Atlantic population on the Falkland Islands/Malvinas consisting entirely of *N. deaurata* was dominated by the Hsp2 allele group. The gradual increase of hybrid *N. magellanica* individuals to the eastern Atlantic opening of the Magellan Strait carrying one or even both alleles of the Hsp2 group suggest that this allele group originated in the Pacific Ocean and dispersed eastward after the LGM ended and surviving Pacific limpet populations recolonized the Magellan Strait. The Hsp2 group, dominant in *N. deaurata*, shows an opposite distribution pattern suggesting an Atlantic origin. The clear genetic differences in the Hsp70 gene on either side of South America are best explained in the way that the geographical separation in times of glacial maxima let to the divergence in Hsp70 alleles by random processes like genetic drift that might have been accelerated by locally different selective pressures. What is now described as *N. magellanica* survived in refuge areas on the Pacific side, whereas *N. deaurata* originated in refuge areas on the Atlantic side.

Taking into account the results of both marker types, neutral and under selection, the best explanation for the evolutionary history of South American limpets is that a genetic divergence was built in phases of allopatry during glacial maxima that did not break down at secondary contact in parts of the genome. The fact that no genetic differentiation between *N. magellanica* and *N. deaurata* can be seen in neutral markers and that hybrid individuals of both morphotypes can be found carrying one or both

Hsp70 alleles typical for the other morphotype indicate that this reproductive isolation is not complete and that hybridization is still possible. The selective pressure may lie on the hybrids, when hybridizing reproduction produces unfit or sterile offspring The divergent habitat use of both morphotypes in the Magellan Strait can also lead to sexual selection by reduced chances of interspecific fertilization. The probability that male and female gametes of the same morphotype meet would be higher than melting with a gamete of the other morphotype. On the other hand, selection could also act on the production of hybrids by restricted gamete recognition (Palumbi 1994). Asynchronous times of gamete release can further decrease the chance of hybridization as it has been shown in sympatric Caribbean corals (Fukami et al. 2004; Levitan et al. 2004). The increase of pre-zygotic, reinforcing selection is thought to be especially important in speciation where initial assortative mating is already high because otherwise high gene flow would possibly break down associations of loci responsible for hybrid fitness (Dobzhansky 1951; Felsenstein 1981; Liou and Price 1994). In the *Nacella* case such an initial assortative mating could have been summoned by divergent evolution in phases of geographical isolation. Figure 4 describes how differences in gene flow levels between neutral markers and those under selection can be facilitated and maintained when natural selection acts on hybrids reducing their reproductive success.

The morphological analyses presented in the chapters 2.2 and 3.2 provide additional evidence for reinforcing selection. On the Falklands/Malvinas *N. deaurata* occupies a broader vertical depth range and reaches much greater shell sizes than in the Magellan Strait, where it is restricted to the shallow subtidal. *N. magellanica*, on the other hand, has considerable smaller shells at Puerto Montt than at Punta Arenas. Concretely, in Punta Arenas, an area where both morphotypes occur in sympatry, body sizes show extreme divergence between both morphotypes opposite to regions where only one of the morphotypes occurs (Fig. 8 in chapter 3.2). In sympatry the survival chances of extreme morphotypes might guarantee best chances for survival as a consequence of habitat specialization and habitat extension of the whole complex highlighting the importance of ecological factors for speciation in sympatry. Gene flow among limpets from different shore levels does not cease completely as seen in the microsatellite data and in the Hsp70 alleles but ecological selection maintains the genetic divergence between *N. magellanica* and *N. deaurata* (Nosil 2008; Nosil et al. 2009).

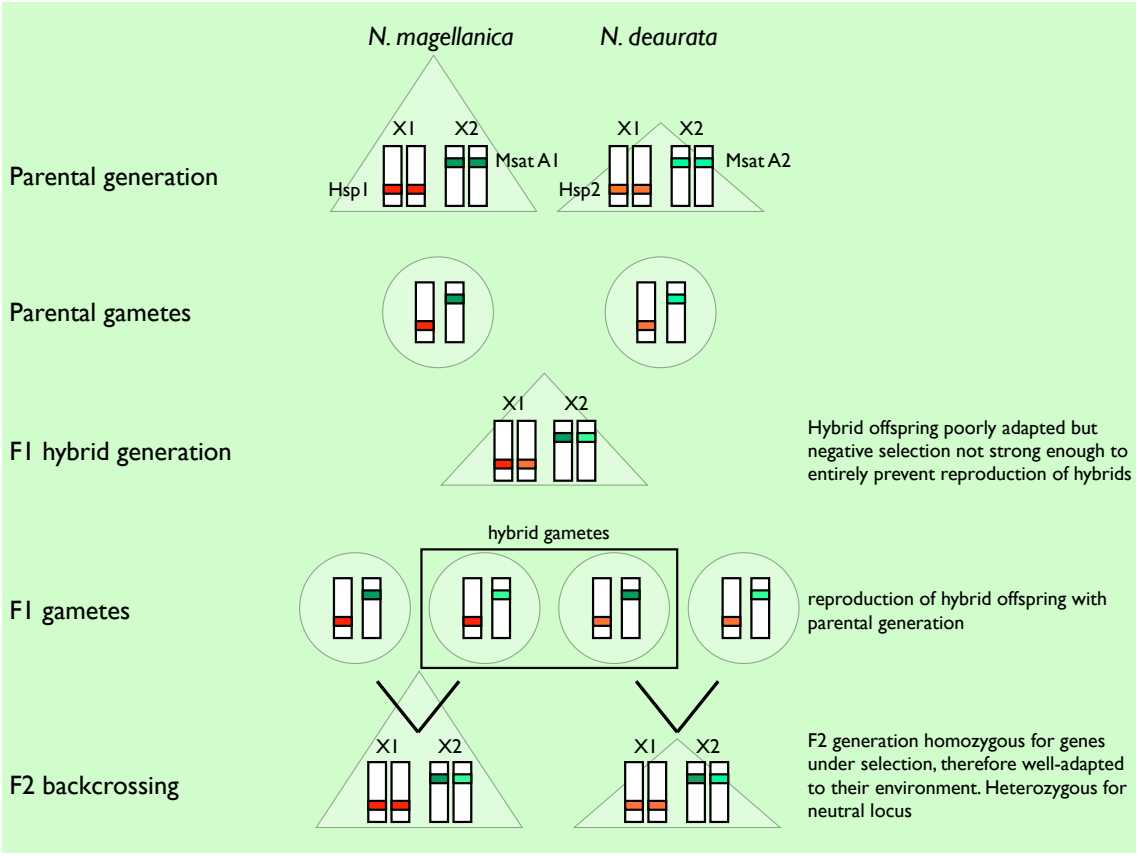

Fig. 4: One possible way of how alleles of neutral loci can be transferred between populations that show strong diverging selection on other alleles. In this example, mating of one homozygote *N. magellanica* carrying Hsp1 (under selection) and MsatA1 (neutral) alleles and one homozygote *N, deaurata* with Hsp2 and MsatA2 would produce hybrid F1 offspring. The gametes that are produced by the F1 hybrid generation can either reflect the parental gametes or be hybrid, carrying the Hsp allele typical for *N. magellanica* and the *N. deaurata* microsatellite allele or vice versa. Melting of a hybrid gamete with a parental gamete would then produce a F2 offspring that will be homozygote for the selected allele, thus adapted to either the intertidal or the subtidal, and heterozygote for the microsatellite locus. That way, neutral loci can be transported between the two ecotypes when gene flow is not totally restricted and the selection against hybrid offspring is not strong enough to entirely prevent them from reproducing themselves. An investigation of only one of the two loci would therefore lead to two different results, where gene under selection describes a clear divergences whereas the neutral locus indicates high gene flow

The physiological stress experiments in chapter 2.2 demonstrated that *N. deaurata* is not adapted to prolonged phases of air exposure during low tides compared to well-adapted *N. magellanica.* After two hours of falling dry, a time frame that is regularly experienced by *N. magellanica*, subtidal *N. deaurata* respond with a significant up-regulation of heat-shock proteins gene expression. Therefore, the ecological stress the animals have to cope with in the shallow intertidal zone prevents subtidal limpets from successfully colonizing that part of the intertidal. Hybrids might be under negative selection because of of maladaptation to the tidal gradients. On the Atlantic side of the Magellan Strait, I found two populations at Bahia Gregorio and Punta Catalina, which

were dominated by hybrid *N. magellanica*, carrying one allele of either Hsp allele cluster. The tidal change in this area is least pronounced in the whole Magellan Strait and ecological selection is probably so little that hybrids can survive and reproduce.

On the other hand, Hsp2 alleles were completely absent In Puerto Montt. When reproduction is not entirely restricted between both morphotypes and also migration between Puerto Montt and populations located in the Magellan Strait is not restricted as suggested by the gene flow analysis in chapter 3.2, we would expect hybrid individuals to be also present at the northern distribution edge. It is possible that hybrid *Nacella* could not be found in this area because the imposed temperature levels set a selective constraint on hybrid individuals rendering them impossible to survive the local situation at Puerto Montt. The combined results of physiology and genetics show that hybrid mating can produce offspring capable of surviving and reproducing in some parts of the distribution area (Eastern Magellan Strait) whereas natural selection is too high in others (Central and Western Magellan Strait, Puerto Montt).

The assumption that natural selection drives genetic divergence between *N. magellanica* and *N. deaurata* does not imply that they will ever reach species level differentiation. We might be witnessing an early state of an incipient speciation process but the observed partial reproductive isolation could as well be reversed when the environmental patterns change again. However, the process of speciation is a continuum and divergent populations of one species can help to identify the barriers that lead to complete speciation in the future, where approaches with well-diverged species do most likely not allow to reveal the initial causes of reproductive isolation during speciation.

Chapter 6

Conclusions and Perspectives

6.1 Conclusions

In the present thesis genetic and physiological markers were investigated to reconstruct the evolutionary history of South American and Antarctic *Nacella* limpets. The superordinate aim was to test the importance of geographical gene flow barriers and natural selection on speciation processes in marine organisms.

The overall results of the thesis highlight that the classical belief that speciation needs geographical isolation (Mayr 1963) is far too simplistic. The creation of genetic divergence is not strictly hampered by gene flow and natural selection is much more important in speciation processes than previously believed.

1) The ACC was not a strict barrier to gene flow between South American and Antarctic populations ever since it came into existence around 35 Ma ago (Lagabrielle et al. 2009). The calculated divergence time of *Nacella* from South America and Antarctica (< 15 Ma) implies that additional forces were necessary to create an efficient gene flow barrier. The calculated speciation date is in line with the second major cooling of the Antarctic and the glaciation of the Antarctic Peninsula (around 15 Ma). This indicates that strongly differing environmental constraints on either side of the Drake Passage were important to establish a gene flow barrier. The necessity for local adaptations to the changing climatic conditions in Antarctica have likely played a major role in the isolation of the Southern Ocean. The true reason for the high endemic rates in the Antarctic is therefore not only summoned by physical isolation but by a more complex interplay most likely including divergent selection.

It might be the most parsimonious conclusion to interpret a causal correlation between identified physical barriers and the origin of presently well-diverged species. The presented results, however, demonstrate that it is indispensable to thoroughly test the actual permeability of assumed gene flow barriers like currents or large geographical distances and to take into account evolutionary forces like natural selection.

2) The population genetic study carried out with the two morphotypes and nominal species *N. magellanica* and *N. deaurata* from South America demonstrate the powerful means provided by multilocus marker approaches. Only the combination of selectively neutral markers and markers under selection revealed a profound picture of the recent

evolutionary history of Patagonian limpets. A study based on the neutral markers microsatellites and COI would have led to the conclusion that *N. magellanica* and *N. deaurata* are not genetically divergent but represent two phenotypically plastic morphotypes. The addition of markers under selection (Hsp70) provided much deeper insights into the evolutionary history of South American *Nacella.* Geographical isolation summoned by glacial maxima resulted in a deep divergence of a Hsp70 gene in separated Atlantic and Pacific populations. Gene flow was not entirely restricted among both morphotypes upon secondary contact as indicated be the neutral markers. The strong divergence in the Hsp70 gene, on the other hand, reveals the presence of divergent selection between the morphotypes. Ecological selection maintains adaptively diverged gene pools and highlights the impact of selection in high gene flow environments. The *Nacella* evolution is an outstanding example how allopatric isolation and sympatric divergence, acting on different time scales, both shaped the evolution of Patagonian limpets.

3) The divergent selection between intertidal *N. magellanica* and subtidal *N. deaurata* is most likely caused by the strong ecological constraints along the vertical intertidal stress gradient. Morphological analyses and physiological experiments showed that subtidal *N. deaurata* are significantly less adapted to the tidal rhythm than the intertidal *N. magellanica*. Shells of *N. magellanica* are higher and in general larger than those of *N. deaurata* providing better adaptation to air exposure upon tidal emersion (Weihe and Abele 2008; Weihe et al. 2010). Larger shells can store more shell water that is used as an oxygen reserve during tidal uncovering. Physiological experiments showed a considerably higher stress response to experimental air exposure in *N. deaurata* that inhabit lower shore levels and are therefore usually not exposed to tidal emersion in the field. *N. deaurata* show an earlier and significantly higher expression of heat-shock proteins and activities of enzymatic antioxidants to cope with the stress summoned by air exposure.

In summary, the recently frequently asked question whether or not *N. magellanica* and *N. deaurata* represent two different species might be too simplistic. The results show that gene flow barriers can be restricted in parts of the genome (Hsp70) whereas it is

ongoing in others (COI, microsatellites). The presented evidence for adaptive divergence in South American limpets does not guarantee that the two investigated morphotypes will ever reach a species level differentiation. It is possible that we are witnessing the early state of incipient speciation that will reach completeness in the future. It is also imaginable that the observed partial reproductive isolation will be reversed when the environmental patterns causing adaptive divergence change again. The more important task is to identify and understand the mechanisms that drive selective divergence and establish initial gene flow barriers between populations. The understanding of these processes might be the major key in understanding the drivers of evolution and the importance of local adaptation in the background of gene flow.

The insights provided by this thesis will hopefully motivate population genetic studies to include multiple markers that are both neutral and under selection. Neutral markers are important to show the background connectivity of populations and the amount of gene flow between them. Markers under selection provide additional information about the presence of selective forces that drive adaptive divergence. The combination of both marker systems and the addition of physiological analyses allow to establish a comprehensive, micro-evolutionary framework of speciation in the marine realms.

6.2 Perspectives

In the following some ideas are outlined for further research on the process of speciation, the evaluation of gene flow barriers and the impacts of natural selection:

(a) The molecular dating approach presented in chapter 3.1 aims at goals similar to that of many geological studies: The investigation of oceanographical barriers. The dating of species divergence times can serve as an independent source of information that might help geological investigations aiming at dating historical events. The present example concerning the onset of the ACC shows that whenever the ACC came first into existence it most likely served as a barrier to genetic exchange not only before 15 Ma ago. The wide variance in published dates for the onset of the ACC indicates the uncertainties geological dating attempts. An interdisciplinary cooperation in future studies where information of molecular biology and geological approaches are discussed in a combined framework can help to reliable characterize geographical barriers in the marine realm.

(b) The emergence of genome wide sequence approaches, so called next generation sequencing, provides the opportunity to investigate the whole genomes of *N. magellanica* and *N. deaurata*. This provides the possibility to identify all regions of the genomes that are actually under divergent selection such, as the chosen Hsp70 gene in this thesis. These additional insights can teach us how many genes are necessary to build and maintain a genetic divergence based on selection. Furthermore it could help to reveal the key genes that are important in adaptive divergence.

(c) The inclusion of populations from the Argentinian Atlantic coast north of the Magellan Strait will be an important task to cover the whole distribution area of investigated *Nacella*. Following question have to be answered yet: Have there also been refuge areas along the Argentinian coast? Do we also only find *N. magellanica* north of the the Magellan Strait in the Atlantic as we do in the Pacific? Which alleles of Hsp70 are dominant in those populations? The answers to these question will help to learn more about the origin of the two different Hsp70 allele groups in *Nacella.*

(d) Deeper studies on the reproduction of *N. magellanica* and *N. deaurata* are necessary to reveal the mode of reproductive barriers between the two morphotypes. Mating experiments and the determination of actual spawning times of both morphotypes will allow profound conclusions on the actual modes that apparently reduce mating success in large parts of the sympatric distribution area.

(e) The direct link between genotypic and phenotypic variation in the investigate Hsp70 gene has to be investigated. As we find individuals of subtidal *N. deaurata* carrying Hsp70 alleles dominant in *N. magellanica* and vice versa, stress experiments could show how hybrid individuals react to air exposure stress. This approach will give more insights into the importance of the Hsp70 regarding the adaptability to the tidal gradient. In-depth protein sequence analyses could reveal which amino acid changes are functionally important and how structural changes in the protein provide better survivability in certain environments.

(f) The low genetic diversity found in Antarctic *N. concinna* has raised concerns about there survival chances under rapid climate change. The physiological tests presented in chapter 2.2 should be carried out with Antarctic limpets to make predictions on the upper stress levels of this species. Although *N. concinna* is considered to be physiological highly adaptive (Weihe and Abele 2008, Weihe et al. 2010), the upper temperature boundaries that would still allow sustained survival are not known. Furthermore, invading South American *Nacella* would probably still be better adapted once certain water and air temperatures are reach, regardless of the physiological plasticity of *N. concinna*.

Summary

The classical belief that speciation needs geographical isolation has raised the question how the huge amount of biodiversity can be explained in an open environment like the ocean that is characterized by the absence of strict barriers to gene flow over large geographical scales. Many theoretical approaches have contested that classical belief showing how speciation can take place in the background of gene flow rendering natural selection as a potent evolutionary force. However, most of the empirical population genetic studies aiming at speciation processes apply neutral molecular markers which do not respond to selective forces and therefore do not allow for statements on the actual roles of selection causing ecologically based barriers to gene flow.

The present study‘s superordinate aim was to reconstruct the evolutionary history of South American and Antarctic patellogastropods of the genus *Nacella*. The central aspects of the thesis were the investigation of the actual roles of gene flow barriers and natural selection in processes causing speciation and population divergence. In order to fulfill this task a wide range of markers, both neutrally evolving and under selection, was applied to reveal gene flow patterns and local adaptation.

The ACC is one of the most prominent physical barriers in the ocean. Its origin around 35 Ma before present should therefore be reflected in the timing of the divergence of *Nacella*. Molecular dating approaches carried out in this thesis with three different nuclear and mitochondrial genes (COI, 16S, 18S) revealed the timing of speciation of *Nacella* to be much younger than the proposed onset of the ACC (~ 10 Ma). Gene flow must, therefore, have been possible after the ACC had already been fully established until the Miocene 10 Ma ago. This time coincides with the second major cooling of Antarctica that led to the glaciation of the Antarctic Peninsula and most likely increased the selection for physiological and genetic adaptation on both sides of the Drake Passage. The present example highlights that the mere occurrence of the ACC alone was not sufficient to promote speciation between Antarctic and South American species. It most likely needed the additional influence of natural selection summoned by climatic changes.

A more detailed view on the impact of natural selection was obtained by population genetic studies of the two South American species *N. magellanica* and *N. deaurata*. They largely occur in sympatry but exhibit differences in vertical zonation along the tidal gradient. *N. magellanica* mainly inhabits the shallow intertidal areas and is exposed to tidal emersion twice a day. *N. deaurata* occurs in the deeper subtidal that is normally not heavily affected by the tidal cycles. Neutral markers (COI and seven microsatellites) reveal Southern South America to be a high gene flow environment without any genetic divergence between both morphotypes rendering them as morphotypes caused by phenotypic plasticity. The addition of genetic markers under selection (Hsp70) revealed a deep divergence between the two morphotypes. These contradicting results show that divergent selection in sympatry can cause ecologically important genomic regions to resist gene exchange, whereas in other parts of the genome gene flow continues. The genetic divergence in Hsp70 has most likely established in phases of geographical isolation of populations on Atlantic and Pacific sides of South America summoned by glacial maxima. Upon secondary contact after the Last Glacial Maximum had ended, gene flow between those populations was not restricted as seen in neutral markers. The divergence in Hsp70, however, displays how divergent selection maintains adaptive divergence in parts of the genomes in order to ensure adaptability to the environmental constraints along a tidal gradient.

Physiological experiments in which individuals of *N. magellanica* and *N. deaurata* were exposed to air furthermore revealed some of the potent drivers of ecological selection that maintain genetically disrupted gene pools. The differences in vertical zonation are reflected in lower adaptability to desiccation stress and elevated temperatures summoned by tidal emersion as seen in higher heat-shock response (HSR) and enzymatic antioxidant defense levels in subtidal *N. deaurata*.

The present study highlights the complex interplay of geographical isolation and natural selection in the evolutionary history of *Nacella*. The addition of genetic markers under selection provides a powerful tool to understand the importance of natural selection and local adaption as common evolutionary forces that are largely undetected by neutral marker approaches.

Zusammenfassung

Das klassische Verständnis von Artbildung sieht die Notwendigkeit von geographischer Isolation vor. Diese Annahme führt dazu, dass die unglaublich große Artenvielfalt im Ozean, welcher charakterisiert ist durch das Fehlen von geographischen Genfluss-barrieren über große geographische Distanzen, nur schwer erklärt werden kann. In jüngerer Vergangenheit haben viele theoretische Ansätze die Erfordernis von geographischer Separation angefechtet. Sie konnten aufzeigen, wie Artbildung geschehen kann, ohne das der Genfluss zwischen divergierenden Populationen gestoppt wird. Die wichtige evolutionäre Kraft ist hierbei natürliche Selektion. Die meisten empirischen Studien der Populationsgenetik verwanden allerdings genetische Marker, die neutral evolvieren und daher nicht das Potential besitzen, die Einflüsse von Selektion aufzuzeigen.

Das übergeordnete Ziel der vorliegenden Arbeit war die Rekonstruktion der evolutionären Geschichte südamerikanischer und antarktischer Napfschnecken der Gattung *Nacella.* Zentrale Aspekte waren dabei, die Rollen von physikalischen Genflussbarrieren und von natürlicher Selektion zu testen, die ein Auseinander-divergieren von Populationen und letztendlich Artbildung zur Folge haben. Ein weites Spektrum an verschiedenen genetischen und physiologischen Markern wurde verwendet, sowohl neutral evolvierende als auch solche, die unter Selektion stehen, um die Demographie und lokale genetische und physiologische Anpassungen aufzudecken.

Eine Untersuchung der Rolle des Antarktischen Zirkumpolarstroms (englisch: ACC) im Artbildungsprozess südamerikanischer und antarktischer Nacelliden sollte die Wichtigkeit von geographischen Barrieren verdeutlichen, da der ACC eine der am besten bekannten physikalischen Barrieren im Meer ist. Die Entstehung eben dieses ACC vor ungefähr 35 Millionen Jahren sollte daher mit der Datierung der Artaufspaltung heutiger Napfschnecken auf beiden Seiten des ACC übereinstimmen. Molekulare Datierungen, durchgeführt in dieser Arbeit anhand von drei verschiedenen Genen (COI, 16S, 18S), zeigten allerdings, dass die tatsächliche Artaufspaltung von *Nacella* deutlich jünger ist (~ 10 Millionen Jahre) als der angenommene Zeitpunkt des Entstehens des ACC. Ein Genfluss über den ACC muss also stattgefunden haben lange

nachdem er schon voll ausgebildet war. Die Aufspaltung vor 10 Millionen Jahren passt zeitlich mit dem zweiten großen Abkühlungsereignis der Antarktis zusammen. Es legt daher die Vermutung nahe, dass eine tatsächliche Barriere für genetischen Austausch zwischen Südeamerika und der Antarktis erst intakt war, als unterschiedliche Klimabedingungen, die Selektionsdrücke auf beiden Seiten des ACC zusätzlich verstärkt haben.

Detailliertere Einblicke in das Wirken von natürlicher Selektion konnten populationsgenetische Studien der beiden südamerikanischen Arten, *N. magellanica* und *N. deaurata*, erbringen. Diese kommen in großen Teilen ihres Verbreitungsgebiets in Sympatrie vor, zeigen aber eine leicht unterschiedliche vertikale Zonierung entlang des Gezeitenbereichs. *N. magellanica* besiedelt überwiegend das Flache Intertidal und fällt regulär zweimal am Tag trocken. wogegen *N. deaurata* das tiefere Subtidal besiedelt und daher kaum von den Gezeiten beeinflusst wird. Auf der einen Seite haben neutral evolvierende Marker, COI und sieben Mikrosatelliten, gezeigt, dass Südamerika ein Gebiet mit hohen Genflussraten zwischen den einzelnen Populationen beider nomineller Arten ist. Außerdem konnte mit beiden Markern keine genetische Divergenz zwischen den beschriebenen Arten festgestellt werden. Dies lässt darauf schließen, dass es sich hier lediglich um zwei Morphotypen einer Art handelt, hervorgerufen durch phänotypische Plastizität. Die Anwendung eines genetischer Markers unter Selektion (ein Heatschock-Protein der Hsp70 Familie) zeigte dagegen eine starke Divergenz der beiden Morphotypen. Diese gegensätzlichen Ergebnisse heben hervor, dass durch divergente Selektion in Sympatrie, ökologisch wichtige Bereiche des Genoms genetischem Austausch widerstehen können, obwohl in anderen Teilen des Genoms Genfluss weiter besteht. Die genetische Divergenz im Hsp70 Gen in *Nacella* entstand höchstwahrscheinlich in Zeiten reproduktiver Isolation von Populationen auf der atlantischen und der pazifischen Seite des Kontinents, hervorgerufen durch glaziale Maxima. Bei sekundärem Kontakt nach dem Ende des letzten glazialen Maximums war Genfluss zwischen den vormals separierten Populationen nicht vollkommen eingeschränkt, wie die neutralen Marker zeigen. Die Divergenz im Hsp70 Gen allerdings verdeutlicht, dass aber ökologische Selektion adaptive Divergenz in Teilen

des Genoms aufrecht erhalten kann und Populationen entlang eines intertidalen Gradienten trotz Genfluss an ihre speziellen Umweltbedingungen angepasst bleiben.

Physiologische Experimente, in denen Individuen von *N. magellanica* und *N. deaurata* Luft ausgesetzt wurden, konnten die möglichen Faktoren aufzeigen, welche ökologische Selektion vorantreiben und genetische Divergenz aufrecht erhalten. Die Unterschiede in der vertikalen Zonierung der beiden untersuchten Morphotypen spiegeln die verringerte Anpassbarkeit der subtidalen *N. deaurata* an das Trockenfallen bei Niedrigwasser wider. Zu sehen ist dies in einer stärkeren Hitzeschock-Stressantwort und höheren Aktivitäten von enzymatischen Antioxidantien in subtidalen *N. deaurata.*

Die vorliegende Arbeit verdeutlicht das komplexe Zusammenspiel von geographischer Separation und natürlicher Selektion im Hinblick auf die evolutionäre Geschichte von *Nacella*. Die Anwendung von genetischen Markern unter Selektion gibt zusätzliche Einblicke in die Wichtigkeit von natürlicher Selektion und lokaler Anpassung als bedeutende evolutionäre Kräfte, deren Ermittlung in wissenschaftlichen Analysen basierend auf rein neutralen Markern größtenteils unentdeckt bleibt.

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit eidesstattlich, dass es sich bei den von mir abgegebenen Arbeiten um 3 identische Exemplare handelt

Bremen, 1. August 2011

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Kevin Pöhlmann