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High levels of realized dispersal and phenotypic plasticity in the polymorphic squat lobster *Munida gregaria* (Decapoda: Anomura: Munididae) in Patagonia and New Zealand - a multi-marker genetic and morphological approach

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Prüfungsausschuss

1. Gutachter:	Prof. Dr. Thomas Brey Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung Funktionelle Ökologie – Bremerhaven
2. Gutachter:	Prof. Dr. Martin Zimmer Leibniz-Zentrum für Marine Tropenökologie Ökologie
1. Prüfer:	Prof. Dr. Kai Bischof Universität Bremen Marine Botanik
2. Prüfer:	Dr. Christoph Held Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung Funktionelle Ökologie - Bremerhaven

For my wife Yameng and our son Yiyuan

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Erklärung

Chen Wang
Borriesstr. 20
27570 Bremerhaven

Erklärung gemäß §6(5) der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche vom 14. März 2007

Hiermit erkläre ich, Chen Wang, dass ich die Doktorarbeit mit dem Titel:

"High levels of realized dispersal and phenotypic plasticity in the polymorphic squat lobster *Munida gregaria* (Decapoda: Anomura: Munididae) in Patagonia and New Zealand - a multi-marker genetic and morphological approach"

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

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Summary

Summary

Successful species delimitation is of fundamental importance in understanding the process of speciation. The tools of molecular genetics provide enormous potential for clarifying the nature of species boundaries, which has challenged the generalization that marine species are often associated with high dispersal and mild genetic differentiation over large spatial scales by unravelling hidden diversity in the form of unrecognized cryptic species. The occurrence of two or more sharply distinct morphotypes, even if currently considered a single nominal species, can either be a case of previously overlooked genetic differentiation or it may be caused by the expression of different morphotypes from essentially the same genetic background.

The squat lobster *Munida gregaria* (Decapoda: Munididae) is a shallow-water species that is widely distributed mainly on the continental shelf off southeastern New Zealand and Patagonia. Its global disjunct distributions are separated by more than 7000 km' expanse of deep Pacific water, which is known as a major marine biogeographical barrier, the East Pacific Barrier (EPB). Under this currently accepted species (i.e., *M. gregaria* sensu lato) there are two ecotypes, *gregaria* sensu stricto and *subrugosa* that are characterized sharp differences in morphology, ecology and behaviour. Therefore, this species provides an excellent case study to test whether present marine major barriers and heterogeneous environmental forces would have resulted in genetic differentiation and further speciation.

The superordinate aim of this study was to reconstruct the evolutionary history of the South American and New Zealand squat lobster *M. gregaria*. The central aspects of the thesis were investigations of i) the relationship between phenotype and genotype; ii) interaction of emerging geographical barriers and oceanographic influence in shaping genetic structure, biogeography of *M. gregaria*. In order to fulfil this task a comprehensive analytical framework based on the combination of nuclear microsatellites and mitochondrial COI genetic markers, as well as range-wide sampling, were applied to address these major tasks.

To avoid potential pitfalls of using a single marker especially the mitochondrial DNA barcoding gene, such as introgression, incomplete lineage sorting and insufficient evolutionary rates to recover recent divergence in potential cryptic speciation, a set of fast-evolving nuclear microsatellite were newly developed for *M. gregaria*. These microsatellites together with conventional DNA barcoding *sensu stricto* (mitochondrial COI) were firstly applied on South

American samples to test whether the different ecotypes arise from differentiated genotypes. Both data sets yielded a congruent result of an undifferentiated genetic background corresponding to either ecotypes or geographic units across the entire South American distribution. The *gregaria* s. str. and *subrugosa* ecotypes are not underpinned by cryptic genotypes, instead the discrete phenotypic differentiation is a representation of environment-driven phenotypic plasticity or developmental variation.

Morphometric analyses were performed in order to see if continental-scale sampling would cover unsampled intermediary morphotypes and then blur the boundaries of the two ecotypes. Results from PCA and using discriminant functions demonstrated the morphology of the two forms remaining discrete in the context of sampling from large-scale geographic locations as well as different ontogenetic composition. Moreover, the inclusion of different life stages of *M.gregaria* demonstrates that a part of the morphometric variance is adequately explained by an ontogenetic transition between moults.

Population genetic analyses based on global sampling of *M. gregaria* detected a genetic structure across the Pacific between New Zealand and South America. But many shared alleles, as well as testing in a Bayesian framework, confirmed gene flow at different timescales across the open water barrier spanning more than 7000km. The dispersal of *M. gregaria* between New Zealand and South America was possibly facilitated by the ACC. This study demonstrated that the EPB is not a strict marine biogeographical barrier to gene flow.

This case study demonstrates a striking case in contrast with the mounting number of cryptic speciation resulted from geographic separation or disruptive environmental selection. The combination of nuclear and mitochondrial genetic markers are highly recommended in order to improve the power of molecular data to test phylogenetic and population genetic hypotheses, especially in the event of homogeneous genetic background that is hard to falsify with easily obtainable but error-prone mitochondrial data alone.

Zusammenfassung

Die korrekte Abgrenzung von Arten untereinander ist eine Grundvoraussetzung für ein besseres Verständnis dafür wie Arten überhaupt entstehen. Die Werkzeuge der molekularen Genetik haben ein enormes Potenzial, die Grenzen zwischen den Arten sichtbar zu machen. Durch den Nachweis bisher übersehener, kryptischer Arten haben sie geholfen, pauschale Verallgemeinerungen wie die Vorstellung, marinen Arten seien insgesamt durch ein hohes Ausbreitungsvermögen und einer nur geringen Differenzierung auch auf großen geografischen Skalen gekennzeichnet. Umgekehrt kann das Auftreten zweier sehr unterschiedlicher Phänotypen entweder die Folge einer zwar erfolgten, aber bisher übersehenen genetischen Differenzierung sein, oder aber auf die Expression zweier Phänotypen aus einem einzigen Genotypen hindeuten.

Der Springkrebs *Munida gregaria* (Decapoda: Munididae) besiedelt flache Küstengewässer vor der Südinsel Neuseelands und ganz Patagoniens. Die beiden Teile des globalen Verbreitungsgebiets sind durch eine Lücke von mehr als 7000km unbesiedelbarer Tiefsee des Pazifischen Ozeans voneinander getrennt, eine bedeutende biogeografische Ausbreitungsgrenze, die als Ostpazifische Barriere (East Pacific Barrier, EPB) bekannt ist.

Innerhalb der taxonomisch akzeptierten Art *Munida gregaria* sensu lato werden zwei Ökotypen (*gregaria* sensu strictu und *subrugosa*) zusammengefasst, die sich durch zwei scharf voneinander abgegrenzte Morphotypen mit ebenso deutlichen Unterschieden in Ökologie und Lebensweise auszeichnen. Diese Art eignet sich daher besonders, um zu untersuchen, ob und wie das Vorhandensein von Ausbreitungsbarrieren und einer heterogenen Umwelt zu einer genetischen Differenzierung und schließlich zur Bildung neuer Arten führen könnte.

Die Rekonstruktion der evolutiven Geschichte von *M. gregaria* in Südamerika und Neuseeland stellt das übergeordnete Ziel dieser Arbeit dar. Wichtige Aspekte waren daher 1) die Beziehung zwischen Phänotyp und Genotyp und 2) das Zusammenwirken geografischer und ozeanografischer Faktoren bei der Herausbildung der genetischen Struktur und Biogeografie von *M. gregaria*. Zu diesem Zweck wurden Mikrosatelliten als Marker aus dem Zellkern ebenso verwendet wie mitochondriale COI Sequenzen und Proben aus dem gesamten Verbreitungsgebiet der Art analysiert.

Um Introgression, ancestrale Polymorphismen und unzureichende Mutationsraten als Fehlerquellen von Studien, die auf einem einzigen mitochondrialen Marker basieren,

ausschließen zu können, wurden im Rahmen dieser Arbeit voneinander unabhängige und hochvariabler Mikrosatelliten als molekulare Marker für *M. gregaria* etabliert.

Mitochondriale Daten sowie die Mikrosatelliten zeigen übereinstimmend, dass es keinen Hinweis auf eine genetische Differenzierung der beiden Ökotypen in Südamerika gibt. Die beiden Ökotypen *gregaria* s. str. und *subrugosa* sind daher keine kryptischen Arten, sondern stellen alternative Phänotypen des gleichen Genotyps dar.

Unser Probenmaterial, das aus dem gesamten südamerikanischen Verbreitungsgebiet stammt, wurde morphometrisch untersucht, um festzustellen, ob die scharfe Unterscheidbarkeit der beschriebenen Ökotypen von *M. gregaria* ein Artefakt einer geografisch begrenzten Untersuchung darstellt. Die morphometrische Analyse zeigt jedoch klar, dass die Unterscheidbarkeit über das gesamte südamerikanische Verbreitungsgebiet hinweg stabil bleibt. Zusätzlich zeigt die Einbeziehung verschiedener Lebensstadien eine ontogenetische Dimension in der morphometrischen Varianz.

Populationsgenetische Analysen zeigen, dass eine schwache genetische Differenzierung zwischen dem neuseeländischen und dem südanerikanischen Teil des Verbreitungsgebiets nachzuweisen ist. Dennoch belegen eine Vielzahl von Allelen mit Nachweisen beiderseits des Pazifik sowie eine genetische Modellierung, dass von einem Genfluss auf verschiedenen Zeitebenen über die 7000km Barriere in Form von unbesiedelbarer pazifischer Tiefsee ausgegangen werden muss. Dieser Austausch wurde wahrscheinlich durch die Verdriftung von Organismen mit dem Antarktischen Zirkumpolarstrom (ACC) begünstigt. Diese Arbeit zeigt, dass die EPB kein unüberwindbares Hindernis für Genfluss ist.

Diese Arbeit zeigt entgegen einem allgemeinen Trend zunehmender Nachweise kryptischer Arten, dass in *M. gregaria* unterschiedliche Phänotypen aus demselben Genotypen entstehen. Ein derartiger Nachweis kann nur mit Hilfe hinreichend variabler molekularer Marker aus dem Kerngenom geführt werden. Die Verwendung ausschließlich mitochondrialer Marker, die leichter zugänglich, aber deutlich fehleranfälliger sind, ist dagegen nicht möglich.

Abbreviations

π	nucleotide diversity
µl	microliter
µM	micromolar
ACC	Antarctic Circumpolar Current
AFLP	amplified fragment length polymorphism
Ar	allelic richness
AMOVA	analysis of molecular variance
bp	Base pairs
BSC	biological species concept
CCP	Central Chilean Patagonia
CI	confidence interval
COI	cytochrome c oxidase subunit I gene
DNA	deoxyribonucleic acid
DV	developmental variation
EPB	East Pacific Barrier
ESU	Evolutionary Significant Unit
FM	Falklands/Malvinas
H_D	haplotype diversity
H_O	observed heterozygosity
H_E	expected heterozygosity
HPD	highest posterior density
HWE	Hardy-Weinberg equilibrium
IBD	isolation-by-distance
K	number of clusters
LD	linkage disequilibrium
Ma	million years ago
MCMC	Markov chain Monte Carlo
MdP	Mar del Plata
Msats	microsatellites
mtDNA	mitochondrial DNA
N_A	number of alleles
NCP	Northern Chilean Patagonia

Abbreviations

NZ	New Zealand
PCR	polymerase chain reaction
PLD	pelagic larval duration
PSP	paralytic shellfish poisoning
SA	South America
SNP	single nucleotide polymorphism
SSD	sum of square deviation
STF	Subtropical front
TdF	Tierra del Fuego archipelago

1. Introduction

1.1 Theory of speciation

Understanding the processes that promote speciation is one of the foremost goals of evolutionary biology. The prerequisite of identifying these processes is to successfully recognize and delimit species or evolutionary significant units (ESUs) (Sites & Marshall 2004), for which a species concept and operational criteria for diagnosing taxonomic limits are required. New insights gained from learning the speciation process and the nature of 'species' in the past decades have broadcasted several species concepts with adjustments to meet different criteria of assessing lineage separation (de Queiroz 2007; Hausdorf 2011), whereas the most influential and broadly accepted species is still the biological species concept (BSC) that defines species as "*groups of interbreeding natural populations that are reproductively isolated from other such groups*" (Mayr 1942, 1963). However, the findings that reproductive barriers are semipermeable to gene flow and speciation may be possible despite ongoing interbreeding (Mallet 2005; Mallet 2008; Rieseberg 2001; Wu & Ting 2004) challenged this delimitation criteria based on reproductive compatibility. In practice, a particular species concept and the associated operational criteria are often adopted from the perspective of research fields and interests (Sites & Marshall 2004). For example, morphological species concepts (phenotypic differences: Cronquist, 1978) are central for paleontologists and museum taxonomists, whereas genetic ones (monophyly of lineages: Donoghue, 1985; Mishler, 1985; genotypic cluster: Mallet, 1995; Guichoux et al, 2013) are key for population geneticists and molecular systematists. In contrast, ecologists tend to apply the ecological species concept and niche differences for delimiting species (Andersson 1990; Van Valen 1976). Exclusive use of a single operational criterion and an outright acceptance of one species concept (e.g., BSC) could underestimate biodiversity by lumping together many well-differentiated and generally accepted species that regularly hybridize (Hausdorf 2011).

Irrespective of the fuzzy nature of species boundaries, the process of speciation is a temporally continuum whereby lineages gradually accumulate ecological, morphological and genetic differences. The process itself is not observable and researchers can only rely on certain evidences or inferences to dictate the evolutionary pathways. With the aid of genetic approaches, species delimitation has been strongly fostered and the diversity of species on the planet continues to astonish biologists. Meanwhile, numerous models of speciation were proposed to accompany the renewed knowledge of species diversity (Coyne & Orr 2004;

Gavrilets 2014). No matter under which model that speciation is designated, the beginning of speciation must be reflected through a substantial reduction in gene flow. As argued by Mayr, the most effective and common form to the reduction of gene flow is geographic isolation, which constitutes the core of allopatric speciation. Physical barriers such as insurmountable mountains, oceans or simply great distances prevent free movement and mating between members from both sides of the barrier, resulting in a spatial separation. The next step is that different evolutionary forces controlling the dynamics of speciation act simultaneously and often have opposite effects. As the intrinsic evolutionary driver, mutation leaves stochastic changes in allele frequencies in local populations and subsequently is accumulated or dropped out through the random genetic drift process, which leads to genetic divergence between the separated populations. As a creative force in evolution, natural selection will tend to adapt a population to local environmental conditions and subsequently lead to genetic differentiation between populations. On the contrary, migration of individuals or dispersal of gametes among populations, which are collectively called gene flow, homogenise the geographic distribution of alleles. A final step of speciation for populations in allopatry is the elimination of gene flow and prevention of the reappearance of intermediate/hybrid genotypes. Allopatric isolation has been well supported by most empirical species diversification and is widely acknowledged to be a common mode of speciation. It was further subdivided into vicariant speciation and peripatric speciation (= founder effect) (Coyne & Orr 2004; Mayr 1954). In the former, a widespread species becomes sundered by an arising geographic barrier, reproductive isolation occurs between the split populations that diverge independently, whereas in the latter a new population is founded by a few individuals without any continuation of genetic exchange with the widely distributed ancestor. Irrespective of the underlying bases for allopatric speciation, geographic separation is the premise in leading to very low level or complete cessation of gene flow.

In contrast with spatial segregation required in allopatric speciation, alternative speciation concepts were developed to account for some unexpected high species richness in a randomly mating (panmictic) population, such as the celebrated cichlids case (Barluenga *et al.* 2006; Dieckmann & Doebeli 1999; Schliewen *et al.* 1994), which can be summarized in the term sympatric speciation. Sympatric speciation is a process taking place in the context of high gene flow, where physical barriers to genetic exchange between diverging populations are absent (Fitzpatrick *et al.* 2008). The initial driver of sympatric speciation despite ongoing gene flow is disruptive (diversifying) ecological selection at one or several loci at which different alleles

confer adaptation to two distinct resources (Maynard Smith 1962, 1966). The difficulty of sympatric speciation is how to establish linkage disequilibrium among a set of genes that together confer reproductive isolation. Compared with tremendous evidences for allopatric speciation, sympatric speciation is hard to demonstrate because evidence must show that a past allopatric phase of genetic differentiation is hardly true (Coyne & Orr 2004). Therefore this mode of speciation is still under debate. For example, Mayr (1942, 1963), the most vigorous and influential critic of the sympatric speciation hypothesis, argued that many supposed cases are rather unconvincing that the precise mechanism of sympatric speciation is a form of microallopatry, whereas geography is of primary importance. Disagreeing with Mayr, some argued that environmentally induced barriers to interbreeding and physical barriers to dispersal can have equivalent effects on gene flow (Butlin *et al.* 2008; Coyne & Orr 2004; Fitzpatrick *et al.* 2009).

Another commonly discussed speciation model that takes growing heed with experimental and conceptual advances is ecological speciation. Ecological speciation refers to the evolution of reproductive isolation between populations or subsets of a single population by adaptation to different environments or ecological niches (Nosil 2012; Rundle & Nosil 2005; Schluter 2001). Distinguished from the traditional models of allopatric and sympatric speciation that were classified by the geographical arrangement, ecological speciation falls into a different category that focuses on the inhibitory effects of gene flow on the evolution of reproductive isolation; that is, it may occur in allopatry or in sympatry (Schluter 2001). Although ecology may contribute to many mechanisms of speciation, the determinant of reproductive isolation must be ecologically based divergent selection in the sense of ecological speciation. Distinguishing ecological from non-ecological selections (e.g., sexual selection, or selection not divergent between environments, see Rundel & Nosil 2005) is of primary importance to determine whether a studied case has undergone ecological speciation, which is yet not that easy to tell them apart because the form of reproductive isolation is usually indirectly implicated by which type of selection (Rundle & Nosil 2005). Ecological speciation might occur indirectly as a consequence of natural selection on morphological, physiological or behavioural traits, or it might be resulted from direct selection on premating isolation (Reinforcement) (Schluter 2001). So far the growing but still limited evidences implicating ecological speciation mainly relied on observational and comparative experiments, whereas the genetic basis for the process of ecological speciation is still underexplored (Schluter & Conte 2009). The widely acknowledged model of ecological speciation provides the knowledge that divergent

environments, such as different resources or habitats, can cause the adaptive divergence of populations, which leads to the evolution of reproductive barriers that decrease, and ultimately cease, gene flow.

1.2 Molecular markers in realizing speciation

1.2.1 *Why use molecular tools?*

In the pre-molecular era, species delimitation and description of taxonomic relationships are mainly based on morphological characters, because the differences of morphology are observable, discernable and available-only. A majority of taxonomic decisions (e.g., synonyms, descriptions) to date still rest only on morphology. However, morphology is a non-neutral marker, it may be subject to difficulties and limitations when dealing with, e.g., poorly-studied organisms with few diagnostic characters, members of cryptic species complexes, eggs and immature stages, damaged or incomplete specimens. Consequently it will lead to under- or over-estimation of biodiversity (Lefébure *et al.* 2006). In the context of accurately identifying a species, which is essential to the performance of ecological and evolutionary research, molecular characters emerge as an important complement to conventional morphology.

Since the discovery of DNA (Watson & Crick 1953), molecular techniques have gone through unprecedented revolution in only a few decades, fueled in part by the discovery of recombinant DNA (Cohen 1993) and the polymerase chain reaction (Mullis *et al.* 1986). The availability of molecular data and capability of access to the intrinsic genetic architecture raise the fundamental question of untangling the relationship between phenotypes and the underlying genotypes, which has been a long-standing challenge since the proposal that phenotype and genotype form two fundamental different levels of biological abstractions (Johannsen 1911). The innumerable molecular data have on the one hand cohered with traditional taxonomic data and validated the systematic position. On the other hand they have challenged morphology-based species delimitation, to which questions arise owing to discrepancies between morphological traits and genotypic diversity: i) whether morphological differences among populations are reflecting corresponding genetic differences, or a consequence of phenotypic plasticity; ii) morphologically indistinguishable populations may represent genetic distinct lineages, i.e., (pesudo)cryptic species.

1.2.2 *Which molecular marker(s)?*

Since the first widespread use of allozymes in the 1970s, advances in DNA marker technology including modern availability of genome-wide sequencing, have resulted in a wealth of genetic markers with potentially broad utility in a variety of ecological and evolutionary endeavours. Depending on their properties of evolving rates, molecular markers are designated to answer questions at different taxonomic rank: phylogenetics and population genetics.

Sequences used in phylogenetic studies aim to deal with evolutionary relationships among deeper clades (usually at the level of orders or families), such as the 18S subunit of eukaryotic cytoplasmic ribosomes. When aiming to recognize divergences among populations and possibly species-level entities, population genetic approaches with faster evolving genes and genotypic markers are of primary importance (Knowles & Carstens 2007; Shaffer & Thomson 2007). Widely used population genetic markers include microsatellites, single nucleotide polymorphism (SNP), and some sequences of mitochondrial markers (mtDNA; such as mitochondrial cytochrome c oxidase subunit I (COI), control region).

DNA barcoding, which is normally referred to as a 658-bp fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene for animals, was proposed by Hebert *et al.* (2003) to serve as a rapid, efficient way to reduce the taxonomic deficit and automate taxon determination for ecological research. Since then DNA barcoding began its golden stage and became a prevailing choice of marker for identifying species and describing taxonomic relationship. The outcome of pervasive utility is the well-established Consortium for the Barcode of Life (CBOL, http://barcoding.si.edu), an international initiative supporting the development of DNA barcoding, which in turn promotes global standards and coordinates research in DNA barcoding. The reasons that COI is chosen over nuclear DNA include uni-parental inheritance (in a majority of animal phyla), elevated mutation rate (an evolutionary rate 5 to 10 times faster than the nuclear genes, Brown *et al.* 1979), lack of introns, large copy numbers in every cell, and lack of recombination (but see Galtier *et al.* 2009). In DNA barcoding, complete data set can be obtained from a single specimen irrespective to morphological or life stage characters. The advent of easily obtainable and affordable DNA barcoding has served to greatly intensify the crosstalk between molecular and taxonomic disciplines by successfully identifying unknown specimens in terms of a preexisting classification and uncovering a large number of previously overlooked (pseudo)cryptic genotypes (Dasmahapatra & Mallet 2006; Frézal & Leblois 2008; Hebert & Gregory 2005; Hebert *et al.* 2004).

Nevertheless, due to such special biological properties of mtDNA, for example, the maternal inheritance makes the whole genome behave as a single, non-recombining locus – all sites share a common genealogy, it is more vulnerable to limitations such as introgressive hybridization, sex-biased dispersal, incomplete lineage sorting and heteroplasmy (Ballard & Whitlock 2004; Ebach & Holdrege 2005; Petit & Excoffier 2009; Rubinoff *et al.* 2006; Wares 2010). If one mitochondrial gene was positively biased towards an incorrect result, adding more mitochondrial markers would likely only increase the support for this incorrect result rather than alleviate the dependence upon the unreliable marker (e.g., Page & Hughes 2010). DNA barcoding was also criticised for an overestimation of species numbers due to possible inadvertent inclusion of nuclear mitochondrial pseudogenes (= non-functional copies of mtDNA in the nucleus, or numts, Song *et al.* 2008). Discrepancies of genetic relationships yielded by mtDNA and nuclear DNA (mito-nuclear discordance) are becoming prominent with increasing utilization of molecular markers (Pons *et al.* 2014; Toews & Brelsford 2012). Relatively lower success rates of identification by mtDNA genes have been reported in certain groups of animals (Meyer & Paulay 2005; Shearer & Coffroth 2008; Vences *et al.* 2005; Whitworth *et al.* 2007; Wiemers & Fiedler 2007). In general, single mitochondrial marker may give first hints to cryptic species, unjustified splitting of species, or oppositely a lack of genetic differentiation. But these results have to be verified by further comparison with multiple independent nuclear loci, which is considered superior to single-gene approaches in removing under- or overestimation of species as a result of incomplete lineage sorting or introgression (Hausdorf & Hennig 2010).

Microsatellites. In order to get more in-depth understanding about the recent evolutionary history of populations of a species, microsatellites have shown their popularity and versatility as a satisfactory choice of nuclear marker in population genetic studies (Guichoux *et al.* 2011; Selkoe & Toonen 2006b). Microsatellites, also known as simple sequence repeats (SSRs), are stretches of DNA comprising short tandem repeats of 2-6 bp repeated up to ~100 times (Litt & Luty 1989; Tautz 1993). These repeats are highly polymorphic due to mutation-caused variation in the number of repeating units. Variations in tandem repeat length accumulate in populations more rapidly than the point mutations and larger insertion/deletion events on that sequence markers are based. The mutation rates of microsatellites are estimated at 10^{-2} - 10^{-6} per locus per generation, which is 100-1000 times faster than that of a single-copy nuclear DNA (Ellegren 2000; Gilbert *et al.* 1990; Schlötterer 2004). They are furthermore biallelic, codominant markers following Mendelian inheritance, thus are considered to be selectively

neutral sequences randomly or almost randomly distributed over the euchromatic genome (Allendorf *et al.* 2010; Schlötterer & Wiehe 1999; Tóth *et al.* 2000). A sufficient number of selectively-neutral microsatellite loci have great potential to promise extrapolation to the entire genome.

The high mutation rates of microsatellites come at the cost of their limited applicability outside the taxon for which they have been established. The high cost and unpredictability of developing microsatellites *de novo* for species used to prevent them from pervasive use (see Sands *et al.* 2009 for a discussion; Zhang 2004). Nevertheless, recent progress of microsatellite extraction protocols have streamlined the process of marker development considerably (Leese 2008; Nolte *et al.* 2005). In particular, next-generation sequencing technologies allow the identification of large numbers of microsatellite loci at reduced cost in non-model species (Abdelkrim *et al.* 2009; Malausa *et al.* 2011; Santana *et al.* 2009). Furthermore, the improvement in methods of multiplexing PCR enables more rapid scoring and higher throughput at low cost across larger number of loci (Ajzenberg *et al.* 2010; Hayden *et al.* 2008). Therefore, these versatile and cost-effective microsatellites are promising in studies of kinship, genetic structure and individual relatedness.

The combination of fast-evolving mitochondrial genes and nuclear genotypic markers (such as microsatellites) promises intensified resolution in 1) providing information of recent evolutionary events; 2) drawing complementary inference of genetic relationship and population differentiation, with greatly reduced chance of biased genetic structure and phylogenies that are not representative of the actual species tree. The combined analysis of multiple independent loci with various evolutionary rates has demonstrated enhanced power than that from using mitochondrial genes only (Griffiths *et al.* 2010; Hausdorf *et al.* 2011; Latinne *et al.* 2012; Vanhaecke *et al.* 2012).

1.3 Genetic divergence of widespread marine species

Over a long period of time marine ecosystems were considered relatively open, with high connectivity among populations (Caley *et al.* 1996; Roughgarden *et al.* 1985), since barriers to gene flow in the sea seem not as conspicuous as that in terrestrial and lacustrine ecosystems. Additionally, most marine species have life histories that include at least one potentially widely dispersal stage (Cowen *et al.* 2000), which seem to favour gene flow over large geographic scales and retain panmixia within population (Palumbi 1994). Recent applications of molecular methods, on the contrary, have uncovered remarkable population genetic structure or cryptic

diversity in a variety of coastal taxa (Cowen & Sponaugle 2009; Hellberg *et al.* 2002; Palumbi 2003), indicating that marine community does not intuitively display geographical and bathymetric uniform and continuum. For example, traditionally it has been assumed that many Southern Ocean marine animal species have a broad circumpolar (Clarke & Johnston 2003; Dayton *et al.* 1994; Knox 2006) and eurybathic (Brey *et al.* 1996) distribution. Yet recently it was found that the circumpolar giant sea spider *Colossendeis megalonyx* comprises a complex of as many as 15-20 cryptic species (Dietz *et al.* 2015). Similar cryptic speciation also exists in the crinoid *Promachocrinus kerguelensis* that has a planktonic dispersal stage (Hemery *et al.* 2012), or several lineages occurring in sympatry (Baird *et al.* 2011; Wilson *et al.* 2013), implicating possible role of ecological speciation.

In their pioneering work of marine biogeography, some biogeographers realized paradoxically common distribution patterns and endemism across various distinct marine fauna that were actually outlined by some 'hard' and 'soft' barriers (Briggs 1974; Ekman 1953; Vermeij 1978). Hard barriers, such as Isthmus of Panama (IOP), were caused by the formation of land bridges that physically split marine populations, while soft barriers involve different forms that impede dispersal. The deep ocean basins with extensive distance serve as a major way for nearshore marine species to overcome (Briggs 1974). Another form of barriers, in spite of relatively short distance being able to cross, is a reflection of historically episodic separation due to glaciations in Pleistocene. The best-known case of such is between the tropical Indian and Pacific Oceans at the Sunda Shelf, aka the marine equivalent of Wallace's Line (Barber *et al.* 2000; Huxley 1868; Whitmore 1981), owing to isolation resulting from Pleistocene low sea-levels. Strong ocean currents provide another kind of barrier. They can on the one hand significantly facilitate the transportation of pelagic propagules, on the other hand prevent dispersal across them. An eminent example is the circum-Antarctic Polar Front that separate Antarctic and sub-Antarctic (see Griffiths et al. 2009 for a review). These soft marine biogeographic barriers clearly act as 'filters' by restricting dispersal to generate and maintain biodiversity; meanwhile they are penetrable as evidenced by the existence of transoceanic marine species, which can be recent diverged sister taxa or truly trans-barrier species (Crandall *et al.* 2008; Dawson *et al.* 2011; González-Wevar *et al.* 2010; Ilves & Taylor 2008; Leese *et al.* 2010; Lessios *et al.* 2003; Luiz *et al.* 2012; Vermeij 2005).

Widespread marine organisms also exhibit high levels of morphological variation (Fowler-Walker *et al.* 2006; Marchinko 2003; Sanford & Kelly 2011; Trussell 1996). Morphological differences may be a phenotypically plastic response to variation of broad environmental

gradients, e.g., environmental tolerance (Ashton *et al.* 2007; Sorte *et al.* 2010), resources and habitat (Ewers & Didham 2006; Guisan & Thuiller 2005), but also they can be attributed to accumulated genetic differences due to disruption of gene flow among populations. The occurrence of two or more sharply distinct morphotypes, even if currently considered a single nominal species, can either be a case of previously overlooked genetic differentiation or it may be caused by the expression of different morphotypes from essentially the same genetic background.

Hence, purportedly widespread marine species may harbour distinct species, which failed to be discriminated or correctly reflected by conventional taxonomy based on morphology. Comprehensive molecular-based framework that can be extrapolated to better reflect genome-wide background is needed in an attempt to reconstruct evolutionary processes of widespread marine species.

1.4 The study case *Munida gregaria*

1.4.1 *Distribution and ecology of Munida gregaria*

South American and New Zealand squat lobster species *Munida gregaria* (Decapoda: Munididae) provides an excellent study case for research addressing ecological and evolutionary processes on various time scales. It allows for testing whether present marine major barrier and environmental forces would have resulted in genetic differentiation and further speciation.

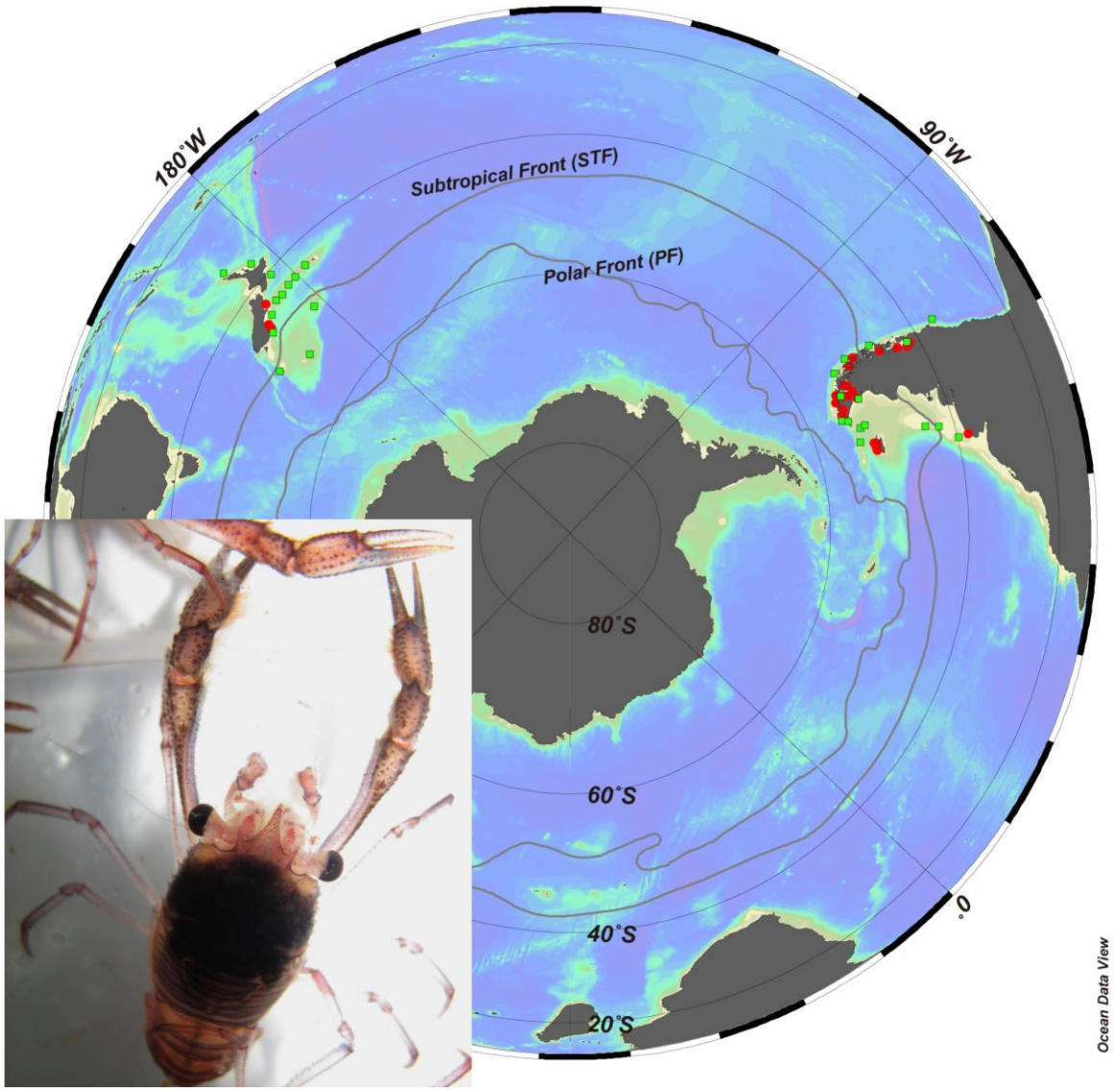

Fig. 1.1: Map showing records of distribution of *Munida gregaria* (in light green rectangles; source: World Register of Marine Species (WoRMS), http://www.marinespecies.org/) and sampling localities in this thesis (in red dots). Photography of *M. gregaria* by Jürgen Laudien.

Munida squat lobster is a highly diversified and speciose genus comprising 274 species at the time of writing (Lee *et al.* 2016), with potentially new endemic species being discovered (Macpherson *et al.* 2010). Out of the genus or even the family Munididae, *M. gregaria* (Fabricius, 1793) is the only widespread species that has a transoceanic distribution (Baba *et al.* 2008; Tapella 2002). It spreads nearshore off south-eastern New Zealand and along Chilean Patagonia from Chiloé (41° S) to Cabo de Hornos (55° S), as well as in the Atlantic shelf off Patagonia, from Uruguay's continental shelf waters (35°S) to Cabo de Hornos (55°S), including Falkland Islands/Islas Malvinas (Boschi *et al.* 1992; Zeldis 1985). Some records show it also occurs around Tasmania (Baba *et al.* 2008) and the islands nearby New Zealand

(Schnabel *et al.* 2011) (Fig. 1.1). Its bathymetric distribution varies from sub-littoral to ca. 1100 m depth (Arntz *et al.* 1999). As one of the few representatives of the family that have planktotrophic developmental stages and swarming behaviours, *M. gregaria* goes through a pelagic post-metamorphic stage (post-larva and juvenile) that can last about four months in life history (Tapella 2002; Williams 1980). During austral spring–summer large swarms of pelagic *M. gregaria* are available inshore and beach stranding has been regionally reported (McClatchie *et al.* 1991; Varisco & Vinuesa 2010) (Fig. 1.2). Later after this period they settle to the bottom (Zeldis 1985). *M. gregaria* occurs in high abundance in its distribution, at densities as high as 27 individuals·m^{-2} in Tierra del Fuego (Gutt *et al.* 1999), or reaching up to 366 individuals·m^{-3} in New Zealand (Zeldis 1985). In benthic samples from the Beagle Channel, *M. gregaria* represent ~90% of decapods (Perez Barros *et al.* 2004). In terms of biomass they constitute ~50% of the macrobenthic community in southern South America (Arntz & Gorny 1996).

Fig. 1.2 Swarming (left) and beach stranding (right) of *M. gregaria* at Otago Peninsula, east of Dunedin, New Zealand in 2016 January. Photos retrieved from http://www.dailymail.co.uk/news/ article-3391383/Swarm-krill-overrun-quiet-New-Zealand-beach-color-water-bright-red.html. © Paul Van Kampen.

Squat lobsters in general and *M. gregaria* in particular play an important role in food webs. *M. gregaria* displays two different and simultaneous feeding habits: as active predator it preys on crustaceans, algae and polychaetes; as scavenger it feeds on the particulate organic matter (POM). In turn, it is predated on by a long list of species at different trophic levels including fish, sea birds and marine mammals such as seals and whales (Romero *et al.* 2004). Thus as a key component *M. gregaria* can directly link primary producers and top predators and then shorten the trophic chains in which it participates with efficient energetic transfer (Tapella *et al.* 2002b).

Because of the special position of *M. gregaria* in the food web and life strategy, it plays an important role in the biological CO_2 pump in the continental shelf (Wada *et al.* 1987), meanwhile it is a potential vector of pathogens and toxins delivery to the higher trophic level. In New Zealand, study demonstrated that *M. gregaria* actively grazes on the toxic *Alexandrium catenella* bloom and thus accumulates toxins such as paralytic shellfish toxin (PST) and analogues (MacKenzie & Harwood 2014). Since June 2015, nearly 400 Sei whales were found to be stranded and dead in the southern Chilean Patagonia, which makes it the biggest single whale stranding event known to science (Fig. 1.3). The causes of the whales' death are under investigation in the Patagonia Projects: Golfo de Penas (https://expedition2016.wordpress.com/) and remain still unclear. But stomach contents analysis showed Sei whales in the region feeds on swarms of *M. gregaria*, which are suspected to be potential vectors of toxins or pathogens causing the whales' death (Vreni Haussermann, personal communication, February 29, 2016).

Fig. 1.3 The first 37 beached whales, out of a total number of 337 whales died in mass stranding on Chilean Patagonian coast in 2015, found in April in Gulf of Penas by a team led by Vreni Haussermann of the Huinay Scientific Field Station. Photography by Vreni Haussermann.

Due to the prevalent abundance, *M. gregaria* is economically of great potential as an alternative fishery resources to the heavily harvested squat lobsters *Pleuroncodes monodon* and *Cervimunida johni* (Tapella *et al.* 2002b; Zeldis 1989). It is the main species in the bycatch of

the trawling fisheries for the shrimp *Pleoticus muelleri* and for the king crab *Lithodes santolla* (Tapella *et al.* 2002b; Varisco *et al.* 2015). Because *M. gregaria* is rich in red astaxanthin pigments, it is useful for colouring the flesh in salmon farming and serving in foodstuffs such as soups, pastes, and surimi (Lovrich *et al.* 1998; Zeldis 1989).

1.4.2 *Taxonomic debate and biogeography of M. gregaria*

The taxonomic status of *M. gregaria* has been long tangled with another squat lobster *M. subrugosa* Dana, 1852. Both *Munida* squat lobsters occur in sympatry (Tapella *et al.* 2002a; Vinuesa 1977) and were originally described as separate species due to some different morphological characters (Lagerberg 1906; Matthews 1932) and supported by Boschi *et al.* (1992); Hendrickx (2003); Retamal (2000); Tapellaand Lovrich (2006) (Fig. 1.4). Chilton (1909) then later Williams (1973 and 1980) and Zeldis (1985) interpreted *M. gregaria* as a transient ontogenetic phase of *M. subrugosa*. Apart from the diagnostic morphological differences, both *Munida* squat lobsters also feature distinctive ecological and behavioural differences at certain developmental stages. *M. gregaria* passes through a pelagic postmetamophic phase, then adults settle benthic but still perform vertical migration in the water column. By contrast, *M. subrugosa* lacks pelagic phase in life history and remains benthic constantly after metamorphosis (Tapella *et al.* 2002a; Zeldis 1985). Specific feeding behaviours that probably led to the morphological adaptations display that *M. gregaria* is a detritivore or scavenger whilst *M. subrugosa* has been found to feed on algae or small crustaceans and worms (Tapella & Lovrich 2006; Vinuesa & Varisco 2007).

Despite profound phenotypic differences, it is still unknown whether the classification of the two different morpho- and eco-types truly reflects reproductively isolated species. A Recent molecular examination using mitochondrial genes failed to reveal genetic differentiation corresponding to their morphology (Pérez-Barros *et al.* 2008), on the grounds of which Baba *et al.* (2008) synonymised *M. subrugosa* as a junior form of *M. gregaria* and in the International Code of Zoological Nomenclature (ICZN) they are currently considered as a single species. For brevity and clarity, in this thesis, I refer to the current recognized species as *Munida gregaria* sensu lato (s. l.), whilst the two ecotypes under this species are termed *gregaria* sensu stricto (s. str.) and *subrugosa*. Even though this molecular result may reflect a true homogeneous genetic background of these two ecotypes, i.e., *M. gregaria* s. l. represents a case of phenotypic plasticity, relying on only mitochondrial evidence and insufficient sampling of geographic coverage can be also risky to overlook the possibilities of such as introgression,

incomplete lineage sorting and heteroplasmy due to the nature of mitochondria, thus wrongly to interpret two different species as one.

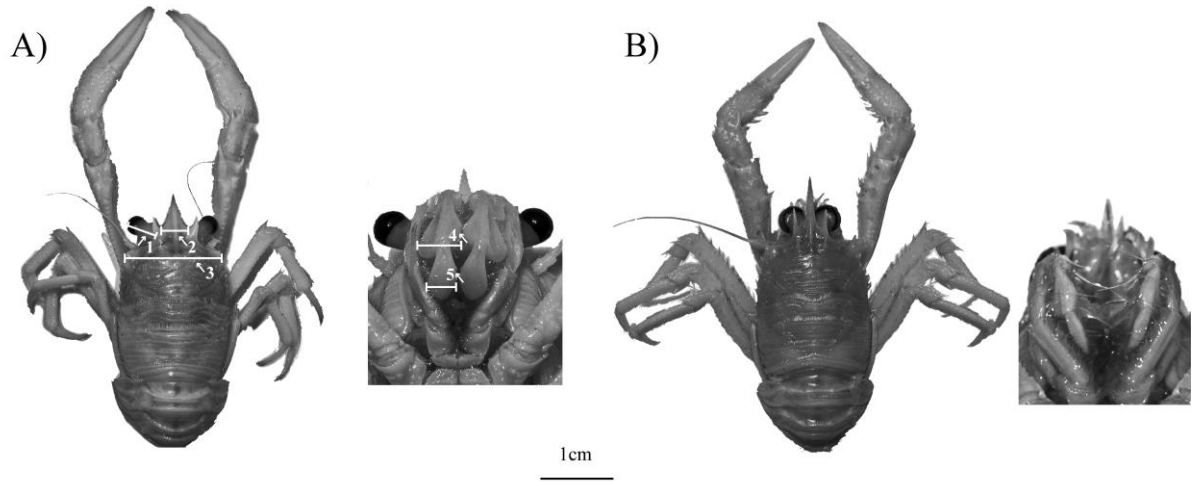

Fig. 1.4: Dorsal views (scale bar: 1 cm) and zoomed ventral views of mouth parts of A) *gregaria* s. str. ecotype and B) *subrugosa* ecotype. Arrows and numbers indicate statistically significant morphometric body parts between ecotypes. 1 - eyestalk, 2 - rostrum basis, 3 - anterior carapace, 4 - propodus of the third maxilliped, 5 - dactylus of the third maxilliped. Photo by Chen Wang.

The case of *gregaria* s. str. and *subrugosa* ecotypes provides a suitable paradigm to testify the existence of cryptic sympatric speciation. Moreover, the widespread *M. gregaria* s. l. might have undergone cryptic allopatric speciation. In its South American range, the extensive latitudinal distribution at both sides of Patagonia and the wide bathymetry occupied by *M. gregaria* s. l. (from water surface down to 1137m recorded for *subrugosa* (Arntz *et al.* 1999)) suggest that the species might be adapted to survive in diverse environmental conditions throughout its life cycle. The Magellan Zoogeographic Province in southern Chile is an extensive estuarine system resulted from glacial erosion during the Quaternary. Adults live inside the fjords associated with areas of detritus accumulation, whereas the larvae reside in the water column. Various environmental conditions (temperature, salinity, oxygen concentration) can act as potential selection leading to local adaptation, which constitutes the prerequisite of ecological speciation. Given the global species' range, *M. gregaria* s. l. spans over huge distances from central Pacific to eastern Pacific, which is at least a 7,000 km' expanse of deep Pacific water. This stretch of open deep water without islands in between is well known as the East Pacific Barrier (EPB). This major marine barrier has been described the most potent ´soft´ (comparing to the closure of land bridge such as the Isthmus of Panama) barrier (Ekman 1953), which is responsible for the long-term separation of assemblages in the Indo-Pacific and East Pacific (Briggs 1974). In spite of the long duration of pelagic phase and

swimming ability of *M. gregaria*, crossing such broad distance without stepping-stone dispersal seems questionable. It is necessary to carry out genetic investigation to find out whether or not ongoing gene flow still exists between ecotypes or geographic regions and which forces could drive a genetic divergence between them.

2. Aims, questions and outline of the thesis

2.1 Aims and questions of the thesis

The superordinate aim of this thesis 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 analysed i) the relationship between phenotypic and genetic patterns and possible existence of sympatric speciation, and ii) interaction of emerging geographical barriers and oceanographic influence in shaping genetic structure, biogeography of the squat lobster *M. gregaria*. The principal strategy of this thesis was to establish a comprehensive analytical framework to shed light on the various aspects of evolutionary processes. Large data sets from genetic and morphological analyses, mitochondrial and nuclear fast-evolving markers were applied to address the following major tasks.

Task 1: Developing a comprehensive multimarker approach to reliably study the evolution of New Zealand and South American squat lobster species *Munida gregaria*.

Despite numerous studies on discovery of cryptic speciation, the vast majority of conclusions are based on evidence from mitochondrial genes, very few studies applied high resolving nuclear markers such as microsatellites. In case of genetic homogeneity suggested by mitochondrial markers, alternative explanations such as introgression and incomplete lineage sorting cannot be ruled out, therefore a more confident conclusion should be based on markers from both mitochondrial and nuclear genome, a prerequisite of which is to provide higher resolution in resolving differentiation among populations.

Questions to this task:

i. Is the method of reporter genome protocol (RGP) (Leese 2008) applicable for *M. gregaria* and what is the success rate?

ii. Are the isolated microsatellites highly variable and suitable for subsequent population genetic and evolutionary studies?

iii. Can the isolated microsatellites be cross-amplified with close-related species so as to provide wider utility?

Task 2: Testing whether different ecotypes arise from differentiated genotypes, in other words, whether morphological and ecological divergences have resulted in genetic adaptation.

Although the *M. gregaria* s. l. ecotypes *gregaria* s. str. and *subrugosa* display clear morphological, ecological and bahavioural divergences, for which they were often considered as separate species, recent molecular evidence seems to support that they belong to a single species (Pérez-Barros *et al.* 2008). This evidence was based on mitochondrial-only markers, as well as geographically very limited sampling scheme. Populations belonging to a so-called 'ring species' might appear sharply distinct in an area of secondary overlap, but appear more gradually changing in morphology or genetics through areas of their distribution that have been more continuously inhabited (see Irwin *et al.* 2005; Liebers *et al.* 2004 and references therein). Inadvertently sampling only in the zone of secondary overlap might overlook potentially intermediary phenotypes and differentiated genotypes from discrete habitats. Meanwhile, in view of the growing reports of mito-nuclear discordance (Toews & Brelsford 2012), the lack of differentiation at mitochondria cannot be confidently extrapolated to genetic homogeneity without checking with multiple independent nuclear loci. I suspect if *M. gregaria* represents a cryptic species complex or involves more hidden ecotypes. Therefore comprehensive molecular and morphological examination with more inclusive sampling coverage is necessary to conclude whether the ecotypes arise from a common genetic background.

i. In contrast to the lack of mitochondrial differentiation, whether nuclear microsatellites would discordantly detect genetic differentiation between the two ecotypes; or both types of markers would yield a coherent result of cryptic genetic divergence among populations/ecotypes with continental scaled sampling?

ii. On the basis of large-scale and more inclusive sampling strategy, if the morphological traits are continuous or still disrupt; are there possible hidden morphotypes?

iii. Whether the nomenclature *M. gregaria* harbours cryptic species, or is a single species with possible phenotypic plasticity?

Task 3: Analysis and discussion of patterns of genetic structure, gene flow, genetic diversity and demographic processes of *M. gregaria* throughout global species' range in the context of the climatological and geological history.

Although the squat lobster *M. gregaria* is shown to have great potential dispersal capability, in view of the great longitudinal and latitudinal distances along Patagonian coastlines and the strong isolation between central and eastern Pacific, it seems implausible that gene flow over large distances can be maintained. Especially the > 7,000 km expanse of open deep water

between New Zealand and Chilean Patagonia forms the continuum of the East Pacific Barrier (EPB) that was a highly effective marine biogeographical barrier separating west-central and eastern Pacific fauna. Alternatively, molecular dating of divergence suggested speciation for residences between sister taxa residing both sides of the EPB was much younger than the proposed onset of the barrier, which implies that the existence of truly trans-EPB specie, if any, would be a complex interplay between vicariance and dispersal.

The questions are in particular:

i. Is the EPB really a strict barrier to gene flow and migration between New Zealand and South America?

ii. Do we find population genetic evidence that *M. gregaria* located off New Zealand and South America has undergone cryptic speciation or remains as one integrated species?

iii. To what extent and in which direction do populations from New Zealand and South America historically and contemporarily exchange genes?

2.2 Outline of the thesis

The thesis consists of the introduction (chapter 1), three chapters corresponding to scientific publications and manuscripts on the development and analysis of molecular markers on the squat lobster *Munida gregaria* (chapter 4-6) and a chapter of synthesis that summarizes the results and points out the importance for present and future research (chapter 7).

The three manuscript chapters discuss the necessity of a microevolutionary framework by developing high informative marker set for the study species (chapter 4) and subsequently using the novel marker set in combination with a traditional mitochondrial marker to provide evidence in delimiting species (chapter 5) and to analyze the population genetic structure, the present and past gene flow and demography of the study species (chapter 6).

3. List of publications and declaration of contribution

This thesis is organized into three chapters, each corresponding to separate research articles. The candidate is the first author of the 3 manuscripts.

List of publications and declaration of contribution:

Publication I

Wang C, Held C

Isolation and characterization of 11 microsatellite markers from the squat lobster *Munida gregaria* (Decapoda: Munididae) around the Falkland Islands/Islas Malvinas

Conservation Genetics Resources (2015) 7, 147-149

DOI: 10.1007/s12686-014-0314-5

The candidate designed the research, performed experiments, analysed the data and prepared the manuscript. CH provided guidance in data analysis and improved manuscript.

Publication II

Wang C, Agrawal S, Laudien J, Häussermann V, Held C

Discrete phenotypes are not underpinned by genome-wide genetic differentiation in the squat lobster *Munida gregaria* (Crustacea: Decapoda: Munididae): a multi-marker study covering the Patagonian shelf

Submitted to *BMC Evolutionary Biology* on 17 August 2016

CH and the candidate conceived and designed the study. The field work was carried out by CH, the candidate, JL and VH. The candidate carried out the experiments and the analyses. SA and CH provided guidance in data analysis. The candidate and CH wrote the manuscript.

Publication III

Wang C, Held C

Crossing the impassable: mitochondrial and microsatellite markers confirmed high level of realized dispersal and gene flow in the transoceanic squat lobster *Munida gregaria* (Decapoda: Munididae) across the East Pacific Barrier

To be submitted to *Molecular Ecology*

The candidate designed the research, performed experiments, analysed the data and prepared the manuscript. CH provided guidance in data analysis and improved draft.

4. Isolation and characterization of eleven microsatellite markers from the squat lobster *Munida gregaria* (Decapoda: Munididae) around the Falkland Islands/Islas Malvinas

4.1 Abstract

Understanding phylogenetic relationship and patterns of contemporary population genetic structure is a prerequisite for conservation and management of potential fishery resources. In this study I report the isolation and characterization of 11 polymorphic microsatellite loci for the squat lobster *Munida gregaria* from around the Falkland Islands/Islas Malvinas. The number of alleles ranged from 3 to 31, observed heterozygosity varied from 0.130 to 0.870. Cross-amplification was 100% successfully in the species/morph *M. subrugosa* and 36.4% in another congeneric species *M. gracilis*. This set of microsatellites is useful for studies focused on taxonomy, genetic diversity and genetic connectivity further may provide stock assessment information for monitoring this important fishery resource.

Keywords: *Munida*, Microsatellite, Falklands/Malvinas, Fishery

4.2 Methods and Results

Munida gregaria (Fabricius, 1793) is an abundant decapod in the benthic fauna and a potential fishery resource in southern South America (Tapella *et al.* 2002b) with further occurrences in the Bass Strait, southern Australia and eastern New Zealand. Although sharp differences in morphological, behavioral and ecological traits have sometimes lead to the recognition of *M. subrugosa* Dana, 1852 as a distinct species, the two forms show no consistent genetic differentiation based on mitochondrial markers (Pérez-Barros *et al.* 2008). Clarifying if they are either (1) recently diverged species or (2) distinct phenotypes arising from a single genotype requires multiple independent, fast-evolving genetic markers from the nuclear genome, therefore I developed and characterized eleven polymorphic microsatellite markers for *M. gregaria*.

Genomic DNA from several specimens from the Falkland Islands/Islas Malvinas was extracted using QIAamp DNA Mini Kit (Qiagen) and subsequently pooled to construct microsatellite enriched libraries according to the reporter genome protocol (Leese *et al.* 2008). Single stranded DNA of *Mus musculus* served as reporter genome to hybridize target genomic DNA. Enriched fragments were amplified, purified and cloned into pCR2.1-TOPO® TA vector then transformed into competent TOP10F' *E. coli* (Invitrogen, Promega). In total, 128 positive clones were sent to GATC-Biotech (Konstanz, Germany) for shotgun sequencing. Microsatellite characterization and primer design was carried out using the STAMP package

(Kraemer *et al.* 2009). Out of 113 clones containing microsatellites, 29 candidate loci were identified and tested in 10 µl reactions containing final concentration of 0.02 U/µl 5-Prime Hotmaster Taq, 1 x PCR-buffer, 0.5 µM of forward and reverse primers, 0.2 mM dNTPs. Out of 21 primer pairs that successfully amplified, forward primers of each locus were synthesized with M13 tails added to their 5' terminus (5'-GTAAAACGACGGCCAG-3'). Optimal annealing temperature of M13-tailed primer pairs were decided after test on gradient PCR. Final amplifications were conducted in two separate, successive PCRs, the first of which contained 0.1 µM M13-tailed forward primers and reverse primers at annealing temperatures optimized for each locus. The second amplification contained 0.2 µM labeled universal M13 primers and unlabeled locus-specific reverse primers with annealing temperature at 53 °C. One microliter product of the first PCRs served as DNA templates in 10 µl reactions of the second PCRs. PCR program was as follows: 94 °C for 2 min, then 36 cycles of 94 °C for 20 s, annealing temperature for 20 s and 65 °C for 1 min, followed by a final elongation at 65 °C for 30 min. Final labeled products were analyzed on an ABI 3130xl using ROX GS500 size standard (Applied Biosystems). Genotyping was performed using GENEMAPPER ® 4.0 (Applied Biosystems).

Allelic variation of eleven successfully genotyped microsatellite loci (Table 1) was assessed on 92 specimens from six stations around the Falkland Islands/Islas Malvinas. Alleles per locus ranged from 3 to 31, observed heterozygosities (H_o) varied from 0.130 to 0.870. No evidence for scoring errors caused by large allelic dropout or stutter bands could be found using MICROCHECKER 2.2.3 (Van Oosterhout *et al.* 2004), while null allele presence was suggested for Locus Mgr63. Significant deviations from Hardy-Weinbeig equilibrium (HWE) were observed for two loci (Mgr63 and Mgr90) after Bonferroni correction ($P < 0.001$), but no linkage disequilibria were detected using GENEPOP 4.0.

Cross-amplification test was successful for all 11 loci in the *M. subrugosa* species/morphotype (n = 10), but only for four loci (mgr4, mgr52, mgr90, mgr120) in another congeneric species *M. gracilis* (n = 3). These polymorphic loci will prove useful to solve the aforementioned taxonomic riddle and study the population genetics of *M. gregaria* in terms of conservation and fisheries management.

4.3 Acknowledgments

I thank Andrea Eschbach for technical assistance. CW was supported by the Chinese Scholarship Council (CSC grant Nr. 2009633009).

Table 1 Characterization of 11 polymorphic microsatellite loci from *M. gregaria* for the Falklands/Malvinas population (n = 92)

Locus	Primer sequence (5' – 3')	Repeat motif	N_A	Size range (bp)	M13-tailed T_a(°C)	H_o/H_e	Genbank accession no.
Mgr4	F: TACATTTGGACAGGTGGCGAC R: TTCACAACATTTTCTCCCGAC	$(ACC)_5$…$(ACC)_4$ …$(ACC)_4$	12	296-338	58	0.717/0.767	KF011233
Mgr8	F: TTCAGGTGACATGTTAGGCA R: AACAGCTCCTGGCAGTACAT	$(AATC)_3$…$(AC)_{13}$	15	310-364	58	0.846/0.806	KF011234
Mgr46	F: CACAGTGAAACAGAGACCCA R: AAGGGAGGATGTGTGAGGTA	$(AG)_8$	3	166-170	60	0.130/0.162	KF011236
Mgr52	F: CCGGAGTTCATTCACAATTT R: CATAAGTAGACGCACACGGA	$(AC)_{10}$	9	160-176	55	0.761/0.733	KF011230
Mgr60	F: CACCTGGACCCATCATTATC R: GTATGGAATGTGTATGGCCG	$(AC)_{29}$	31	170-258	56	0.750/0.867	KF011231
Mgr62	F: TAAGTTCGACTCCTGGCATT R: TGCTGCCTTTATCCAGTTCT	$(AC)_{11}$	6	155-167	60	0.527/0.473	KF011232
Mgr63	F: ATGAGACTGTCTGGGTCTGG R: GACAAACAAGGAGAGACCGA	$(ACAG)_7$...$(ACAG)_3$…$(ACAG)_4$	21	161-217	57	0.522/0.829	KF011235
Mgr81	F: TTACAACACAACAGTGGCTAC R: CCGTGTTCACATTGTATCAG	$(AC)_9$	8	304-322	58	0.674/0.622	KF011229
Mgr90	F: GCTGCAGTAGTAGTCTTGGC R: ACATGAGCAGCACGTACAGT	$(AGC)_8$	8	232-253	60	0.870/0.815	KF011238
Mgr105	F: CTTGGTAAGAAAATGAAGGTA R: TTGTTTATGTGTCAAATGCTG	$(AC)_6$…$(ACAG)_{10}$	26	267-353	57	0.802/0.929	KF011228
Mgr120	F: GGCAGATAACGTGACAAGGAC R: CCGTCTTACTCGTCACATTCAT	$(AAGAGG)_4$	10	249-318	58	0.283/0.304	KF011237

N_A number of alleles, T_a annealing temperature, H_o/H_e observed and expected heterozygosity

5. Discrete phenotypes are not underpinned by genome-wide genetic differentiation in the squat lobster *Munida gregaria* (Crustacea: Decapoda: Munididae): a multi-marker study covering the Patagonian shelf

5.1 Abstract

Background: DNA barcoding has demonstrated that many discrete phenotypes are in fact genetically distinct (pseudo)cryptic species. Similarly different phenotypes, however, can also be expressed by genetically identical, isogenic individuals depending on environmental conditions. This alternative explanation to cryptic speciation often remains untested because it is harder to ascertain.

The widespread squat lobster *Munida gregaria* comprises two discrete ecotypes, *gregaria* s. str. and *subrugosa*, which were regarded as different species by some due to marked differences in their morphological, ecological and behavioral traits. I studied the morphometry and genetics of *M. gregaria* and tested (1) whether the phenotypic differences remain stable after continental-scale sampling and inclusion of different life stages, (2) and whether each phenotype is underpinned by a specific genotype.

Results: A total number of 219 *gregaria* s. str. and *subrugosa* individuals from 25 stations encompassing almost entire range in South America was included to perform morphological and genetic analyses using nine unlinked hypervariable microsatellites and new COI sequences. Results from the PCA and using discriminant functions demonstrated that the morphology of the two forms remains discrete. The mitochondrial data showed shallow, star-like haplotype network and complete overlap of intra- and inter-morphotypic genetic distances. Although all microsatellite markers possess sufficient genetic variation, AMOVA, PCoA and Bayesian clustering approaches revealed no genetic clusters corresponding to ecotypes or geographic units across the entire South-American distribution. No evidence of isolation-by-distance could be detected for this species in South America.

Conclusions: Despite their pronounced bimodal morphological and different lifestyles, the *gregaria* s. str. and *subrugosa* ecotypes form a single, dimorphic species *M. gregaria* s. l.. Based on adequate geographic coverage and multiple independent polymorphic loci, there is no indication that the two phenotypes have a genetic basis, leaving phenotypic plasticity or localized genomic islands of speciation as possible explanations.

Keywords: phenotypic plasticity, genetic homogeneity, squat lobster, microsatellites, gene flow

5.2 Background

Different species have different morphologies and lifestyles, which is commonly taken (but not often tested) to reflect different underlying genotypes. The advent of affordable DNA sequencing and molecular barcoding has served to greatly intensify the crosstalk between molecular and taxonomic disciplines by uncovering a large number of previously overlooked (pseudo)cryptic genotypes (Bickford *et al.* 2007; Hebert *et al.* 2004; Knowlton 2000), many of which could be shown to be associated with equally overlooked morphotypes (Krabbe *et al.* 2009; Saez & Lozano 2005; Vanelslander *et al.* 2009).

However, the popularity and large number of cryptic species currently being discovered have led to an under-appreciation of the notion that sharply distinct morphotypes are not always the consequence of genetic differences but can also be invoked from the same genotype, often called by environmental triggers. The differences between associated morphotypes and lifestyles of ecotypes within the same species can be surprisingly pronounced (Harris *et al.* 2012; Jarrett 2009; Padilla 1998; Römer & Beisenherz 1996; Zakas & Wares 2012).

Proving polyphenism and rejecting cryptic speciation as an explanation is harder than sequencing a mitochondrial gene fragment, which may in part explain the relatively lower number of well-studied cases of polyphenism. Consistent differences among representatives of different morphotypes in a single mitochondrial marker may suffice to at least flag these clades to be checked for the presence of cryptic species, sometimes the mitochondrial differentiation is taken at face value with no additional test carried out. But to demonstrate that no genetic differences are responsible for the differences between ecotypes requires much more extensive molecular evidence from multiple unlinked nuclear loci with sufficiently high substitution rates in order to avoid common artefacts leading to the absence of demonstrable variation in the molecular markers, in view of the commonly reported mito-nuclear discordance (Toews & Brelsford 2012). Such extensive *a posteriori* knowledge is rare (e.g. *Daphnia pulex* (Colbourne *et al.* 2011; Spanier *et al.* 2010; Tollrian & Leese 2010)), hence it is mostly experimental studies in which the genetic identity of individuals is known *a priori* that contribute greatly to our understanding of the importance of polyphenism and morphological plasticity, e.g. parthenogenetic aphids (Dombrovsky *et al.* 2009; Müller *et al.* 2001), marbled crayfish (Vogt *et al.* 2008), polyembryonic armadillos (Loughry & McDonough 2002), inbred lines of *Drosophila* (Astauroff 1930), cloned swine (Archer *et al.* 2003a).

It is unclear if the small number of confirmed polyphenism resulting from similar or identical genetic backgrounds is a condition truly rare in nature or whether it reflects mostly a discovery and/or publication bias.

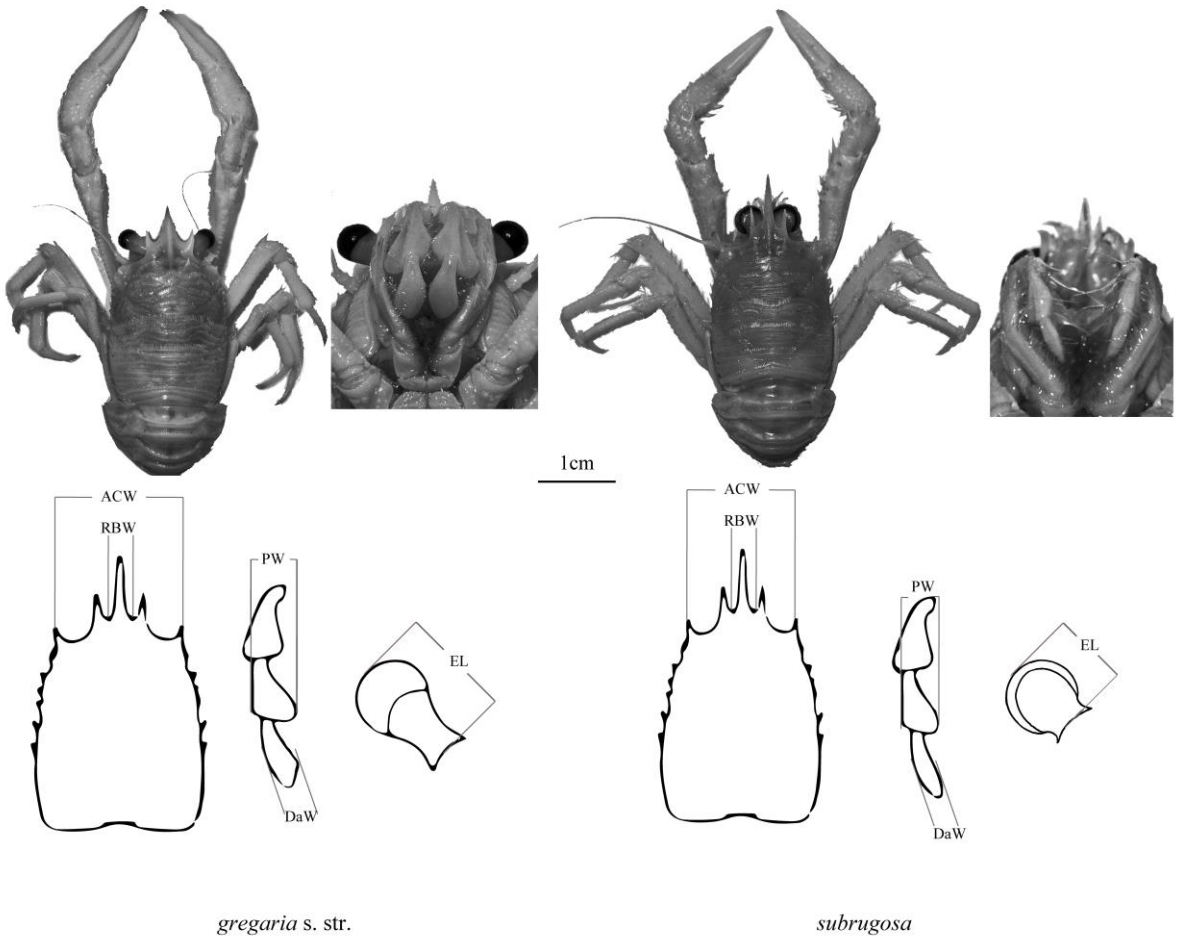

Fig. 5.1 General views of *gregaria* s. str. and *subrugosa* ecotypes (above) and schematic diagrams showing morphometric measurements (below). For the dorsal view of both ecotypes, scale bar represents 1 cm. Measurements are made on: ACW, anterior carapace width; RBW, rostrum basis width; DaW, width of dactylopodus of third maxilliped; PW, width of propodus of the third maxilliped; EL, eyestalk length.

In this paper I investigate the dimorphic squat lobster, *Munida gregaria* sensu lato (Fabricius, 1793), which is currently considered to comprise the ecotypes *M. gregaria* sensu stricto Miers 1881 as well as its junior synonym *M. subrugosa* Dana, 1852 (see Baba *et al.* 2008). For clarity and brevity, hereafter I refer to them as *Munida gregaria* s.l. comprising the two ecotypes *gregaria* s.str. and *subrugosa*. In South America, *M. gregaria* s. l. occurs in shallow marine waters off Patagonia, including Tierra del Fuego and the Falkland Islands/Islas Malvinas, while in the southwestern Pacific *M. gregaria* s. l. are reported from off eastern New Zealand and

Tasmania (Baba *et al.* 2008; Zeldis 1985). The taxonomic status of *gregaria* s. str. and *subrugosa* ecotypes has been subject to conflicting interpretations. Both ecotypes were often regarded as different species because of morphological differences in adult specimen (Fig. 5.1) as well as different behaviors at certain developmental stages (Boschi *et al.* 1992; Hendrickx 2003; Retamal 1981; Tapella & Lovrich 2006). Williams (1973) on the other hand interpreted *gregaria* s. str. as a transient, pelagic ontogenetic stage that would later in life gain the physical features of *subrugosa* upon adopting a permanently benthic lifestyle. Regardless of the taxonomic ramifications, g*regaria* s. str. is often found in huge pelagic swarms that *subrugosa* lacks (Tapella *et al.* 2002a; Zeldis 1985). These differences persist even where both ecotypes co-exist in the same habitat. Nevertheless, on the basis of a lack of mitochondrial DNA differentiation (Pérez-Barros *et al.* 2008), these two ecotypes are currently treated as a single polymorphic species under the name of *M. gregaria* in the most recent taxonomic revision of the family (Baba *et al.* 2008). But this evidence must be considered insufficient because the sampled region (Beagle Channel) represents a very small part of the species' distribution and the molecular evidence rest exclusively on two linked mitochondrial markers (COI and ND1), whereas the results of the only nuclear marker (ITS-1) had to be excluded from the final analysis of the only molecular study (Pérez-Barros *et al.* 2008).

In order to test whether *gregaria* s. str. and *subrugosa* ecotypes correspond to different species of *Munida* or represent a single species with variable phenotypes, I employed multiple independent, fast-evolving nuclear microsatellite markers (Wang & Held 2015) and an expanded set of mtDNA sequences. The sampled area encompasses nearly the entire distribution of *gregaria* s. str. and *subrugosa* ecotypes in South America. In addition, I analysed morphological differences of both ecotypes and different ontogenetic stages following the method of Tapella and Lovrich (2006) in order to test if the more complete geographic sampling continues to support the discrete morphological clusters or if the boundaries between the two ecotypes vanish under more complete geographic coverage.

Table 5.1 Sampling sites and number of adult and juvenile (in parentheses) *gregaria* s. str. and *subrugosa* ecotypes

			gregaria s. str.	*subrugosa*
Station	Latitude	Longitude	adults (juveniles)	adults (juveniles)
			N_{mtDNA}/N_{MSAT}	N_{mtDNA}/N_{MSAT}
Falklands/Malvinas (FM)				

12OT12	-52.110	-59.595	3/16 (0/0)	0/0 (0/0)
13OT13	-52.285	-59.546	4/12 (0/0)	0/0 (0/0)
15OT18	-52.391	-59.131	2/16 (0/0)	0/0 (0/0)
16OT19	-52.354	-58.592	2/16 (0/0)	0/0 (0/0)
17OT20	-52.359	-58.889	2/16* (0/0)	0/0 (0/0)
21OT16	-52.408	-59.976	2/16* (0/0)	0/0 (0/0)
12OT15	-52.189	-59.975	1/1 (0/0)	0/0 (0/0)
Total			16/93 (0/0)	0/0 (0/0)
Northern Chilean Patagonia (NCP)				
Punta Metri	-41.595	-72.712	0/0 (4/7)	0/0 (0/0)
Huinay	-42.354	-72.463	3/3 (13/13)	0/0 (0/0)
Isla Dring	-46.442	-73.957	0/0 (7/7)	0/0 (0/0)
Punta Añihue	-43.793	-72.925	0/0 (4/16)	0/0 (0/0)
Islas Pajaros	-43.783	-72.997	0/0 (0/0)	0/0 (5/5)
Isla Lopez-mine	-50.361	-75.332	0/0 (4/7)	0/0 (0/0)
Isla Solaris	-51.330	-74.311	5/5 (0/0)	7/7 (0/0)
Total			8/8 (32/50)	7/7 (5/5)
Tierra del Fuego archipelago (TdF)				
Punta Dungenes	-52.436	-68.568	0/0 (0/0)	1/1 (0/0)
Bahia Gregorio	-52.685	-70.142	5/10 (0/0)	0/0 (0/0)
Seno Otway	-52.918	-71.347	0/0 (0/0)	4/13 (0/0)
Silva Palma	-53.347	-71.800	0/0 (0/0)	0/0 (2/7)
Bahia Inutil	-53.556	-69.695	0/0 (0/0)	2/5 (0/0)
Bahia Nassau	-55.383	-69.450	0/0 (0/0)	1/1 (0/0)
Pt. Engaño	-54.929	-70.709	0/0 (0/0)	2/2 (0/0)
Bahia Virginia	-54.911	-67.726	0/0 (0/0)	8/12 (0/0)
Isla Picton	-55.174	-66.721	0/0 (0/0)	0/2 (0/0)
Isla Lennox	-55.393	-66.679	0/0 (0/0)	2/2 (0/0)
Total			5/10 (0/0)	20/38 (2/7)
Mar del Plata (MdP)				
CCDB 2374	-38.003	-57.469	0/0 (0/0)	1/0 (0/0)
Grand total			29/111 (32/50)	28/45 (7/12)

N_{mtDNA}, the number of specimens used with mitochondrial marker; N_{MSAT}, the number of specimens used with microsatellites

* refers to one specimen with missing genotype at a certain microsatellite locus

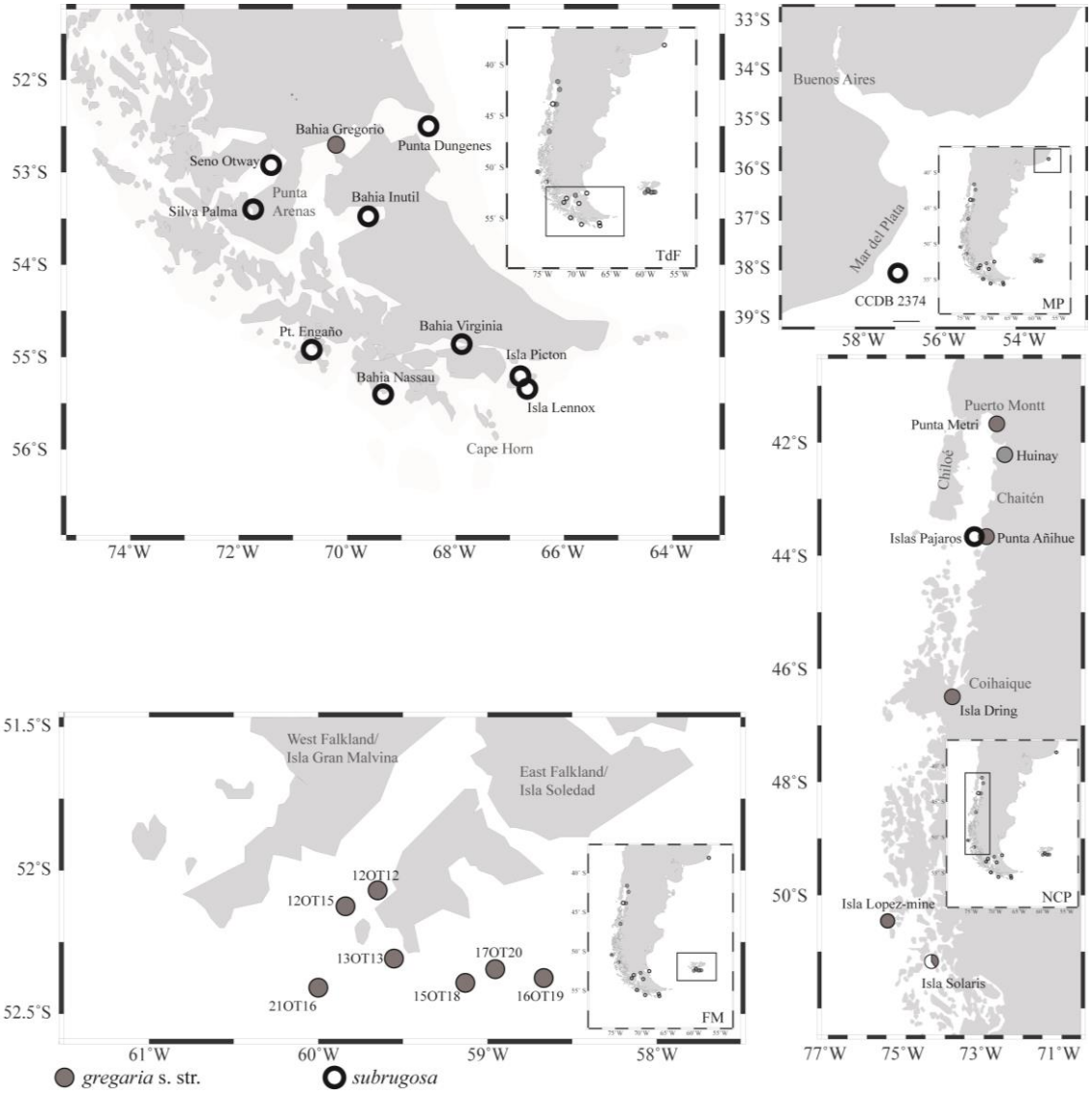

Fig. 5.2 Sampling sites of *gregaria* s. str. (solid circle) and *subrugosa* (open circle) ecotypes. FM, Falklands/Malvinas; TdF, Tierra del Fuego archipelago; NCP, Northern Chilean Patagonia; MP, Mar del Plata.

5.3 Methods

Study population

A total number of 219 individuals were used for both morphological and molecular analyses in this study. These samples were collected at 25 stations from Patagonia and off the Falkland Islands/Islas Malvinas ranging from 38°S to 55°S from the shallow subtidal area to 179 meters water depth by mid-water or bottom trawls (Table 5.1, Fig. 5.2). Sexual maturity was identified

as presence of eggs and/or sexually dimorphic pleopods. In adult males the first two pairs of pleopods are modified to form gonopods, the remaining three pairs are flap-like; in adult females the first pair is missing and the remaining four pairs are elongated with long setae for egg-carrying (Tapella *et al.* 2002a).

Morphological analysis

Specimens were classified as *gregaria* s. str. rather than *subrugosa* ecotypes based on the following characteristics: longer eyestalk length (EL), wider rostrum basis (RBW) and broader and blunter dactylus of the third maxilliped (DaW) (Fig. 5.1). These three morphometric characteristics together with anterior carapace width (ACW) and width of propodus of the third maxilliped (PW) were statistically significant in discriminating ecotypes (Tapella & Lovrich 2006). I measured my samples using a Leica MZ-12.5 microscope with intraocular scale to the nearest 0.1 mm. To determine patterns emerging from the morphometric measurements of these five body parts, principal components analysis (PCA) was plotted using the statistical package PAST 3 (PAlaeontological STatistics, Hammer *et al.* 2001). Applying discriminant functions (DF1 and DF2) introduced in (Tapella & Lovrich 2006), ΔDF (DF1-DF2) values were calculated based on these measurements and subsequently plotted using R (R Core Team 2015).

mtDNA analysis

DNA was extracted from ethanol-preserved abdominal or cheliped muscle tissue using QIAamp DNA Mini Kit (QIAGEN, Germany). For mtDNA analysis, a region of the COI gene was amplified using the universal primers HCO2198 and LCO1490 (Folmer *et al.* 1994) for 96 individuals (Table 1). The 10 µl reactions consisted of 0.02U/µl Hotmaster Taq (5 Prime), 0.2 mM dNTPs, 0.5 µM of forward and reverse primers, 1 x PCR-buffer and 1 µl (about 30 ng) of template DNA. PCR was conducted using an initial denaturation at 94°C for 2 minutes, followed by 36 cycles of 94°C for 20 seconds, annealing at 47°C for 20 seconds, 65°C for 1 minute, and a final extension at 65°C for 10 minutes. Size and quality of amplified products were checked on a 2% agarose gel in TAE buffer, and then 1 µl of purified PCR product was used for cycle sequencing with the HCO primer. Sanger sequencing was conducted on an ABI

3130xl sequencer. Alignment was done using CODONCODE ALIGNER 4.0 (CodonCode Corp.) and checked for the presence of ambiguities and stop codons.

DNA polymorphism was examined as haplotype diversity (H_D) and nucleotide diversity (π) for each ecotype and all samples using DnaSP 5.10 (Librado & Rozas 2009). Genealogical relationships among haplotypes were inferred using statistical parsimony implemented in TCS 1.21 (Clement *et al.* 2000). Pairwise genetic divergences measured as number of nucleotide differences were calculated within and between the two ecotypes in MEGA 5.2 (Tamura *et al.* 2011). For a better understanding of the genetic distances and barcoding gap analysis, I added three congeneric species to my analysis: *M. rutllanti* (n = 5; GenBank accession numbers: JQ306226-JQ306230), *M. quadrispina* (n = 3; GenBank accession numbers: DQ882090-DQ882092), and *M. gracilis* from my own collection (n = 2; GenBank accession numbers: KJ544249-KJ544250). Pairwise genetic distances were calculated within *M. gregaria* s. l. (pooled *gregaria* s. str. and *subrugosa*) and versus the other three *Munida* species.

Microsatellite analysis

In total, 218 individuals were screened for genetic variation at 11 microsatellite loci that were originally designed for *M. gregaria* s. l. (Wang & Held 2015) (Table 5.1). Allele sizes were manually binned and genotypes were assessed in GENEMAPPER 4.0 (Applied Biosystems). Null alleles, stuttering and large allele dropout were tested using MICROCHECKER (Van Oosterhout *et al.* 2004). Because of too many missing data and possible null alleles, locus Mgr63 and Mgr105 were excluded from subsequent analyses. Genetic diversity within each ecotype was summarized as allelic richness (*Ar*) in FSTAT 2.9.3.2 (Goudet 2001) using the rarefaction approach, which was also used to determine the number of private alleles using standardized sample sizes in ADZE 1.0 (Szpiech *et al.* 2008). Detection of linkage disequilibrium between loci and deviations from Hardy-Weinberg equilibrium (HWE) per ecotype were performed using GENEPOP 4.2 (Raymond & Rousset 1995). All loci were tested for positive/diversifying or balancing selection using LOSITAN (Antao *et al.* 2008), which simulates an expected distribution of F_{ST} as a function of expected heterozygosity under an island model of migration. The statistical power of this set of microsatellite loci to detect significant genetic differentiation between populations/ecotypes was tested with POWSIM 4.1 (Ryman & Palm 2006) using both Chi-square (χ^2) and Fisher's exact test analysis. Various levels of differentiation (measured as F_{ST} in the range from 0.001 to 0.01) were determined by combining different effective population size (N_e) and times since divergence (t). In addition,

POWSIM allows calculating type I error probability, which is the probability of rejecting the null hypothesis of genetic homogeneity although it was true by drawing the alleles directly from the base population ($t = 0$).

The genetic differentiation among the three major sampling areas, i.e., FM, NCP and TdF (see Table 1), was assessed for each ecotype separately with AMOVA in ARLEQUIN 3.5 (Excoffier & Lischer 2010). Both F_{ST} and R_{ST} estimators were calculated over all nine loci with 1,000 permutations. To provide a visual representation of species separation and potential subdivision, Principal Coordinate Analysis (PCoA) was performed in GENALEX 6.5 (Peakall & Smouse 2006).

Bayesian assignment tests were used to evaluate the level of genetic clustering. I used STRUCTURE 2.3.4 (Pritchard *et al.* 2000) first without giving any prior population information, letting K range from 1 to 5. I also checked whether individuals could be assigned correctly to clusters if the number of ecotypes was given *a priori* ($K = 2$). Both conditions were run with the correlated allele frequencies option under the non-admixture model, i.e. under the assumption that there is no gene flow between ecotypes, as well as under an admixture model, i.e. allowing limited introgression between clusters. Twenty runs with 200,000 Markov chain Monte Carlo (MCMC) iterations after a burn-in period of 25,000 steps were carried out for each K. The results were uploaded onto STRUCTURE HARVESTER (Earl & von Holdt 2012) and K was determined using the *ad hoc* statistic ΔK (Evanno *et al.* 2005), as well as mean estimates of posterior probability $L(K)$ (Pritchard *et al.* 2000). Results from the 20 replicates of the most likely value for K were averaged using the software CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007) and the output was visualized using DISTRUCT 1.1 (Rosenberg 2004).

Since $L(K)$ does not always provide the correct number of clusters and the ΔK statistic cannot evaluate $K = 1$ or the largest value chosen for K (Evanno *et al.* 2005), I also applied STRUCTURAMA 2.0 (Huelsenbeck & Andolfatto 2007), which can directly estimate the number of clusters in which a sample can be subdivided. I allowed the number of populations to be a random variable following a Dirichlet process prior, ran the MCMC analysis for 1,000,000 cycles, sampled every 100th cycle, and discarded the first 400 samples as burn-in.

The impact of isolation by distance (IBD) across southern South America on genetic differentiation was estimated by Mantel tests as implemented in IBD Web Service 3.23 (Jensen *et al.* 2005). For this purpose, only the geographic position but not the ecotype of the samples

(mitochondrial and microsatellite data) were used (Table 5.1; Fig. 5.2). Pairwise F_{ST} values for mitochondrial data and pairwise $(\delta\mu)^2$ genetic distance (Goldstein *et al.* 1995) for microsatellites were obtained in ARLEQUIN 3.5, where spatial distances were calculated using the Geographic Distance Matrix Generator 1.2.3 (http://biodiversityinformatics.amnh.org/open_source/gdmg/index.php). Geographical distances were log-transformed to account for two-dimensional habitat distribution (Rousset 1997), and the significance of the slope of the reduced major axis (RMA) regression was assessed by 30 000 randomizations.

5.4 Results

Morphological analysis

PCA comparison of five key morphometric characteristics (Tapella & Lovrich 2006) revealed clearly distinct groups corresponding to ecotype and age, with the first principal component explaining 92.48% of the variation. Samples of adult and juvenile *gregaria* s. str. formed two isolated groups, both of which were clearly distinct from the *subrugosa* samples. The *subrugosa* individuals comprise the adult and juvenile sub-groups that overlap in part (Fig. 5.3). The discriminant functions with these five morphometric characteristics yielded result coherent with the PCA. Samples of adult *gregaria* s. str. and juvenile *gregaria* s. str. clustered separately, while due to the limited number of *subrugosa* juveniles (n = 12) it is hard to ascertain whether juvenile and adult *subrugosa* show statistically significant differences (Fig. 5.4). Results of morphological analyses ascertain that even among samples from the entire South American distribution *gregaria* s. str. and *subrugosa* are distinct ecotypes at different ontogenetic stages with discrete morphological traits rather than forming the extremes of a continuous distribution.

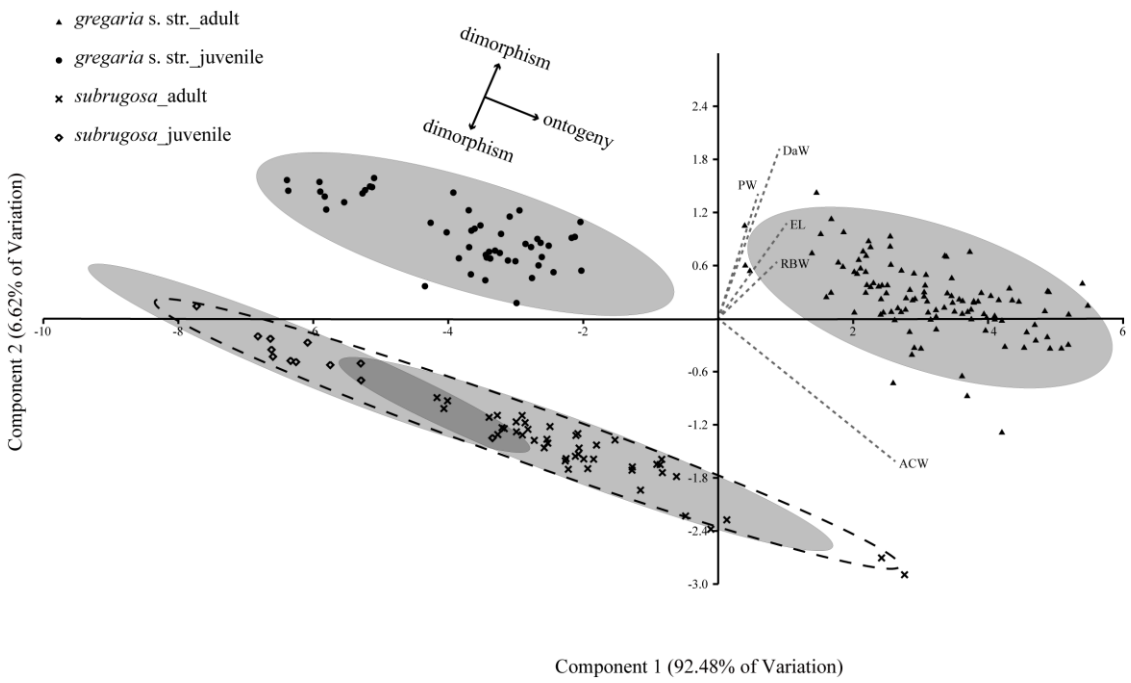

Fig. 5.3 Principal component analysis (PCA) biplot for the morphometric measurements of all the samples. Dashed lines show vectors from the five representative body characteristics that are statistically significant in discriminating ecotypes. The 95% concentration ellipses are given for juvenile and adult *gregaria* s. str. as well as juvenile and adult *subrugosa*. A dashed 95% concentration ellipse represents all *subrugosa* individuals. Solid arrows indicate suggested interpretation as ontogenetic transition within ecotype (horizontal) and morphological discreteness between ecotypes (vertical; see discussion).

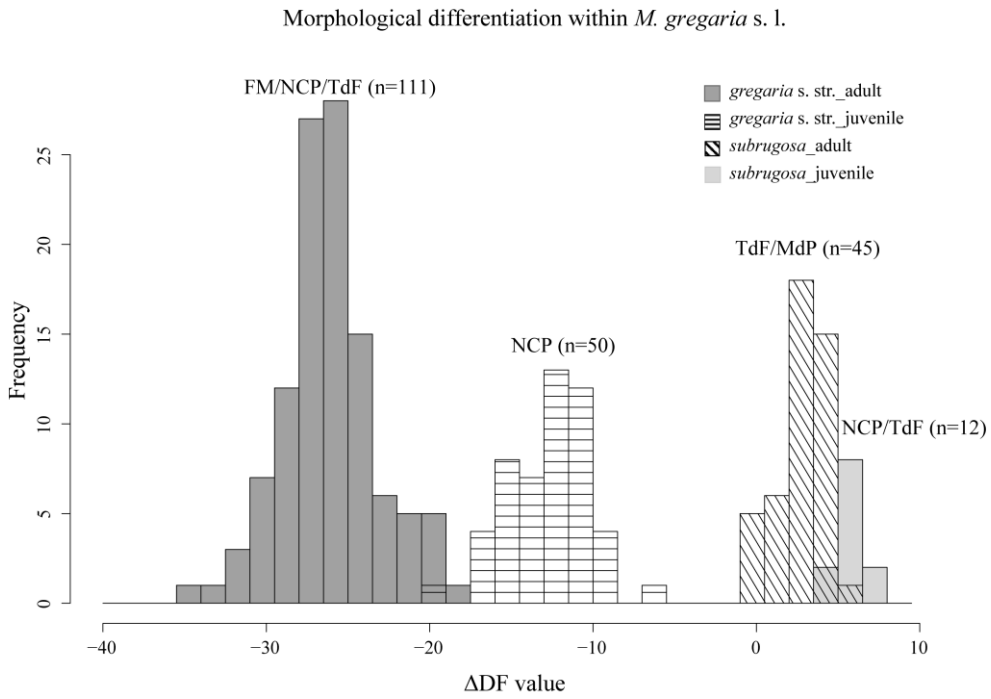

Fig. 5.4 Frequency distribution of ΔDF scores based on the morphometric measurements.

Mitochondrial COI sequence variation

A total number of 96 COI sequences from 61 *gregaria* s. str. and 35 *subrugosa* individuals were obtained as an alignment of 618 bp (GenBank accession numbers: KJ544251 - KJ544346). These sequences were collapsed into 30 different haplotypes, possessing 29 variable (segregating) sites, of which 11 were parsimony-informative. The *subrugosa* ecotype showed slightly higher genetic diversity than *gregaria* s. str. (Additional file 1: Table S5.1). In the 206 codons of the alignment, the 29 variable sites were all synonymous substitutions and no stop codons were found.

Genealogical relationships among haplotypes showed a very shallow, star-like structure. The most common haplotype (n = 59) was shared by both ecotypes as well as by all the sampling regions, which differed from the other haplotypes in 1 to 3 mutational steps (Additional file 2: Fig. S5.1).

Extent of intraspecific and interspecific COI divergence

The mean number of differences among sequences within each ecotype was 0.809 for *gregaria* s. str. and 1.408 for *subrugosa*, between ecotypes it was 1.107. The plotted pairwise genetic distances show complete overlap of distributions within and between ecotypes (Fig. 5.5). The maximal number of differences was six base pairs and no barcoding gap between ecotypes could be identified. By contrast, pronounced barcoding gaps exist between *M. gregaria* s. l. and each of the three other *Munida* species. These interspecific distances were at least ten times larger than distances between *gregaria* s. str. and *subrugosa* ecotypes (Fig. 5.5). The results of COI data including samples from almost the entire South American distribution provide no evidence of genetic separation between *gregaria* s. str. and *subrugosa* ecotypes.

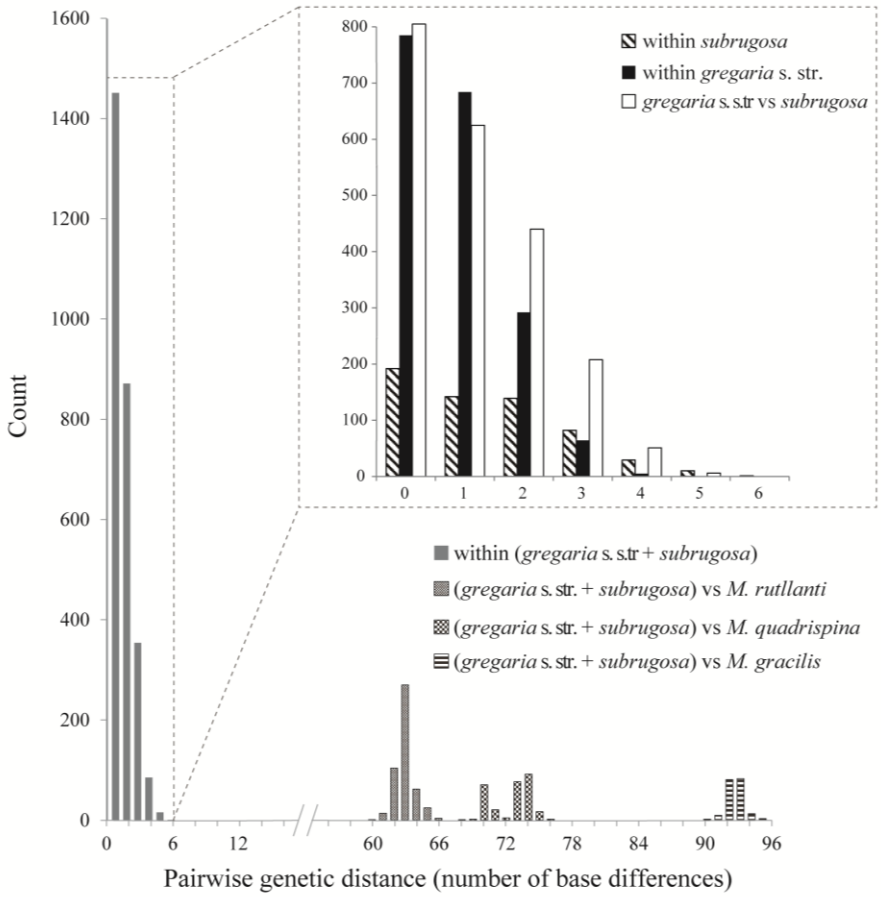

Fig. 5.5 Estimates of divergence between sequences among the two ecotypes and three other *Munida* species. Pairwise comparisons were performed using number of base differences.

Microsatellite diversity

Overall, the number of alleles per locus ranged between six (Mgr46) and 38 (Mgr60) with an average of 14.9. For each locus the number of alleles, allelic size range, allelic richness as well as observed and expected heterozygosities per population and ecotype are reported in Additional file 3: Table S2. The mean number of private alleles per locus when sample size was standardized was slightly higher for *subrugosa* (0.198±0.120) compared to *gregaria* s. str. (0.111±0.065), both of which were very low, reflecting a high degree of allelic sharing between the two ecotypes. Compared to the limited variation among COI sequences, the nine microsatellite loci exhibited broader allelic ranges and orders of magnitude higher allelic variation (Additional file 4: Fig. S5.2). All loci showed no linkage disequilibrium. Locus Mgr90 showed significantly higher heterozygosity than expected, but since excluding Mgr90 had only minor effect on the results, it was kept in this study. The power test suggested that my microsatellite dataset was sensitive enough to detect very weak genetic differentiation (F_{ST} =

0.005) in probabilities of close to 100% using both chi-square (χ^2) and Fisher's exact test (Additional file 5: Fig. S5.3). The F_{ST} outlier analysis showed that none of the loci was under potential selection at 95% confidence level thus they were regarded as neutral in my interpretation of the results.

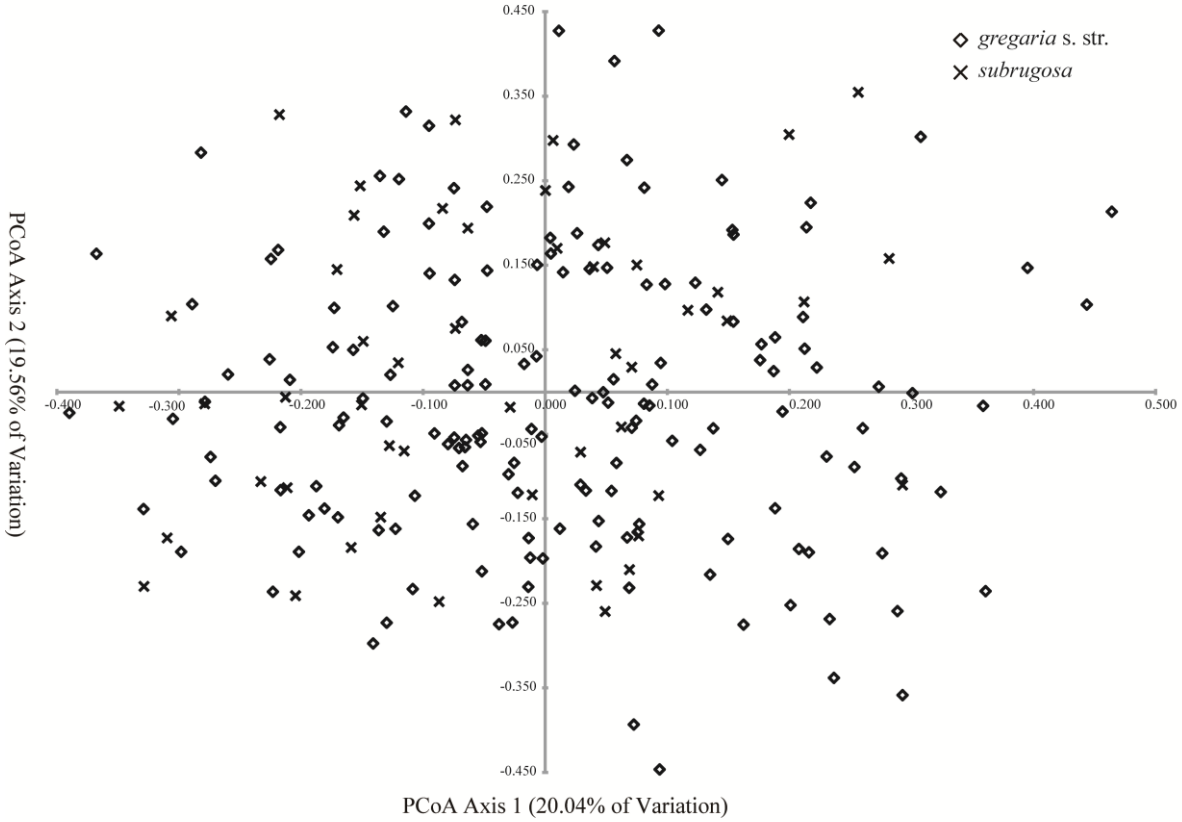

Fig. 5.6 Principal coordinate analysis (PCoA) of 218 individuals of *gregaria* s. str. and *subrugosa* ecotypes based on microsatellites.

Genetic differentiation and individual assignment inferred by microsatellites

Hierarchical AMOVA showed almost all the genetic variance distributed among individuals within sampling areas (Table 5.2), thus corroborating the weak geographic structure in the distribution of mitochondrial haplotypes. PCoA showed approximately equal distributions along the first three axes, which accounted for 20.04%, 19.56% and 18.76% of the total genetic variance, respectively (Fig. 5.6). This result indicates that there is no single factor (ecotype or other) that would dominate the distribution of total genetic variance for the high-resolution microsatellite data.

Table 5.2 Hierarchical analysis of molecular variance based on microsatellites for both ecotypes

Source of variation	d.f.	Sum of squares	variation components	variation [%]	*F*-statistics	*P*	Sum of squares	variation components	variation [%]	*R*-statistics	*P*
Among ecotypes	1	3.487	-0.00290	-0.10	F_{CT}=-0.00103	0.507	167.743	-1.10788	-0.45	R_{CT}=-0.00448	1.00
Among sampling areas within ecotypes	3	10.977	0.01229	0.44	F_{SC}= 0.00437	0.0426	976.391	1.11995	0.45	R_{SC}= 0.00451	0.204
Within sampling areas	431	1207.396	2.80138	99.67	F_{ST}=0.0334	0.0255	106578.987	247.28303	100	R_{ST}=0.00005	0.277
Total	435	1221.860	2.81077	100			107723.122	247.29510	100		

Bayesian cluster analyses with STRUCTURE suggested the best *K* was 1 according to average log probability (*L(K)*), but *K* = 2 was indicated by the highest statistic Δ*K*. This is because the change in log probability does not account for the smallest and largest *K*. Even under *K* = 2, each individual possessed a roughly equal probability of being assigned to the first versus the second cluster, which indicates that all individuals belong to one single group (Fig. 5.7). Congruent distribution of posterior probabilities for each individual was obtained given the *a priori* assumed number of putative populations (=ecotypes).

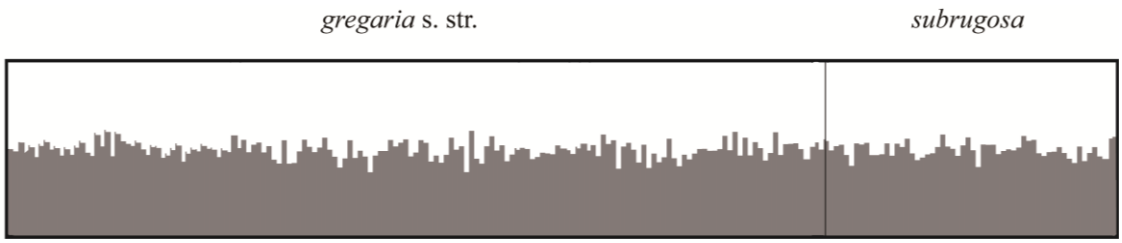

Fig. 5.7 Individual probabilities of cluster assignments from the software STRUCTURE. The most likely number of clusters *K* = 2 is shown for *gregaria* s. str. (n = 161) and *subrugosa* (n = 57) using nine microsatellite loci. Each vertical line represents the probabilities for a single individual to be assigned to one of the clusters (*K*).

The STRUCTURAMA analysis corroborated the inferences of the STRUCTURE analysis. The sampled individuals all belonged to one group with a posterior probability of 1. Eventually, both Bayesian analyses showed no correlation of my microsatellite data with either ecotypes or geographical units.

An absence of genetic structure over the entire distribution range in South America was also found in the IBD tests. Based on both mitochondrial and microsatellite data sets, Mantel tests indicated no significant correlation (for COI, *r* = 0.0608, *P* = 0.229; for microsatellites, *r* = -

0.005, $P = 0.508$) between genetic and log-transformed geographic distances (Additional file 6: Fig. S5.4).

5.5 Discussion

Reality of morphological dimorphism in M. gregaria s. l.

In theory, populations belonging to a so-called 'ring species' might appear sharply distinct in an area of secondary overlap, but appear more gradually changing in morphology or genetics through areas of their distribution that have been more continuously inhabited (see (Irwin *et al.* 2005; Liebers *et al.* 2004) and references therein). Inadvertently sampling only in the zone of secondary overlap might therefore create the incorrect impression of discrete morphotypes when populations with intermediary morphotypes remain unsampled.

Increasing the sampling area from a single location in the Beagle Channel (Pérez-Barros *et al.* 2008) to a continental scale, my data suggest that the boundary between two morphotypes (*gregaria* s. str. and *subrugosa*) is not blurred across the South American shelf (Figs. 5.3 and 5.4).

The expanded morphometric analysis further suggests an ontogenetic dimension in the morphometry. It may be expected that the gap between adults and Northern Chilean Patagonia (NCP) juveniles in *gregaria* s. str. might be closed by inclusion of juveniles from other populations and reveal a continuous ontogenetic transition as can already be found in *subrugosa* (Fig. 5.3). The discreteness of the *subrugosa* and *gregaria* s. str. morphotypes, however, is not a sampling artefact and stable with respect to a more representative sampling scheme as well as inclusion of different life stages.

Phenotype-genotype relationship

Since the proposal that phenotype and genotype form two fundamental different levels of biological abstractions (Johannsen 1911), untangling the relationship between phenotypes and the underlying genotypes has long been challenging and intriguing. The advent of molecular techniques has greatly fostered studies of phenotype-genotype interaction, especially in the venue of ubiquitous application of DNA barcoding. The short mitochondrial fragments (e.g. Held 2003) successfully discovered (pseudo)cryptic genetic divergence whereas corresponding phenotypes appeared identical. Such unexpected genetic diversity, which was later often corroborated by other independent evidences from morphology (Mobley *et al.* 2011), breeding

behavior (Gómez *et al.* 2007) or multiple, independent and informative nuclear markers (Leese & Held 2008), has become an important supplement for the phenotypic identification of an organism to species or sub-species level in taxonomic practice (Hajibabaei *et al.* 2007; Hebert & Gregory 2005; Valentini *et al.* 2009).

Alternatively, molecular marker-based examination found no genetic differentiation matching discrete phenotypes, which is exemplified by the present *Munida* case. Nonetheless, the lack of differentiation at a single mitochondrial marker is insufficient to extrapolate to the entire genome. A previous molecular study used only mitochondrial evidence and found no consistent genetic differentiation associated with each ecotype (Pérez-Barros *et al.* 2008) but failed to demonstrate genetic homogeneity in the nuclear genome. The only nuclear locus (ITS 1) was excluded from the final analysis in (Pérez-Barros *et al.* 2008) due to conflicting information and possible paralogy of sequences. The inference of genetic homogeneity in (Pérez-Barros *et al.* 2008) thus rested exclusively on two fully linked mitochondrial markers, COI and ND1 (the third mitochondrial marker 16S yielded identical sequences among all individuals). In the absence of recombination, mitochondrial genes are vulnerable to introgressive hybridization, sex-biased dispersal, incomplete lineage sorting and heteroplasmy (Ballard & Whitlock 2004; Ebach & Holdrege 2005; Rubinoff *et al.* 2006; Toews & Brelsford 2012; Wares 2010). The determination of a 'barcoding gap' (i.e., significant difference between inter- and intraspecific variation) may fail in case of close phylogenetic relationship or recent divergence (Meyer & Paulay 2005; Moritz & Cicero 2004; Wiemers & Fiedler 2007).

However, the shortcomings of (Pérez-Barros *et al.* 2008) were addressed by my more expansive sampling and the inclusion of multiple unlinked microsatellites, thus suggesting that the distinct phenotypes in *M. gregaria* s. l. are not caused by different genotypes.

A case of phenotypic plasticity

A common caveat to marker-based population genetic studies in case of no differentiation detected among populations (i.e., different phenotypes in this case) is that there may be still unsampled isolated regions of differentiation within genome. Such 'genomic islands' of differentiation (Harr 2006; Turner *et al.* 2005) are usually associated with genes under divergent selection, whilst selectively neutral markers are not involved (Gavrilets & Vose 2005; Nosil *et al.* 2009; Wu & Ting 2004). This alternative is hard to falsify and might be true for any marker-based study in organisms with incompletely known genomes.

Given the geographic and genomic sampling scope of my study, we can eliminate the possibility of genetic differentiation underpinning different phenotypes, then the different ecotypes within *M. gregaria* s. l. represent phenotypic plasticity, which is determined by variations of certain environmental factors. In similar examples from parthenogenetic *Daphnia* and aphids, sharply distinct morphotypes arise from the same genetic background (Dombrovsky *et al.* 2009; Müller *et al.* 2001; Stibor & Lüning 1994) and in some examples the environmental triggers controlling which phenotype is preferentially expressed are known. For example, the sex of offspring from one clutch was found to be determined by temperature among various gonochoristic organisms (those having separate sexes), e.g., invertebrates (Cline 1976; Klass *et al.* 1976), fishes (Baras *et al.* 2000; Koumoundouros *et al.* 2002; Römer & Beisenherz 1996), turtles (Bull & Vogt 1979) and crocodilians (Lang & Andrews 1994). Dramatically different morphology can be expressed in presence or absence of predators in *Daphnia* water fleas (Green 1967; Parejko & Dodson 1991; Tollrian & Harvell 1999), barnacle *Chthamalus fissus* (Jarrett 2009), whereas little genetic correspondence is involved in the predator-induced morphological changes (Jarrett 2008; Spanier *et al.* 2010; Tollrian & Leese 2010).

The determination of *Munida* ecotypes are very likely induced and controlled by one or several as yet unknown environmental factors. In its South American distribution, *Munida gregaria* s. l. occupies extensive latitudinal distribution along both coasts of Patagonia and wide bathymetry (from water surface down to 1137m recorded for *subrugosa* (Arntz *et al.* 1999)), which involves a strong gradient of environmental conditions (temperature, salinity, oxygen concentration and food resources). In some species the feeding performance and diet composition during larval phases can induce development into different morphotypes or sex reversal (Parsons & Robinson 2007; Zupo 2000). Since *gregaria* s. str. and *subrugosa* differ in feeding habit as deposit feeders and predator, respectively (Romero *et al.* 2004), changes in environmental food composition may affect the metamorphosis of *M. gregaria* s. l. in an adaptive way, favouring its development into one ecotype rather than the other. Long-term observations of the proportion of both *gregaria* s. str. and *subrugosa* ecotypes in the Beagle Channel and San Jorge Gulf demonstrate the existence of ecotypes is patchy (see Diez *et al.* 2016 and references therein). Recent hydroacoustical evidence postulates that major pelagic swarms of *gregaria* s. str. on the Argentine continental shelf are associated with productive areas such as frontal zones that vary considerably in spatial and temporal scales (Diez *et al.*

2016), implying the availability of phytoplankton in frontal zones might favor the expression of *gregaria* s. str. ecotype.

Heterochrony, which is generally defined as a developmental change in relation to size and shape in the timing or rate of ontogenic events (see review in Smith 2001), might be a possible mechanism involved in the observed plasticity in *M. gregaria* s. l.. Heterochronic process such as paedomorphic plasticity was postulated in a widespread squat lobster in the Pacific of South America, *Pleuroncodes monodon* (Haye *et al.* 2010). A clear boundary exists in its distribution where to the north it is a smaller, pelagic form and to the south it is a larger, benthic form. These two forms showed no mitochondrial DNA differentiation. A similar developmental variation might be involved in *M. gregaria* s. l., since the population from San Jorge Gulf was shown to have faster growth rate and earlier reproductive investment in its early life history than the southern populations from Beagle Channel and Strait of Magellan (Varisco & Vinuesa 2015).

5.6 Conclusions

Based on extensive sampling of the species' distribution in South America, and using nine independent polymorphic nuclear microsatellite loci in addition to new mitochondrial COI sequences, I were able to show that the lack of genetic differentiation between distinct *gregaria s. str.* and *subrugosa* ecotypes is not an artefact due to insufficient genomic and geographic sampling, instead they are expressed from a single underlying genotype. Morphological tests affirmed the boundaries between the two ecotypes were not blurred with continental-scale geographic sampling, and remain stable despite an ontogenetic dimension in the data. My findings serve as evidence in the taxonomy of species *M. gregaria* s. l. (Fabricius, 1793), which is a species with phenotypic plasticity. Its broad distribution, lack of genetic differentiation and high levels of phenotypic divergence render *M. gregaria* s. l. an appropriate model to study ecologically driven evolution, adaptive radiation and adaptive speciation. In view of the increasing numbers of reported discordance between mtDNA and nuclear DNA, which questions the validity of many species boundaries defined only by fast DNA barcoding, it is necessary and strongly suggested to incorporated nuclear multi-locus markers in the attempt of accurate taxonomy and reflection of genotype-phenotype relationship.

5.7 Acknowledgments

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5.8 Additional files

Table S5.1 Genetic diversity of COI sequences per ecotype

	Sequences	Haplotypes	Private Haplotypes	H_D*	π*
gregaria s. str.	61	18	15	0.571	0.00131
subrugosa	35	14	12	0.677	0.00228
overall	96	30		0.622	0.00169

* H_D, haplotype diversity (Nei 1987, equation 8.4); π, nucleotide diversity (Nei 1987, equation 10.5)
Nei M (1987) *Molecular evolutionary genetics*. Columbia University Press, New York, USA.

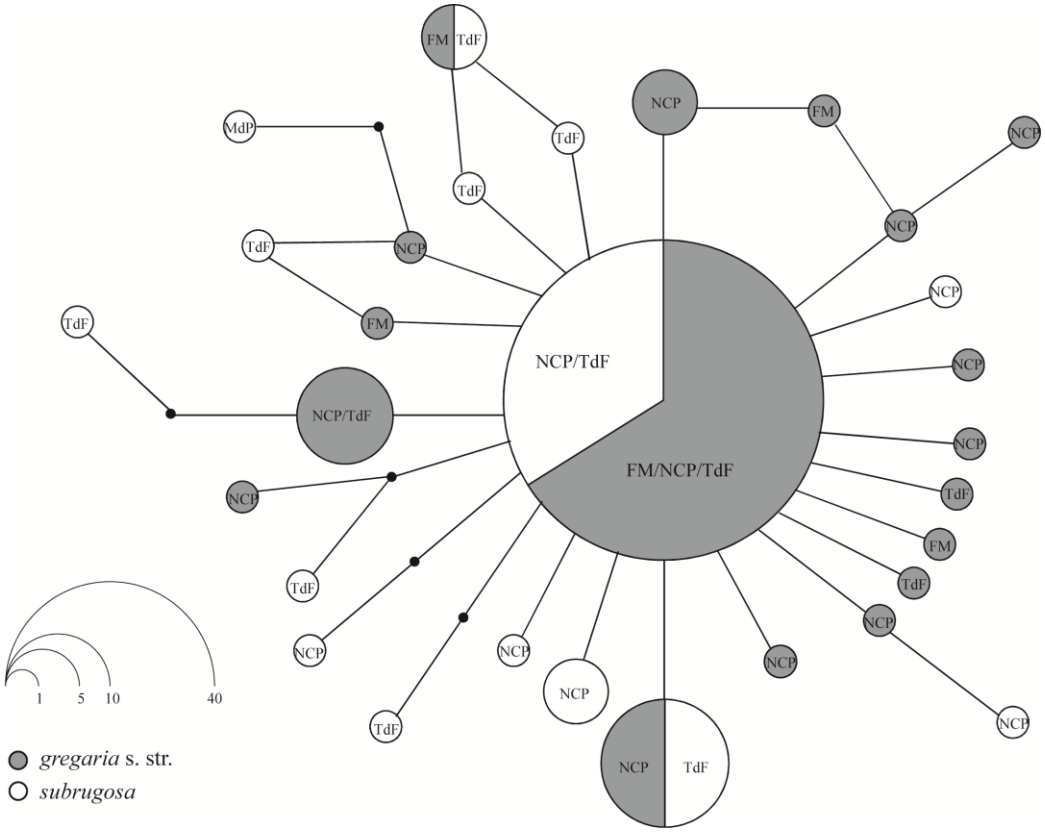


Fig. S5.1 Haplotype genealogies for *gregaria* s. str. and *subrugosa* ecotypes. Each branch represents one substitution; small filled circles represent hypothetical, unsampled haplotypes. Radii reflect number of individuals that share a particular haplotype.

Table S5.2 Diversity indices of nine microsatellite loci for the two ecotypes. Reported are number of alleles N_A, fragment size range, observed heterozygosity H_O, expected

heterozygosity H_E and allelic richness *Ar*. Significant deviation from Hardy-Weinberg equilibruim ($P < 0.05$, based on 10,000 permutations) after Bonferroni correction were labeled in bold.

	N_A	Size range	*subrugosa*_NCP		*subrugosa*_TdF		*subrugosa*_Total	
			H_O/ H_E	*Ar*	H_O/ H_E	*Ar*	H_O/ H_E	*Ar*
mgr4	18	266-341	0.75/0.706	4.13	0.533/0.615	6.02	0.579/0.634	13.03
mgr8	21	284-364	0.916/0.793	7.53	0.844/0.838	6.93	0.860/0.832	13.68
mgr46	6	148-172	0.166/0.159	2.79	0.133/0.208	2.43	0.140/0.198	4.39
mgr52	11	160-180	0.833/0.753	5.32	0.666/0.753	5.07	0.702/0.747	7.93
mgr60	38	170-274	1/0.913	11.91	**0.755/0.895**	11.36	0.807/0.897	29.44
mgr62	7	155-167	0.5/0.489	3.38	0.377/0.406	3.25	0.404/0.424	5.24
mgr81	9	296-322	0.666/0.594	3.53	0.644/0.611	4.05	0.649/0.607	7.09
mgr90	9	232-256	0.75/0.807	6.16	0.8/0.822	6.22	**0.789/0.832**	7.88
mgr120	15	249-318	0.333/0.307	4.12	0.355/0.339	3.95	0.351/0.332	9.59

	gregaria. s. str._FM		*gregaria*. s. str.__NCP		*gregaria*. s. str.__TdF		*gregaria*. s. str.__Total	
	H_O/ H_E	*Ar*	H_O/ H_E	*Ar*	H_O/ H_E	*Ar*	H_O/ H_E	*Ar*
mgr4	0.720/0.768	6.98	0.637/0.676	5.45	0.7/0.805	7.00	0.689/0.737	13.54
mgr8	0.838/0.816	6.62	0.810/0.829	7.46	1/0.773	6.77	0.839/0.816	13.70
mgr46	0.129/0.161	2.16	0.206/0.249	2.67	monomorphic	1.98	0149/0.184	3.68
mgr52	0.763/0.735	5.12	0.689/0.762	5.07	0.7/0.7	4.81	0.733/0.744	7.62
mgr60	0.752/0.870	11.17	0.896/0.887	11.08	0.7/0.905	11.60	0.801/0.880	29.43
mgr62	0.516/0.483	3.17	0.534/0.589	3.53	0.8/0.573	2.83	0.540/0.536	5.47
mgr81	0.677/0.627	4.03	0.620/0.626	4.54	0.4/0.673	2.98	0.640/0.633	7.56
mgr90	**0.870/0.820**	6.32	**0.844/0.830**	6.18	1/0.805	4.98	**0.870/0.824**	8.07
mgr120	0.290/0.311	3.77	0.396/0.376	4.03	0.1/0.1	3.65	0.317/0.324	9.40

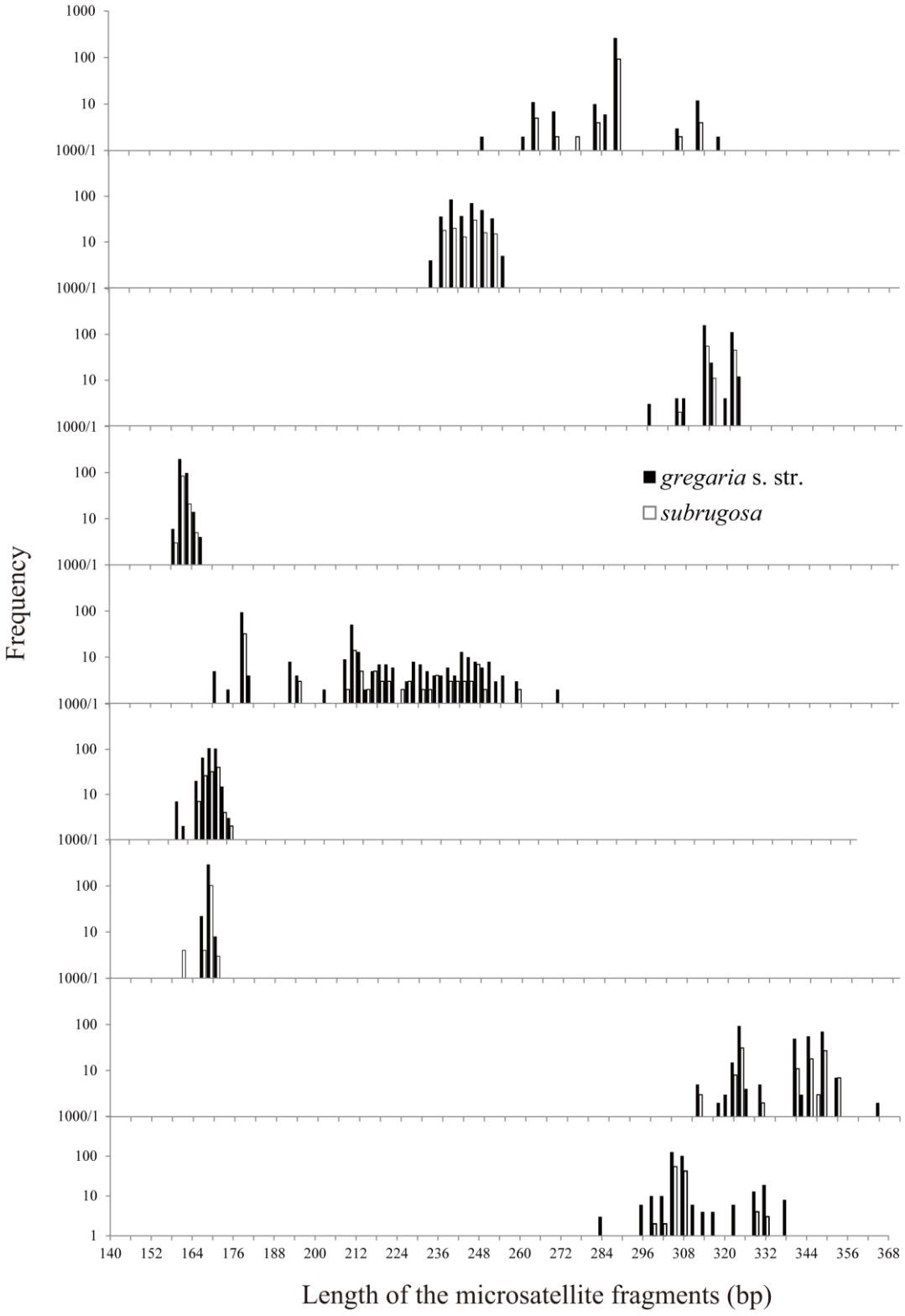


Fig. S5.2 Allelic frequencies (in logarithm) of the nine microsatellite loci polymorphism.

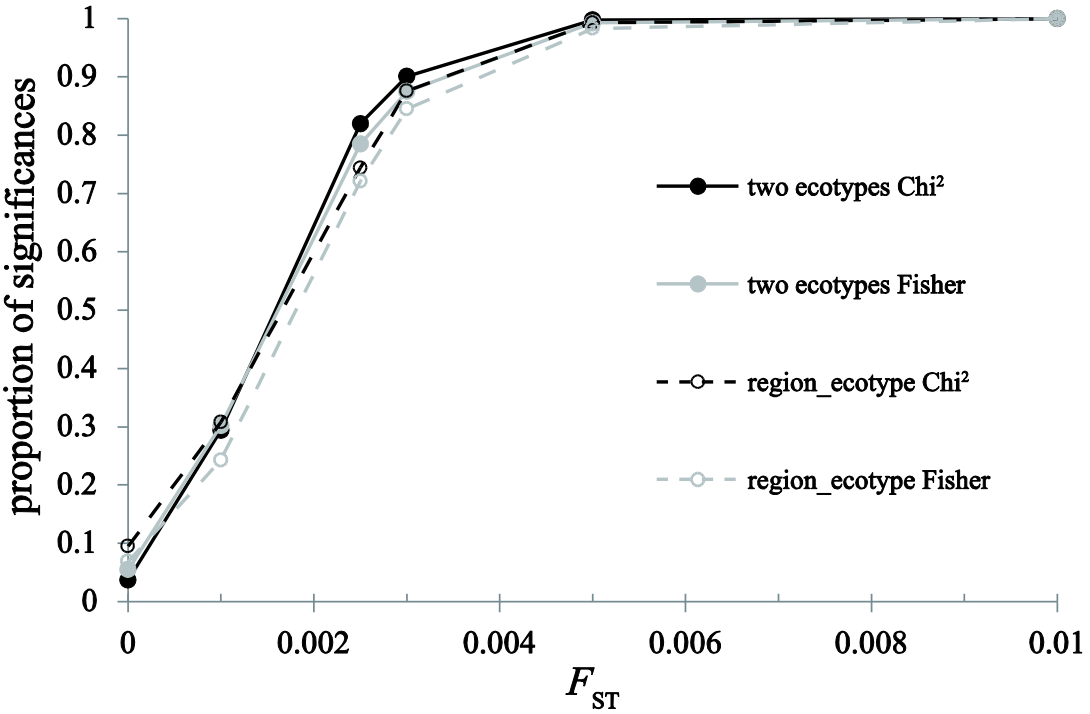


Fig. S5.3 Tests of statistical power for microsatellite data set as inferred with POWSIM 4.1.

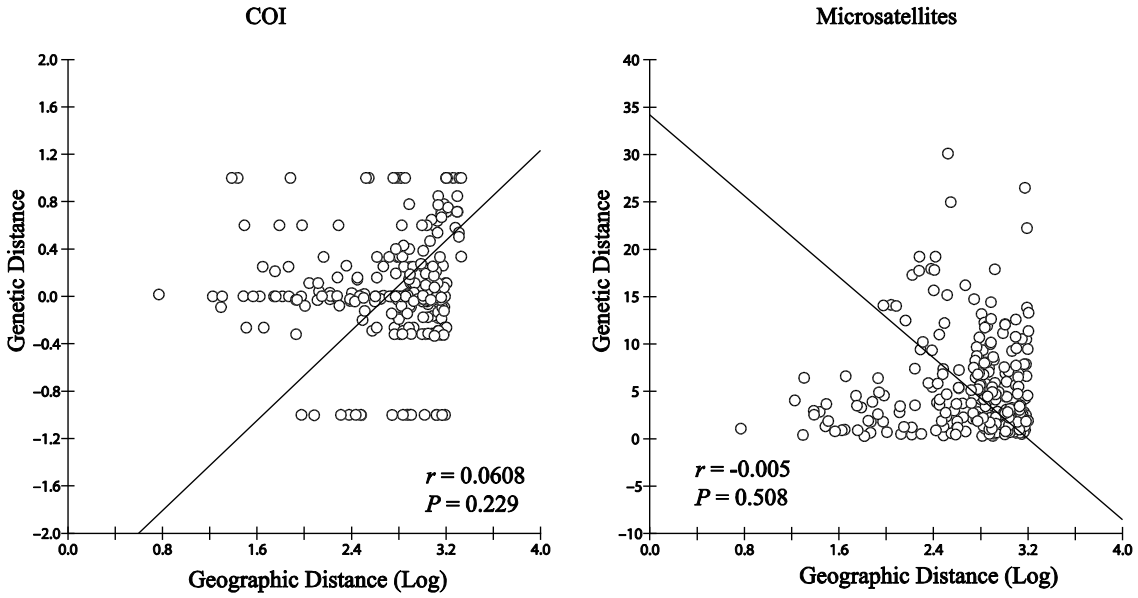


Fig. S5.4 Correlation between genetic distances (Pairwise F_{ST} values for COI and pairwise $(\delta\mu)^2$ values for microsatellites) and log-transformed geographical distances for mitochondrial and microsatellite data for specimens from 25 sampling sites listed in Table 1.

6. Crossing the impassable: mitochondrial and microsatellite markers confirmed high level of realized dispersal and gene flow in the transoceanic squat lobster *Munida gregaria* (Decapoda: Munididae) across the East Pacific Barrier

6.1 Abstract

The Eastern Pacific Barrier (EPB) has been recognized the world´s most potent biogeographic barriers to dispersal and gene flow from various taxa in the tropics. By contrast, in the higher southern latitude (35° -55° S) where stretches of open water are even broader without stopover islands to dispersal, there is a dearth of studies that describe whether the EPB is a strict barrier to gene flow. The widespread shallow-water squat lobster *Munida gregaria* is described the only transpacific species in the family Munididae with disjunct distribution mainly off New Zealand and Patagonia, yet cryptic speciation may present within this nominal species due to separation by the EPB. Population genetic and phylogeographic analyses were performed using mitochondrial COI as well as multiple independent microsatellites in a range-wide sampling scope. Genetic structure was shown across the Pacific between New Zealand (NZ) and South America (SA), but this differentiation was only the reflection of intraspecific polymorphism due to many shared alleles between populations. Testing in a Bayesian framework consistently confirmed eastward gene flow at different timescales by COI and microsatellites. Incorporating a substitution rate of 2 % per Myr for COI sequences in Crustacea, divergence between NZ and SA populations was estimated 0.254 Ma, which was much younger than the installation of the EPB (ca. 65 Ma). My study proved that *M. gregaria* is a truly transpacific species, i.e., the EPB is not a strict marine barrier to dispersal and gene flow.

Keywords: East Pacific Barrier, microsatellite, dispersal, gene flow, *Munida*

6.2 Introduction

The advent of molecular techniques and improved accessibility and sampling of realtively untouched regions such as the deep sea and the Antarctic have refreshed our knowledge of global species diversity and endemism which were previously underestimated (Brandt *et al.* 2007; Griffiths 2010; Gutt *et al.* 2004). Even though there is still a great deal of uncertainty and controversy about how new species arise, the influential concept of allopatric speciation still plays a dominant role in explaining the remarkable biodiversity. The initial step of that one continuous gene pool underwent allopatric speciation and eventually split into two genetically isolated subsets is geographic separation due to emergence of physical barriers, such as mountain formation, island formation, or glaciations. For marine shallow water species, the stretches of open deep water provides as formidable barriers to dispersal.

The East Pacific Barrier (EPB) is an extensive, uninterrupted 4 000-7 000 km stretch of deep water that draws a division line between the biogeographic provinces of the eastern Pacific and the central-western Pacific (sensu Briggs 1974). As early as more than one hundred years ago, this broad space of open ocean extends without any island as transition has already been recognized its zoogeographic importance and remarked as 'impassable' in Darwin (1872). Later Ekman (1953) emphasized that the EPB was the world's most potent marine biogeographic barrier. The effectiveness of the EPB has been well documented through molecular studies in corals (Baums *et al.* 2012), sea urchins (McCartney *et al.* 2000), calyptraeid gastropods (Collin 2003), spiny lobster (Chow *et al.* 2011) and reef fishes (Craig *et al.* 2007; Fitzpatrick *et al.* 2011). This barrier started to come in effect about 65 Ma (Rosen & Smith 1988), and if a previously widespread population was separated by this barrier and subsequently became vicariant, deep genetic divergence between separated populations should be evident. Even though a few examples of fish and invertebrate lineages have been demonstrated to cross the barrier (Lessios *et al.* 2003; Lessios & Robertson 2006), it still remains largely unknown whether the EPB is a strict barrier that interrupts gene flow, thus is responsible for the consequence of allopatric speciation for the pronounced genetic break between the two sides of the barrier. Moreover, current case studies have exclusively centered on circumtropical area, while in the higher latitude of southern Pacific where the distance of open deep water is even broader without intermediate shallow water habitats and oceanographic regimes are different, it remains poorly studied in comparison.

The squat lobster *Munida gregaria* (Decapoda: Munididae) is a nearshore benthic species with amphi-Pacific distribution off southeastern New Zealand and Chilean Patagonia between 41° S and 55° S, as well as in the Atlantic shelf off Patagonia (Boschi *et al.* 1992; Zeldis 1985). Some unique characters of this squat lobster are thought to be related to its extensive area of distribution. It is one of the few species that have a post-larval pelagic phase in the life cycles within the highly diversified and speciose family Munididae, and this pelagic larval phase can last about four months (Tapella *et al.* 2002b). The larval strategies and potential dispersal capability of pelagic juvenile make it the only transpacific species out of its family (Baba *et al.* 2008; Tapella 2002). This species is also featuring sympatric phenotypes that display sharp morphological, ecological and behavioral divergence. Using multiple, unlinked mitochondrial and nuclear molecular markers and sampling almost the entire species' range in South America, Wang et al. (unpublished) confirmed that despite its distinct morphological and ecological characteristics, *M. subrugosa* Dana, 1852 is a junior synonym of *M. gregaria*. Even though its

high dispersal potential was highly impressive due to the absence of an isolation-by-distance pattern in South America, the > 7 000-km stretches of deep sea between New Zealand and southern Chile most likely represent a significant barrier to dispersal for this shallow water species.

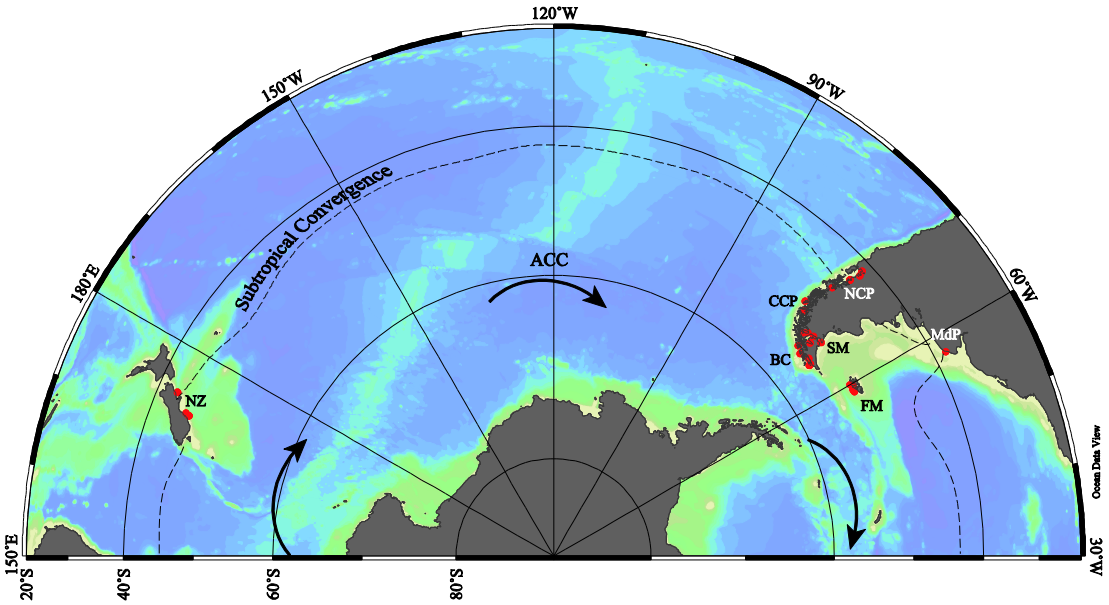


Fig. 6.1 Map of the study area including sampling locations (red dots), the northern boundary (i.e., Subtropical Convergence) of the Antarctica Circumpolar Current (ACC) (dashed lines), and the flowing direction of the ACC (black arrows). NZ, New Zealand; NCP, Northern Chilean Patagonia; CCP, Central Chilean Patagonia; BC, Beagle Channel; SM, Strait of Magellan; FM, Falklands/Malvinas; MdP, Mar del Plata.

In this study I added the New Zealand component of the global species' range to assess the genetic connectivity of *M. gregaria* among its highly disjunct distribution using mitochondrial COI and nuclear microsatellites specific to *M. gregaria* (Wang & Held 2015) as two different molecular marker systems (see discussion in Held & Leese 2007). The utility of different types of markers enabled rigorous analyses in determining genetic structure, directionality of gene flow and constructing multiple time-scaled evolutionary history. Ultimately I would like to answer 1) if the EPB is effective to impede gene flow for *M. gregaria* populations, i.e., if *M. gregaria* is indeed a single interbreeding species maintaining its integrity across the major barrier, or long isolated relicts of vicariance; 2) was dispersal evident across the biogeographic barrier and how was dispersal, if any, influenced by geological or oceanographic events and 3) if residences on both sides of the EPB belong to the same species, what is the pattern of historical and contemporary gene flow?

Table 6.1 Sampling sites and number of specimens used with mitochondrial marker (N_{mtDNA}) and microsatellite markers (N_{MSAT}). FM, Falklands/Malvinas; NCP, Northern Chilean Patagonia; CCP, Central Chilean Patagonia; SM, Strait of Magellan; BC, Beagle Channel; MdP, Mar del Plata; NZ, New Zealand.

Region	Station	Latitude	Longitude	N_{mtDNA}	N_{MSAT}	Region	Station	Latitude	Longitude	N_{mtDNA}	N_{MSAT}
FM	12OT12	-52.110	-59.595	3	16	NCP	Punta Metri	-41.595	-72.712	4	7
	13OT13	-52.285	-59.546	4	12		Huinay	-42.354	-72.463	16	16
	15OT18	-52.391	-59.131	2	16		Isla Dring	-46.442	-73.957	7	7
	16OT19	-52.354	-58.592	2	16		Punta Añihue	-43.793	-72.925	4	16
	17OT20	-52.359	-58.889	2	16*		Islas Pajaros	-43.783	-72.997	5	5
	21OT16	-52.408	-59.976	2	16*	CCP	Isla Lopez-mine	-50.361	-75.332	4	7
	12OT15	-52.189	-59.975	1	1		Isla Solaris	-51.330	-74.311	12	12
SM	Punta Dungenes	-52.436	-68.568	1	1	BC	Bahia Nassau	-55.383	-69.450	1	1
	Bahia Gregorio	-52.685	-70.142	5	10		Pt. Engaño	-54.929	-70.709	2	2
	Seno Otway	-52.918	-71.347	4	13		Bahia Virginia	-54.911	-67.726	8	12
	Silva Palma	-53.347	-71.800	2	7		Isla Picton	-55.174	-66.721	0	2
	Bahia Inutil	-53.556	-69.695	2	5		Isla Lennox	-55.393	-66.679	2	2
NZ	North Canterbury	-43.03	173.55	33	32	MdP	CCDB 2374	-38.003	-57.469	1	0
	Otago Shelf	-45.90	170.97	17	19	**Total of SA**				**96**	**218**
	Haypaddock	-45.34	171.27	26	27	**Total of NZ**				**76**	**78**
						Grand total				**172**	**296**

* refers to one specimen with missing genotype at a certain microsatellite locus

6.3 Materials and Methods

Sampling and DNA extraction

A total number of 297 *Munida gregaria* adults or juveniles were collected from 28 locations that were grouped into six geographic regions. This sampling scope represents almost the entire global range of this species. Samples were mainly obtained via trawling from 0m - 179m (Table 6.1, Fig. 6.1). Total genomic DNA was extracted from ethanol-preserved abdomen or cheliped

muscle tissue using QIAamp DNA Mini Kit (QIAGEN, Germany) following the manufacturer's instructions.

Mitochondrial DNA amplification and sequencing

The cytochrome *c* oxidase subunit I (COI) gene was amplified using the universal primers HCO2198 and LCO1490 (Folmer *et al.* 1994) for 172 individuals from all the six geographic regions. The 10 µl reactions consisted of 0.02 U/µl Hotmaster Taq (5 Prime, Germany), 0.2 mM dNTPs, 0.5 µM of forward and reverse primers, 1 x PCR-buffer and 1 µl (about 30 ng) of template DNA. PCR was conducted using an initial denaturation at 94 °C for 2 minutes, followed by 36 cycles of 94 °C for 20 seconds, annealing at 47 °C for 20 seconds, 65 °C for 1 minute, and a final extension at 65°C for 10 minutes. Size and quality of amplified products were checked on a 2 % agarose gel, then 5 µl product was subsequently purified using 0.25 µl Exo I and 1 µl FastAP enzymes (Thermo Scientific). Cycle sequencing using 1 µM HCO primers was conducted in 10 µl reaction volumes using 1 µl of the purified template DNA and the BigDye Termiator Kit 3.1 (Applied Biosystems) according to the recommendations. Reactions were purified using the Qiagen DyEx Kit (QIAGEN, Germany). Sequencing was conducted on an ABI 3130xl sequencer. Sequences were aligned using CODONCODE ALIGNER 4.2.7 (CodonCode Corp.) and checked for ambiguities by eye.

Microsatellite genotyping

In total, 296 individuals (without the single individual from Mar del Plata) were screened for genetic variation at nine loci (microsatellite markers Mgr4, Mgr8, Mgr 46, Mgr 52, Mgr60, Mgr 62, Mgr81, Mgr90, Mgr120), which were originally designed for *M. gregaria* (Wang & Held 2015). PCR amplification was achieved in two steps. A 10 µl reaction volume containing 30 ng DNA, 0.3 U HotMaster™ *Taq* (5 PRIME), 2 nmol dNTPs, 1 µl HotMaster™ *Taq* Buffer, 1 pmol M13-tailed forward primer and 1 pmol reverse primer were used in the first-step PCR, one microliter product of which together with 2 pmol universal M13 primer labeled with fluorescent dye FAM or Hex and 2 pmol reverse primer were used in the second-step PCR. Fragment lengths were analyzed with ROX GS500 size standard (Applied Biosystems) on an ABI 3130 x 1 sequencer, and scored with GENEMAPPER 4.0 (Applied Biosystems).

Genetic polymorphism

Mitochondrial DNA Polymorphism was examined as haplotype diversity (H_D) and nucleotide diversity (π) for each sampling region , the combined group South America and total samples

using DnaSP 5.10.1 (Librado & Rozas 2009). A statistical parsimony network was constructed with TCS 1.21 (Clement *et al.* 2000) using default settings. The substitution model that best fits the data was determined using jModelTest 2.1.5 (Darriba *et al.* 2012; Guindon & Gascuel 2003).

For the microsatellite loci, deviations from Hardy-Weinberg equilibrium (HWE) for each geographic region at each locus and Linkage disequilibrium between each pair of loci were tested with 5 x 10^7 Markov chain iterations respectively using GENEPOP 4.2 (Raymond & Rousset 1995). Bonferroni correction was applied for multiple comparisons (Rice 1989). The program ANIMALFARM 1.0 (Landry *et al.* 2002) was used to test for loci with significantly disproportionate variances that may bias allele-size based distance estimates such as Slatkin's R_{ST} estimates (Slatkin 1995). Sample statistics including the number of alleles observed per locus (N_A), expected Heterozygosity (H_E) and expected Heterozygosity (H_O) was calculated in ARLEQUIN 3.5. Standardized allelic richness (*Ar*) (measured as the mean number of alleles per locus independent of sample size) in FSTAT 2.9.3.2 (Goudet 2001) using the rarefaction approach (Hurlbert 1971; Petit *et al.* 1998).

The statistical power of the COI and microsatellite data sets was assessed using POWSIM 4.1 (Ryman & Palm 2006). This software simulates a random distribution of the marker alleles over subpopulations at various levels of expected divergence, which is determined by combinations of N_e (effective population size) and t (generations since divergence). It also allows to calculate α error (type I error), which is the probability of rejecting the null hypothesis of genetic homogeneity although it was true by drawing the alleles directly from the base population (t = 0). Simulations were run on a global level including all populations, and between pooled South America (SA) and New Zealand (NZ) populations. For every simulation, 1 000 replicates were run using both Chi-square (χ^2) and Fisher's exact test analysis, the number of dememorizations, batches, and iterations per batch were set to 10^4, 10^3, 10^4, respectively.

Population structure

Levels of genetic divergence among geographic regions were calculated as pairwise Φ_{ST} (Excoffier *et al.* 1992) for COI and F_{ST} (Wright 1965) for microsatellites using ARLEQUIN 3.5 (Excoffier & Lischer 2010). According to the best substitution model suggested by jModelTest, a more inclusive Tamura-Nei (TN, Tamura & Nei 1993) model with the same ti/tv rate was used. The interpretation of the F_{ST} or F_{ST}-analogues (e.g., G_{ST} (Nei 1987), θ (Weir & Cockerham 1984), R_{ST} (Slatkin 1995)) values from highly polymorphic genetic markers, such

as microsatellites is problematic because their maximum values are inversely proportional to the within-population heterozygosity and even in the absence of any shared allele often fail to reach the theoretical maximum of 1 (Hedrick 1999, 2005; Whitlock & McCauley 1999). Following Hedrick (2005), I, therefore, standardized F_{ST} values by applying the sampling bias correction using RECODEDATA (Meirmans 2006), the output of which was used to calculate $F_{ST(max)}$ in ARLEQUIN 3.5. The corrected pairwise F_{ST}' was subsequently calculated by dividing F_{ST} by $F_{ST(max)}$. Significance of the derived pairwise differences was assessed using 10^4 permutations.

The hierarchical genetic structure of both data sets was described using the analysis of molecular variance (AMOVA) implemented in GENALEX 6.5 (Peakall & Smouse 2012). Genetic variation was partitioned into among-group, within-group and within-population components. The same standardization procedure of F_{ST} values was applied for microsatellites. Significance of results for both data sets were assessed with 10^4 permutations.

The Bayesian clustering method implemented in STRUCTURE 2.3.4 (Falush *et al.* 2007; Pritchard *et al.* 2000) was used to infer *M. gregaria* global population structure. STRUCTURE was first run without any *a priori* population/location information using 1) uncorrelated allele frequencies option and non-admixture model, that is, under the assumption that there is no gene flow between populations; 2) correlated allele frequencies option and admixture model, acknowledging that the grouping applied here is only roughly based on their geography. The analyses were conducted with a burn-in length of 5 x 10^4 replications followed by 2 x 10^5 MCMC replications. For each value of K (from one to the number of geographic groups plus two in the present study) that was assessed, 20 independent runs were conducted for each model. The results were uploaded onto STRUCTURE HARVESTER (Earl & von Holdt 2012), best K was determined using the ad hoc statistic ΔK, based on the rate of change in the log probability of the data between successive K values (Evanno *et al.* 2005). Results from 20 replicates of the most likely K were averaged using the software CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007) and the output was visualized using DISTRUCT 1.1 (Rosenberg 2004). I further used prior population information derived from the classification of aforementioned analyses to detect potential immigrants using the USEPOPINFO option, which assumes that most individuals classified as belonging to one group have pure ancestry from that group, while a small percentage of individuals may be immigrants to their supposed group, or have recent immigrant ancestors (Pritchard *et al.* 2000). The MIGRPRIOR option was run at three different values of

immigration rates (0.01, 0.05 and 0.10) to assess the robustness of the assignment as suggested by Pritchard *et al.* (2000).

Demographic history and divergence time estimation

Considering the realized genetic populations (New Zealand (NZ) and South America (SA), see "Results"), a two-population scenario was adopted in the following analyses of ancestral reconstruction, demographic history and gene flow.

Signatures of population demographic changes were first examined by Tajima's *D* (Tajima 1989), Fu's *Fs* (Fu 1997) and R_2 statistics (Ramos-Onsins & Rozas 2002) using DnaSP 5.10.1. The latter two statistics are considered most powerful for detecting expansions on nonrecombining genomic regions (Ramírez-Soriano *et al.* 2008). Significant deviations from neutrality of these tests might be evident for historical population expansion or bottleneck. Statistical significance of these tests was based on 1 000 coalescent simulations in DnaSP. Second, the distribution of the frequencies of nucleotide pairs between individuals were compared with the expected distribution of a model of population expansion as implemented in ARLEQUIN 3.5. Populations that have been stable over time are expected to have a bimodal or multimodal mismatch distribution, whereas a unimodal distribution is typical in populations having undergone demographic expansion (Rogers & Harpending 1992).

BOTTLENECK 1.2.02 (Cornuet & Luikart 1996) was used for microsatellites to determine if data suggested the occurrence of severe past population contraction in both genetic groups.

I used the Bayesian analysis to date the tree with the mitochondrial sequences in BEAST 2.1.3 (Bouckaert *et al.* 2014; Drummond & Rambaut 2007). A relaxed lognormal model of lineage variation and a coalescent prior with constant size were assumed. Without fossil calibration, I implemented the general substitution rates of 2 % per Myr for COI sequences in Crustacea (Schubart *et al.* 1998; Wares & Cunningham 2001), while a rate as high as 7 % was set as the upper limit of the prior distribution to include the highest clock rate of 6.6 % reported by Crandall *et al.* (2012). The best-fit model was selected using jModelTest 2.1.5. Two independent runs with 10^8 MCMC generations and a sampling interval of every 10^4 generations for each run were performed. The total sampled 2 x 10^4 trees were combined in LOGCOMBINER 1.7.4 (http://beast.bio.ed.ac.uk/LogCombiner) with 10 % discarded as burn-in. Convergence of the stationary distribution was checked by monitoring the effective sample sizes of each parameter (>200) in TRACER 1.6 (Drummond & Rambaut 2007). A consensus

tree with the maximum clade credibility was summarized using TREEANOTATOR 1.6.1 (Drummond & Rambaut 2007) with the PP limit set to 0 and summarizing mean node heights. Visualization and polishing of the consensus tree was performed using FIGTREE 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Migration and gene flow

Using the coalescent-based Bayesian framework in MIGRATE-N 3.6.1 (Beerli & Felsenstein 2001), I investigated gene flow between the recognized two genetic populations, NZ and SA. Three models were hypothesized, i.e., oppositely two unidirectional and one bidirectional gene flow. Analyses were conducted based on both mtDNA and microsatellite data sets. In order to avoid bias in gene flow directionality caused by unequal sample sizes, sub sets of SA were randomly chosen to match the size of NZ (for mtDNA, n=76; for microsatellites, n=78). Preliminary runs based on the more complex model (bidirectional migration) were used to determine optimal parameter settings and run lengths. For the final analyses, employing 16 static chains of default temperatures, five independent runs of 10^5 recorded steps sampled every 10^2 steps, with a burn-in length of 5 x 10^3 steps were performed. Parameter ranges for uniform priors were empirically set to $\theta \in [0, 0.1]$, M ∈ [0, 5 000] for mtDNA and $\theta \in [0, 1\ 000]$, M ∈ [0, 1 000] for microsatellites. The Brownian motion model and sequence model were employed for microsatellites and mtDNA, respectively. Parameter starting values were based on F_{ST} estimates. In order to determine the optimal gene flow directionality model, the Bezier corrected marginal likelihood was used to calculate log Bayes Factors and model probabilities (Beerli & Palczewski 2010). Log Bayes Factor differences of > 10 units provide very strong support for one model over another (Kass & Raftery 1995). For the most probable gene flow, I estimated theta ($\theta = 4N_e\mu$ for microsatellites or $\theta = N_e\mu$ for mtDNA, where N_e is the long-term effective population size and μ is the mutation rate per generation) and historical gene flow M (M = m/μ, where m is the immigration rate per generation). The number of migrants per generation was calculated as $N_em = \theta M/4$ for microsatellites or $N_em = \theta M$ for mtDNA.

Contemporary rates of migration were investigated through several analyses. A Bayesian approach implemented in BAYESASS 3.0.3 (Wilson & Rannala 2003) was first performed to estimate migration rates and directions of dispersal that occurred in the past one to three generations. This software relaxes several assumptions (e.g. HWE) of other methods of gene flow estimation thus provides more robust results. Recent immigrants were identified as individuals that display genotypic disequilibrium relative to the population from where they

were sampled. The analyses were performed with 10^7 iterations and a sampling frequency of 100 and 10^6 iterations were discarded as burn-in. Delta values were adjusted to ensure that proposed changes between chains were between 40 % and 60 % of the total number of iterations (Wilson & Rannala 2003). Convergence was examined by comparison of 4 runs with different random starting seeds, as well as monitoring the trace files for the log-probability in TRACER 1.6. Detection of first-generation migrants using resampling algorithm of Paetkau *et al.* (2004) were performed using GeneClass2 (Piry *et al.* 2004). I computed a likelihood ratio test comparing the population where the individual was sampled over the highest likelihood value among all available populations (L=L_home/L_max) with default critical probability level of 0.01.

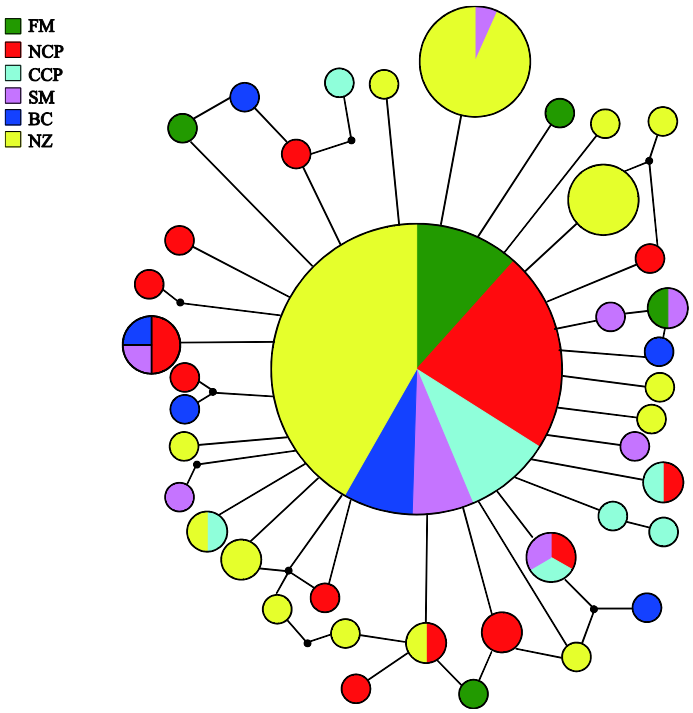


Fig. 6.2 Statistical parsimony network of COI haplotypes. Each branch represents one substitution; small filled circles represent hypothetical, unsampled haplotypes. Radii reflect number of individuals that share a particular haplotype.

6.4 Results

Genetic polymorphism

Alignment of the COI sequences from 172 individuals yielded a consensus sequence of 618 bp (GenBank accession numbers: KJ544251 - KJ544346 and KU728379 - KU728454). These sequences were collapsed into 40 different haplotypes, determined by 36 variable (segregating) sites, of which 16 were parsimony-informative. Haplotype diversity (H_D) and nucleotide

diversity (π) are reported in Table 6.2 for each sampling area, as well as summarized as NZ and SA. The resulting haplotype genealogy (Fig 6.2) was in star-like shape. The most common haplotype (found in 103 individuals) is present in all areas, and the remaining haplotypes differed from the most common one in one or a few mutational steps (Fig 6.2). Mean sequence divergence within NZ or SA is 0.144 and 0.166%, respectively and between them is 0.162%. The maximum sequence divergence between NZ and SA is 0.971%.

Table 6.2 Genetic diversity of COI sequences per population and region. H_D, haplotype diversity; π, nucleotide diversity.

	Sequences	Haplotypes	Private haplotypes	H_D	π
FM	16	5	3	0.450	0.00121
BC	13	6	4	0.641	0.00224
SM	14	7	3	0.731	0.00195
CCP	17	8	5	0.669	0.00207
NCP	36	12	7	0.587	0.00124
SA	96	29	25	0.601	0.00164
NZ	76	15	12	0.650	0.00145
Total	172	40		0.634	0.00160

For the microsatellite data, there were 7 to 39 alleles per locus across all populations, the overall allelic richness ranged from 4.251 to 17.39 independent of sample size. The observed (H_O) and expected heterozygosity (H_E) ranged from 0.053 to 0.895 and 0.053 to 0.915, respectively (Table S6.1, Additional files). There was no evidence of linkage disequilibrium after adjusting the significance level for multiple comparisons ($P < 0.05$). Three locus-population combinations showed significant deviation from the Hardy-Weinberg expectation after Bonferroni correction (Table S6.1, Additional files) at locus mgr90. But since excluding Mgr90 had only minor effect on the results, it was kept in this study. Test of disproportionate variances among loci confirmed that none of the loci contributed disproportionally to distance-based differentiation estimates after Bonferroni or Sidak adjustment of the significance level.

Power test showed that the mtDNA data set had the probability of 94.5 % for the χ^2 test and 86.7 % for Fisher's exact test to detect a global population structure with a true F_{ST} value of 0.01. With pooled SA and NZ populations, this degree of divergence would be detected with

probabilities of 94.4 % for the χ^2 test and 97.3 % for Fisher's exact test. Compared to the mtDNA data, there were smaller deviations at different groupings for the microsatellites, which provide higher statistical power than mtDNA sequences, and the proportion of significances reached 1 already for F_{ST} values as small as 0.005 (Fig S6.1, Additional files).

Table 6.3 Pairwise population differentiation, based on microsatellites (F_{ST}', above diagonal) and mtDNA (Φ_{ST}, below diagonal) respectively. Statistically significant pairwise F_{ST}' or Φ_{ST} values are labeled with level of significance signified by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

Populations	FM	SM	BC	CCP	NCP	NZ
FM		-0.00196	0.0201*	0.0219*	0.00742	0.229***
SM	-0.01377		0.0513**	0.0200	0.00932	0.274***
BC	-0.01942	-0.0168		0.0661**	0.0342*	0.217***
CCP	-0.00095	0.00795	-0.01488		0.000878	0.238***
NCP	-0.01071	0.01262	-0.00913	0.00143		0.192***
NZ	0.03725*	0.03747	0.05428*	0.05224*	0.04454**	

Table 6.4 Results of hierarchical analysis of molecular variance (AMOVA) measured by microsatellites and COI. Populations from South America were categorized as group SA.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Statistic	F_{XX}/ $F_{XX(max)}$	F_{XX}'	*P*
Microsatellites								
Among groups	1	65.740	27.546	8.807	F_{CT}	0.088/0.402	F_{CT}'= 0.219	0.000
Among populations within groups	4	15.727	0.917	0.513	F_{SC}	0.0056/0.384	F_{SC}' = 0.015	0.003
Within populations	586	1595.249	225.753	90.680	F_{ST}	0.093/n.a.	n.a.	0.000
Total	591	1676.716						
COI								
Among groups	1	2.264	0.0212	4.19	Φ_{CT}	0.0419	n.a.	0.175
Among populations	4	1.914	-0.0004	-0.08	Φ_{SC}	-0.00085	n.a.	0.675

within groups								
Within populations	166	80.675	0.4860	95.89	Φ_{ST}	0.0411	n.a.	0.001
Total	171	84.853	0.5068	100				

Genetic population structure

The genetic population differentiations estimated with Φ_{ST} for mtDNA and F_{ST}' for microsatellites were congruent. Both data sets showed significant pairwise population differentiation with the highest degree of divergence for the NZ population. Especially for microsatellites, F_{ST}' values between NZ and any population in SA were at least one order of magnitude larger than values among SA populations and highly significant ($P < 0.001$; Table 6.3). Also, the microsatellite data revealed substructure between regions of Pacific coasts and regions of Atlantic coasts. Significantly greater F_{ST}' values were found for both Northern and Central Chilean Patagonia (NCP and CCP) versus the Atlantic groups (SM and BC) than that between Chilean groups (NCP vs. CCP). Results of hierarchical AMOVA based on both data sets indicate most variation is distributed within populations (Table 6.4), whereas small proportions of variation (4.19 % for COI and 8.8 % for microsatellites) were distributed between continents (SA vs. NZ) with the highest divergence (measured as F_{CT}'/Φ_{CT} values).

The genetic classification indicated by the above findings was corroborated by the Bayesian clustering method implemented in STRUCTURE. Log-likelihood values were calculated for the existence of $1 \leq K \leq 8$ clusters for the microsatellite data set. Under both schemes assuming no-admixture and admixture, the scenario of two clusters was consistently suggested for the highest ΔK values and $L(K)$. One is NZ and the other is a cluster comprising all the sampled groups from SA (Fig 6.3). Noticeably, both clusters contained specimens with shared genotype proportions, where these from SA seemed to incorporate more sharing from NZ (Fig 6.3).

Demographic history and divergence time estimation

Neutrality tests for the mitochondrial data revealed significant negative values for Tajima's D and Fu's Fs, and significant positive R_2 values for both identified SA and NZ genetic populations, which congruently suggest a possible past expansion for both populations (Table 6.5). According to mismatch distributions analysis, the hypothesis of population expansion

could not be rejected for SA (SSD = 0.0002, $P = 0.915$, Table 6.5), whereas small but significant SSD and raggedness index values were observed for NZ (Fig. S6.2, Additional files). The Bottleneck analysis of the microsatellite data revealed nonsignificant P values for Wilcoxon tests of heterozygosity excess (indicative of a recent founder event) under both mutation models, thus suggesting no recent significant reductions in effective population size for either NZ or SA population (Table S6.2, Additional files).

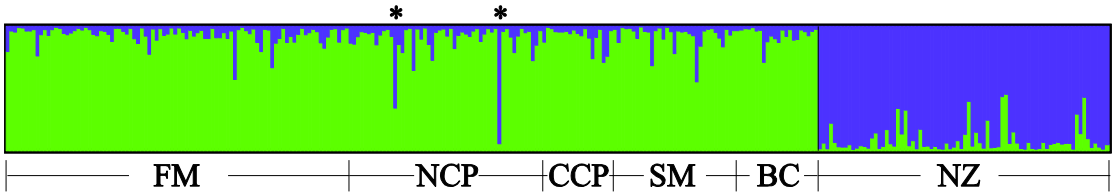


Fig. 6.3 Results of cluster analyses performed with STRUCTURE (non-admixture model, allele frequencies uncorrelated) with the highest ΔK value as well as log likelihood probability $L(K)$. The graphs display the consensus membership coefficients matrices (Q-matrices) for 296 individuals from six populations of *Munida gregaria* using nine microsatellite loci. The genotype of each individual is represented by a single bar, where the proportion of the colour refers to the probability of assignment to a certain cluster. Potential immigrants were labeled with asterisk (USEPOPINFO option, MIGRPRIOR = 0.1).

Different selection criteria (Akaike and Bayesian information criterion) identified the same best-fit substitution model, i.e., HKY+I model (Hasegawa *et al.* 1985) for my COI data. Bayesian inference of phylogenetic relationships using this substitution model suggested a huge monophyletic clade of all haplotypes occurring exclusively from NZ. The four haplotypes shared by SA and NZ also formed a monophyletic clade that was nested in the group of SA haplotypes (Fig. 6.4). The low Bayesian posterior probabilities of most nodes is expected when referring to the very structure of haplotype network, resulting from the low sequence variability. The divergence time between NZ and SA lineages was estimated to be 0.254 (95 %HPD: 0.171 - 0.481) Ma, which corresponded to the late Pleistocene glaciations but much younger than the date when the EPB came into effect (ca. 65 Ma) (Fig. 6.4).

Table 6.5 Neutrality tests on COI data. Parameters of time since the population expansion (Tau), sum of square deviations (SSD) for test of the validity of the sudden expansion model as well as Harpending's Raggedness index (Raggedness) for the test of goodness-of-fit are shown. Significance level: * P<0.05, ** P<0.01, *** P<0.001.

Population	Tajima's *D*	Fu's *Fs*	R_2	Tau (95%CI)	SSD (P_{SSD})	Raggedness (P_r)
SA	-2.492**	-40.543***	0.0202***	1.035 (0-3.918)	0.0002 (0.915)	0.038 (0.780)
NZ	-2.037*	-12.948***	0.0349*	1 (0.719-1.801)	0.009 (0.034*)	0.116 (0.009**)
Total	- 2.523***	-63.866***	0.0144**			

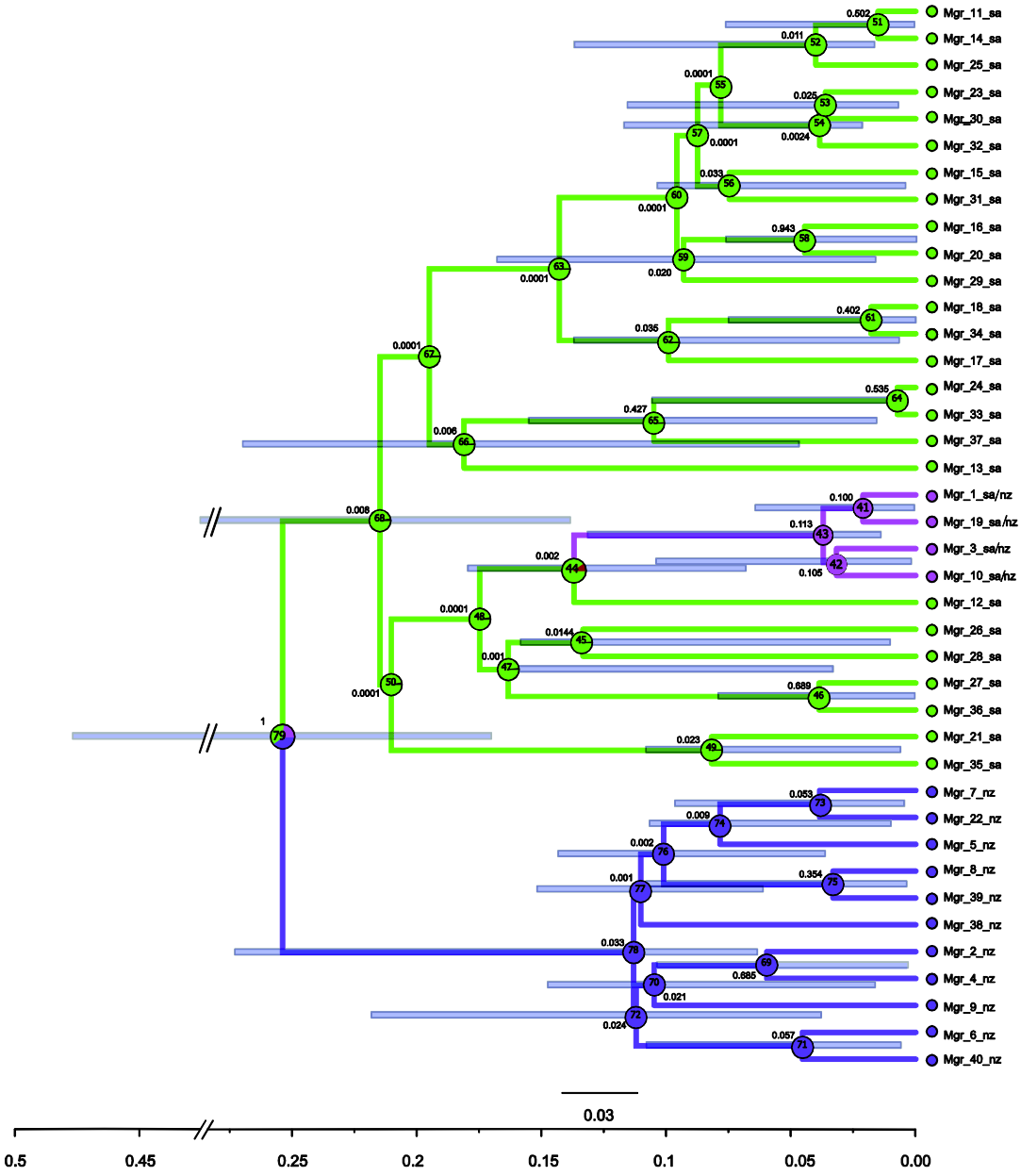


Fig. 6.4 Bayesian phylogenetic tree based on 172 COI sequences. Colors indicate the distributions of haplotypes in relation to SA (light green), NZ (violet) or share of both (magenta). Each node was labeled with posterior support value and bar of 95 % HPD node heights.

Migration and gene flow

Patterns of historical gene flow estimated by Migrate-N employing the mitochondrial and microsatellite data were congruent in favoring the eastward unidirectional gene flow model, i.e., gene flow from NZ to SA (Table 6.6). The log Bayesian Factor difference for microsatellites was more than three orders of magnitude comparing to the other two gene flow models, thus resulting an absolute favor of the eastward gene flow model (probability = 1;

Table 6.6), while the mitochondrial data also suggest a probability of 0.897 for the eastward gene flow model. The estimated value of θ consistently suggested higher value of NZ than that of SA by both data. For microsatellites θ_{NZ} was about two folds of θ_{SA}, although both of them had wide and overlapped 95% confidence intervals. For mtDNA, both the peak and 95% CI values of θ_{NZ} was about an order larger than those of θ_{SA} (Table 6.7). The mutation-scaled migration rate (M) had the values much greater than one suggested by both data, indicating that migration (and not mutation) was the main factor contributing genetic connectivity and variation between these two populations (Prospero *et al.* 2009). The effective number of immigrants per generation N_em (=θM/4) moving from NZ to SA was 15.944 (95% CI: 0-41.173) for microsatellites and 13.288 (95% CI: 8.183-20.4) (Table 6.6).

Table 6.6 Bayes factors and log marginal likelihoods of different models for microsatellites and COI.

	Microsatellites			**Mitochondrial COI**		
Model	SA↔ NZ	SA ← NZ	SA→ NZ	SA ↔ NZ	SA ← NZ	SA → NZ
Bezier ImL	-22948.13	-5135.37	-13859.51	-1389.28	-1387.12	-1400.50
LBF(Bezier)	-35625.52	0	-17448.28	-4.32	0	--26.76
Model probability	0	1	0	0.103	0.897	0

Multiple runs of Bayesass yielded low levels of contemporary gene flow (measured as fraction of individuals that are immigrants) between the two regions. Although the migration rate of SA into NZ (0.0092) was slightly higher than that of NZ into SA (0.0076), they both are minor and overlapped broadly with one another at the 95% CI (Fig. 6.5).

Table 6.7 Estimates of mutation-scaled effective population size (θ), mutation-scaled migration rate ($M_{NZ\rightarrow SA}$) and effective number of immigrants per generation ($N_em = \theta_{SA}M_{NZ\rightarrow SA}/4$ for microsatellites or $N_em = \theta_{SA}M_{NZ\rightarrow SA}$ for mtDNA) from New Zealand (NZ) to South America (SA) based on Bayesian analysis in MIGRATE-N. Data are modes with the lower 2.5 and upper 97.5 percentiles in parentheses.

Data	θ_{SA}	θ_{NZ}	$M_{NZ\rightarrow SA}$	N_em
mtDNA	0.0075 (0.0044-0.0103)	0.0704 (0.0392-0.0925)	1771.67 (1091.13-2720)	13.288 (8.183-20.4)
Msats	5.799 (0-25.732)	13.734 (0-28.4)	10.998(0-28.4)	15.944 (0-41.173)

Migrant detection performed with three different methods (Structure, GeneClass and Bayesass) all suggested potential immigrants from NZ to SA, but no immigrants from SA to NZ. One individual, NCP40, was suggested as immigrant by all these three methods (Table 6.8). Individual NCP12 was detected by Structure and Bayesass, while NCP 17 was suggested by

GeneClass and Bayesass (Structure also showed that this individual had a proportion of 35.4% admixture from NZ) (Fig. 6.3 and Table 6.8). Interestingly, all these three potential immigrants are from the Pacific coast of Patagonia, implying a migration route from NZ to Chile.

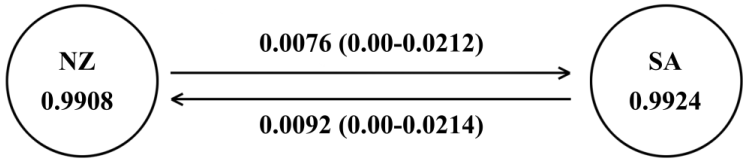


Fig. 6.5 Recent migration rates (mBA) between SA and NZ using BayesAss. The numbers within the circles denote the proportion of non-immigrants within populations.

Table 6.8 Migrant detection performed using STRUCTURE, GENECLASS and Bayesass

Geographical origin	GeneClass F_0 migrants (*P*-value)	Bayesass nonmigrant/1st/2nd Gen migrant	Structure q value
SA	NCP12 (0.012)	0.492/0.499/0.009	0.345/0.655
SA	NCP17 (0.0092)	0.891/0.062/0.046	0.646/0.354
SA	NCP40 (0.0018)	0.154/0.814/0.033	0.055/0.945

6.5 Discussion

A truly single species across the Pacific

Based on sampling of the global distribution of *M. gregaria* using mtDNA and multi-locus nuclear microsatellite markers, my results demonstrate this squat lobster species is a truly single species with high mobility frequently cross the EPB. The maximum uncorrected pairwise sequence distances between the different *M. gregaria* COI haplotypes were 0.971%, which is much smaller than the upper limit expected for a single interbreeding species (3% sequence divergence threshold typically used in the barcoding studies; Song *et al.* 2008). This was further supported by the shared haplotypes between NZ and SA, as well as the star-like shape of maximum parsimony network, which was beyond the 95% parsimony connection limit as a rule of thumb to delimit species (Hart & Sunday 2007). Having higher evolutionary rates relative to coding genes as well as a fourfold greater effective population size compared to mitochondrial genes that enables them less susceptible to random genetic drift, the microsatellites are expected to exhibit more recent genetic differentiation which is not contradicting the mitochondrial data but instead complementing them. In contrast to COI, the genetic structure revealed by microsatellites are more evident as shown in pairwise population comparison as well as the STRUCTURE result. Yet allele sharing are present among all loci

between SA and NZ, which is reflected as the ubiquitous admixture in STRUCTURE even under the model of non-admixture. Thus the level of genetic differentiation between NZ and SA demonstrates intraspecific variance within an interbreeding species.

The revealed gene flow homogenizing distant populations across the seemingly insurmountable EPB makes *M. gregaria* unique in view of none transoceanic relatives in the remarkably diversified family Munididae (Baba *et al.* 2008). High levels of fauna affinities are particularly clear on both sides of the EPB (Briggs 1974; Castilla & Guiñez 2000; Cowman & Bellwood 2013; Glynn & Ault 2000; Macpherson *et al.* 2010). The traditional interpretation of these affinities involves vicariance due to the effective separation by the EPB. In our study, the divergence time between populations NZ and SA (ca. 0.25 Ma) is extremely young compared to the installation of the EPB (ca. 65 Ma), after which further recurrent gene flow occurred regularly to maintain species integrity. An alternative interpretation to the timing of divergence might arise that the species had undergone vicariant speciation since the onset of the EPB, later secondary contact enabled distant populations to hybridize. However, this is hardly true due to no hint of historical secondary contact shown as the unimodal mismatch distribution. In the end, my results highlight 1) the EPB may not always be a strict barrier to dispersal, it is permeable in space and time; 2) the species studied is too young to have been affected by the presumed vicariant barrier, suggesting a recent history of dispersal, rather than vicariance. My estimated divergence time of populations NZ and SA is in line with the finding of recent (less than 0.2 Ma) separations of eastern Pacific and central Pacific populations of 14 transpacific shore fishes (Lessios & Robertson 2006). This congruence may imply that the recent divergence between populations of transpacific species at both sides of the EPB is accompanied with climatic and oceanographic oscillations during geological events of late Pleistocene.

Gene flow from NZ to SA

The mitochondrial COI and nuclear microsatellites applied in my study demonstrates a powerful combination in recovering a more complete evolutionary history of a species. In general, mtDNA evolves 5−10 times faster than single-copy nuclear DNA (scnDNA) (Jansen 2000), thus it is typically used to reconstruct a species' demographic history at scales of tens of thousands to millions of years (Avise 2004). By contrast, microsatellites are generally considered fast-evolving and independent markers, they evolve 100−1 000 times faster than scnDNA (Gilbert *et al.* 1990). Therefore they are expected to detect near-contemporary demographic events at a scale as recent as tens to hundreds of generations (Nance *et al.* 2011;

Raeymaekers *et al.* 2005; Selkoe & Toonen 2006a). The results of MIGRATE-N employing both mtDNA and microsatellites consistently suggest gene flow from west to east at different time scales, which should be interpreted from NZ to SA in view of the ocean currents, life history traits of this organism. The suggested eastward gene flow conforms to the direction of the Antarctic Circumpolar Current (ACC), which extends its influence north to ca. 45^{o} S known as the Subantarctic Front (SAF). As the mightiest current system, it provides overwhelming transportation of pelagic larvae. The lack of genetic differentiation at circum-subantarctic scale of southern bull kelp *Durvillaea antarctica* well exemplified the homogenizing effect of the ACC, whereas differentiation was contrarily shown at a smaller geographic scale along the Chilean coast (Fraser *et al.* 2009). Interestingly, the separation between central Chilean lineage and Patagonian lineage of *D. antarctica* conformed to the split of the ACC by South American continent as the north-flowing Humboldt Current and south-flowing Cape Horn Current (Fraser *et al.* 2010). Unexpected low genetic structure was evident even for Antarctic benthic invertebrates, e.g. the brooding brittle Star *Astrotoma agassizii* (Hunter & Halanych 2008), the Antarctic brooding isopod *Septemserolis septemcarinata* (Leese *et al.* 2010). All of these immobile species displayed extensive genetic continuity across a large geographic range (>500 km) owing to the transport of the ACC.

Besides the aid of the ACC, the long pelagic larval duration (PLD) of *M. gregaria* is also taken into account for the successive migration across the EPB within one generation. *M. gregaria* has a unique pelagic post-larval stage out of its family that lasts about 90-120 days (Tapella *et al.* 2002b). Even though adults settle benthic, they still perform migration through water column (Tapella 2002; Zeldis 1985). Further swarming behavior of both juveniles and adults may promote their dispersal in larger areas (Diez *et al.* 2012; Zeldis 1985). Although under certain circumstances the pelagic larval duration (PLD) may not correlate well with geographic range size (Bowen *et al.* 2006; Selkoe & Toonen 2011), it is still broadly considered to play a key role in population connectivity (Cowen & Sponaugle 2009; Lester *et al.* 2007; Shanks 2009). Reckoning a 7900km-distance between NZ and SA (at 45°S) and a surface speed of 77 cm s^{-1} (2.8 km h^{-1}) inside the jets of the ACC (Whitworth 1988), a voyage from NZ to SA lasted about 117days, whereas the time travelling eastward from SA to NZ (ca. 18 000 km) would need about 266 days. The maximum dispersal in a single generation only allows the short trip on ecological time scale. The plausible interpretation of the eastward gene flow thus would be from NZ to SA. Nevertheless, we cannot explicitly exclude the alternative that gene flow still went eastward but from SA to NZ. Flexibility on evolutionary scale might enable change of

migration patterns according to ecological and biogeographic factors. Although contemporarily *M. gregaria* does not occur in South Africa or around islands in the Southern Indian Ocean (E. Macpherson, personal communication, November 24, 2014) to form a circum-subantarctic distribution via stepping-stone dispersal, which might be due to incompatible oceanographical conditions, but the possibility of historically broader distributions through oscillated climate should not be neglected. During glaciation the cooled seawater temperature might shift the species' range to lower latitudes, such as southern South Africa. A paleobiogeography study based on comprehensive fossil records of decapods from the Southern Hemisphere postulates that the high southern latitudes was a site of origin of several generic-level taxa, which were later found in lower latitude regions (Feldmann & Schweitzer 2006). Furthermore, reconstruction of the ACC in the past 500 kyr suggest that flow speeds were faster during glacial epochs and slower during warm stages (Mazaud *et al.* 2010). Therefore dispersal duration from coast to coast might be shortened during cold periods. Given drastic difference of climatic and oceanographic conditions in the past, migration encircling the globe via the ACC might be historically possible.

Possible ancestor population

Although populations NZ and SA was estimated to have diverged about 0.25 Ma, current data do not allow to articulate which is the ancestral population of *M. gregaria*. Yet patterns of species richness at this temperate latitudes might be indicative. Following the common Gondwanan vicariance and marine dispersal the Indo-West Pacific (IWP) has been proposed to be the global center of both marine and terrestrial biodiversity (Hughes *et al.* 2002; Lohman *et al.* 2011). Coastal species showed maximum diversity in the Western Pacific (Tittensor *et al.* 2010). In southern higher latitude, biogeographers explicitly developed hypotheses involving ACC-mediated transport of marine larvae. Described as 'a diminishing trail of species eastwards' (Fleming 1979), a trend of progressive decline in species richness clockwise from Australia to South Africa was hypothesized as in echinoderms (Fell 1967; Fell 1962), galaxiid fishes (Cussac *et al.* 2004; McDowall 1970; Mcdowall 1978). These lines suggest that South Africa is a most unlikely source of origin and dispersal, and rather, is the end of a chain of dispersal areas (McDowall 1970). Similar pattern of cline was also shown for squat lobsters in the Pacific Ocean, where the Coral Sea (Solomon-Vanuatu-New Caledonia islands) and Indo-Malay-Philippines archipelago (IMPA) comprised the highest number of squat lobster species (Macpherson *et al.* 2010). Exhaustive sorting of species' distributions within the speciose squat

lobster genus *Munida* (comprises 275 species at the time of this writing) showed that 200 species live in the Pacific Ocean, 48 species in the Atlantic Ocean and 27 species in the Indian Ocean. Out of the 200 species in the Pacific Ocean, in the southern hemisphere, 142 species are distributed in IWP and central Pacific, where only 5 and 11 species are in the Pacific and Atlantic coast of South America, respectively (Baba *et al.* 2008; WoRMS Editorial Board 2016). *M. gregaria* is the only species out of the family Munididae that occurs simultaneously in the central and eastern Pacific as well as western Atlantic. According to this fashion of species origin, we hypothesize that NZ might be a reasonable ancestral population at this time point.

6.6 Conclusion

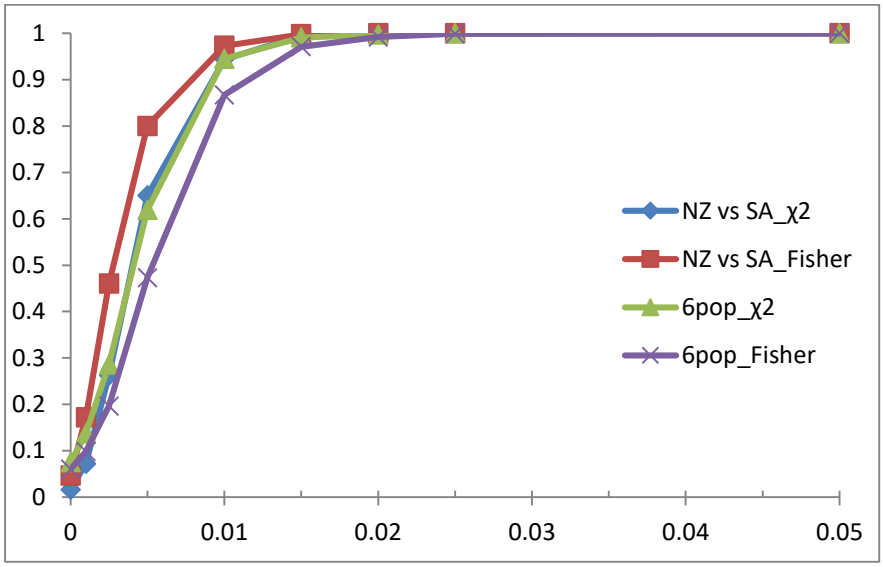
On the basis of adequate scales of geographic and genomic sampling that incorporate mitochondrial COI and nine independent microsatellite loci, present study provided substantial evidence in concluding that the widespread squat lobster *M. gregaria* residing on both sides of the EPB as well as Atlantic Patagonian coasts retains a single species, in which an intraspecific genetic structure was realized between NZ and SA. Analyses under Bayesian framework confirmed historical and contemporary gene flow between the two populations. Both types of marker concordantly suggested the direction of historical gene flow be eastward and in concert with the ACC, implying dispersal was probably facilitated by this powerful ocean current. Phylogenetic analysis based on COI estimated the divergence between NZ and SA was ca. 0.25 Ma. This further suggested that it is dispersal, rather than vicariance of the barrier, in shaping the connectivity and divergence between populations. Our study demonstrates that although the EPB has shown its potent in determining a biogeographic pattern among many various marine fauna, it is rather not a strict barrier to gene flow.

6.7 Additional files

Table S6.1 Genetic diversity indices of nine microsatellite loci. H_O, observed heterozygosity; H_E, expected heterozygosity; *Ar*, allelic richness. Significant deviation from Hardy-Weinberg equilibruim ($P < 0.05$, based on 10,000 permutations) after Bonferroni correction were labeled with asterisk.

		mgr4	**mgr8**	**mgr46**	**mgr52**	**mgr60**	**mgr62**	**mgr81**	**mgr90**	**mgr120**
N_A		18	21	8	11	39	7	10	9	16
Size range		266-341	284-364	148-172	160-180	170-274	155-167	296-322	232-256	249-318
Ar (overall)		7.634	8.637	3.389	5.897	17.39	4.251	4.669	6.731	5.907
FM	H_O	0.720	0.838	0.129	0.763	0.753	0.516	0.677	0.871	0.344
	H_E	0.769	0.816	0.161	0.736	0.871	0.483	0.628	0.821	0.329
	P	0.013	0.276	0.017	0.027	0.021	0.138	0.264	0.000*	0.832
	Ar	9.320	8.433	2.611	6.277	17.040	3.888	4.921	7.071	5.750

NCP	*Ho*	0.667	0.843	0.255	0.686	0.941	0.510	0.647	0.804	0.373
	HE	0.700	0.838	0.294	0.764	0.891	0.578	0.600	0.841	0.350
	P	0.185	0.252	0.439	0.021	0.856	0.249	0.424	0.002	0.806
	Ar	8.519	10.080	3.500	5.761	16.950	4.447	5.214	6.968	6.069
CCP	*Ho*	0.632	0.789	0.053	0.789	0.842	0.579	0.579	0.895	0.421
	HE	0.623	0.771	0.053	0.741	0.899	0.562	0.693	0.825	0.360
	P	0.498	0.108	1.000	0.594	0.069	0.483	0.082	0.011	1.000
	Ar	8.000	8.000	2.000	5.000	17.000	3.000	6.000	6.000	4.000
SM	*Ho*	0.556	0.889	0.111	0.722	0.750	0.528	0.611	0.889	0.250
	HE	0.664	0.817	0.208	0.729	0.872	0.520	0.619	0.808	0.259
	P	0.081	0.994	0.003	0.748	0.144	0.971	0.177	0.010	0.373
	Ar	6.388	8.007	3.516	6.438	16.550	4.342	4.816	6.424	5.453
BC	*Ho*	0.579	0.842	0.105	0.579	0.737	0.316	0.579	0.737	0.316
	HE	0.576	0.851	0.104	0.724	0.915	0.289	0.650	0.845	0.333
	P	1.000	0.424	1.000	0.174	0.002	1.000	0.721	0.194	0.386
	Ar	7.000	11.000	3.000	7.000	15.000	4.000	4.000	7.000	5.000
SA	***Ho***	0.661	0.848	0.147	0.725	0.803	0.507	0.642	0.849	0.339
	HE	0.710	0.816	0.187	0.742	0.882	0.506	0.624	0.824	0.325
	P	0.000*	0.021	0.000*	0.064	0.049	0.948	0.015	0.000*	0.921
	Ar	14.210	13.537	4.754	8.500	32.156	5.581	7.661	8.170	10.764
NZ	*Ho*	0.577	0.427	0.481	0.538	0.805	0.533	0.065	0.551	0.274
	HE	0.617	0.421	0.518	0.561	0.905	0.575	0.638	0.797	0.319
	P	0.400	0.126	0.010	0.048	0.121	0.071	1.000	0.000*	0.003
	Ar	7.962	9.949	5.974	7.000	28.884	6.000	4.000	7.000	11.923



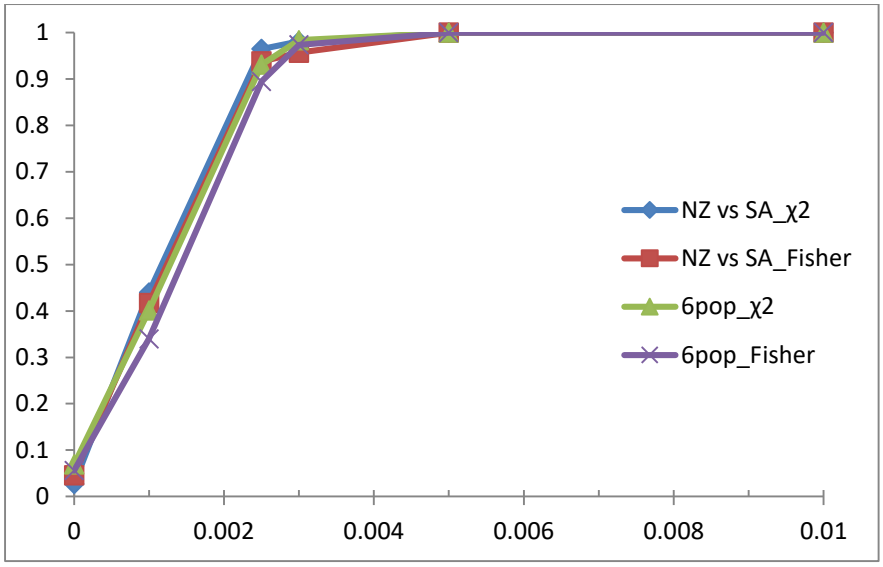


Fig. S6.1 Tests of statistical power for COI (upper) and microsatellite (lower) data sets using POWSIM 4.1. Analyses were conducted among six geographic populations as well as between NZ and SA.

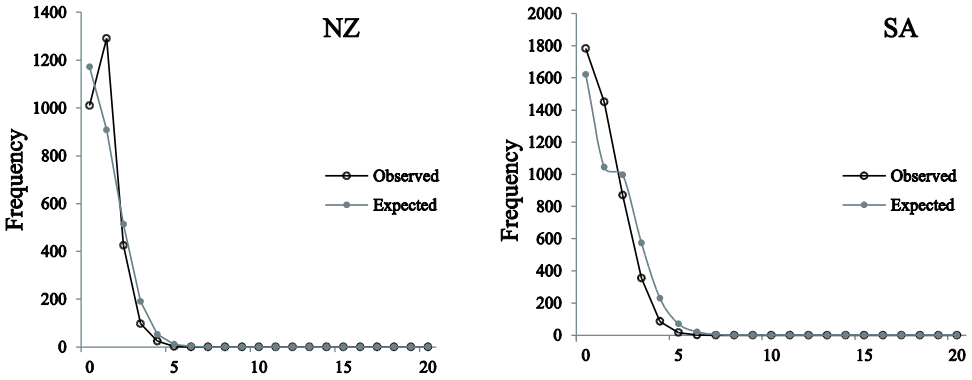


Fig. S6.2 Mismatch distribution over 172 *Munida gregaria* individuals, based on COI sequences (618 bp).

Table S6.2 Bottleneck analyses for the identified populations, South America (SA) and New Zealand (NZ). *P* values are shown for 1-tailed Wilcoxon test of heterozygosity excess, under both the stepwise mutation model (SMM) and the two-phase mutation model (TPM), along with the distribution shape of alleles inferred from the mode-shift test. An L-shaped distribution of alleles suggests absence of bottleneck.

Population	***N***	**Wilcoxon test (one-tail for Heterozygosity excess)**		**Model-shift test**
		TPM	SMM	
SA	218	0.999	0.999	L-shape
NZ	78	0.998	0.999	L-shape

7. Synthesis

In this sythesis the major advances of results of present thesis will be highlighted divided in seven major aspects. First, an efficient protocol of developing new microsatellites for non-model organism is introduced to streamline this process and yield sufficiently many independent candidate loci from unknown genome. In the second and third part I will highlight the importance and the power of multimarker approaches in the attempt of accurate taxonomy, as well as getting deeper insights into the various evolutionary and ecological factors that contribute in understanding genotype-phenotype relationship by illustrating possible mechanisms of the case at hand. The fourth and fifth part of the synthesis are elaborated to provide a striking view of high level of dispersal and gene flow in maintaining species integration rather than prevalent speciation over major biogeographic barrier in marine organisms with a comprehensive reconstruction of the evolutionary history of *M. gregaria*. The last two parts are concluding remarks and future perspectives.

7.1 The power of combined molecular markers in evolutionary studies

One of the major keys to understanding the evolutionary processes and ecological mechanisms by which biological diversity arises is the process of speciation (Coyne & Orr 2004; Turelli *et al.* 2001). Realization of speciation is usually inferred from differences in morphological and/or molecular characters, which are sometimes hard to identify and unify. Among the commonly applied molecular markers in population genetics, microsatellites are most extensively utilized, thanks to their high polymorphism thus great information content (Selkoe & Toonen 2006b). In the past, a major obstacle preventing their use in evolutionary, ecological and conservation studies of non-model organisms was the need to isolate a reasonable number of microsatellite loci from an often unpredictably difficult isolation procedure. In this thesis, the laboratory strategy using the Reporter Genome Protocol (Nolte *et al.* 2005) and the software pipeline based on the STAMP package (Kraemer *et al.* 2009) have shown the promise of successfully isolating and initiating a highly informative set of microsatellite markers within a short period of time.

The set of microsatellites yielded in Chapter 4 shows great polymorphism at each locus and provides solid methodological support as well as successful utilization in Chapters 5 and 6. Their highly elevated mutational rates than single locus coding genes (including the mitochondrial genome) have the potential to detect subtle genetic differentiation (see the significant F_{ST}' values between FI and CCP, FI and BC, Table 6.2, Chapter 6). In addition, these

microsatellites are selectively neutral and thus randomly distributed throughout the genome. The results yielded from them and DNA barcoding (mitochondrial COI gene) provide a sufficient representation that is able to extrapolate to the entire genome. Another advantage of applying such combination of multiple loci is that they can identify evolutionary lineages before monophyly is acquired at any individual locus (Knowles & Carstens 2007). In this way, my choices of molecular markers have the potential to delineate evolutionary lineages that constitute species even at the incipient stage of divergence.

7.2 *M. gregaria* represents a single transoceanic species with discrete phenotypes

Based on variable multi-locus nuclear and mitochondrial markers and continental-scale sampling strategy, I did not detect strong signals of genetic divergence suggesting cryptic speciation neither between different ecotypes (Chapter 5) nor associated with major marine barriers (Chapter 6). I can exclude that it is a hidden diversity of genetic distinctness which is commonly found within cryptic species, thus the different ecotypes truly belong to one single transpacific species *M. gregaria*. Nevertheless, it is a caveat that neutral molecular markers (the focus of most population genetic studies) do not allow predictions on adaptive genetic variation accumulated at certain areas of genome (Reed & Frankham 2001). Such isolated regions of differentiation within genome, also known as 'genomic islands' (Harr 2006; Turner *et al.* 2005), are usually associated with a set of genes under divergent selection, which remains an explanatory underlying genetic basis of the phenotypic differentiation. This alternative is hard to falsify and might be true for any marker-based study in organisms with incompletely known genomes. Assuming the existence of such divergent spots and locating them without indicative information is at the cost of exhaustive genetic survey of candidate loci, which may be eventually still at the risk of failure and is out of the scope of my study. Even though targeted genes under selection could be identified, suggesting that the phenotypic variation between ecotypes are produced by genetic differences, a new question would subsequently arise that whether this part of genetic differentiation should be rather classified as outliers or be interpreted as provisional species (and how small provisional species are allowed to be). Although it has been evidenced that certain loci subject to selection had led to adaptive population divergence in the stasis of neutral markers (Hemmer-Hansen *et al.* 2007; Larmuseau *et al.* 2010), it is doubtful that whether strong local selection could be able to override the effect of drift and gene flow, resulting in isolation in reproduction, which constitutes the main criteria of the BSC. So far, it is known from laboratory experiment that prezygotic behavioural barrier to random mating is absent between *gregaria* s. str. and *subrugosa* ecotypes (Perez-Barros *et*

al. 2011), that is, reproductive isolation should have not formed. In this context, whether the existence of localized genomic divergence does not preclude the conclusion that discrete ecotypes belong to a single species following genetic criteria and the BSC.

With advances in molecular techniques and prevailing implementation of genetic markers, molecular evidences are gaining more weights in taxonomic practices, for which the former species *M. subrugosa* was considered synonymy of *M. gregaria* in the International Code of Zoological Nomenclature (ICZN) (Baba *et al.* 2008). Yet the sharp differences in morphology, ecology and bahaviour of the two ecotypes that are acting as well as truly separate species raise thoughts on how they should be acknowledged and further the long-standing issue of species definition. Ever since Mendel's laws it became gradually realized that the genetic heritable materials determine the observable phenotypic traits, and the ideal that the macroevolutionary phenomenon of speciation is the result of the microevolutionary process of ecologically-based divergent selection dates back to the modern evolutionary synthesis (Dobzhansky 1951; Mayr 1942, 1947). The lack of genetic differentiation at species-level classifies different ecotypes or geographic populations of *M. gregaria* as a single species in the view of population geneticists under the current taxonomic framework, but from the perspective of ecologists, the prominent distinction in all aspects of ecology would keep them valid as separate units that equal to status of species, which should not be overlooked or downplayed. Due to the fuzzy nature of species boundaries and limits of single character classification, it has been recommended using multiple sources of information (e.g., behaviour, ecology, molecules, and morphology) for species discovery and validation (Carstens *et al.* 2013; Raxworthy *et al.* 2007). Current different species concepts are often incompatible with one another, resulting in frequent conflictions in species delimitation, such as the case of *M. gregaria*. Therefore, development of a universal species concept that incorporates and deals with multi-aspects of evidences are strongly needed, meanwhile challenging. In addition, another important aspect must be considered in developing this universal species concept, that speciation must be viewed as a continuum, rather than a discrete event (Coyne & Orr 2004). The process of speciation may contain various stages, but the boundaries between stages are neither abrupt nor obvious and transitions among stages are not inevitable or necessarily unidirectional (Hendry *et al.* 2009; Mallet 2008). To fully appreciate the speciation process, examinations at different stages and levels are necessary.

7.3 Possible mechanisms underlying the discrete phenotypes

Investigating the mechanisms of variation of phenotypes is one of the central issues in biology because it is tightly linked with individuality, adaptation of populations to environmental fluctuations, and the evolution of biodiversity (Crawford & Oleksiak 2007; Pigliucci *et al.* 2006; West-Eberhard 2003). Generally phenotypic variation can be produced by genetic differences, environmental influences and stochastic developmental events. Outcomes from the present study have excluded the explanation of cryptic speciation, that is, the same genotype gives rise to the very different-looking phenotypes. Thus the possible mechanism remains as phenotypic plasticity or developmental variation.

Environmentally induced phenotypic plasticity

Phenotypic plasticity, defined as the capacity of a single genotype to produce different phenotypes in response to varying environmental conditions (Whitman & Agrawal 2009), is a common phenomenon across various taxa in plants and animals. The definition of phenotypic plasticity implies that environmental factors, rather than genetic factors, are playing the role in the observed high phenotypic differentiation. Environmental conditions include biotic and abiotic factors that cannot only vary in time but also vary in space. *M. gregaria* s. l. spans extensive both latitudinal and latitudinal distributions and occupies wide bathymetry (from water surface to 1137m recorded for *subrugosa* (Arntz *et al.* 1999)), which involves a great gradient of heterogeneous environmental conditions (e.g., temperature, salinity, oxygen concentration and food resources). It is known in some species that their feeding performance and diet composition during larval phases can induce the development into different morphotypes or sex reversal (Parsons & Robinson 2007; Zupo 2000). Since *gregaria* s. str. and *subrugosa* differs in feeding habit as deposit feeders and predator, respectively (Romero *et al.* 2004), changes in environmental food composition may likely affect the metamorphosis of *M. gregaria* s. l. in an adaptive way, favouring its development into one ecotype rather than the other. Long-term observations of the proportion of both *gregaria* s. str. and *subrugosa* ecotypes in the Beagle Channel and San Jorge Gulf demonstrates the existence of ecotypes on the sea-bottom are not even and the dominant ecotype can exchange from one to the other (see Diez *et al.* 2016 and references therein). In addition, recent hydroacoustical evidence postulates that major pelagic swarms of *M. gregaria* on the Argentine continental shelf are associated to productive areas such as frontal zones that vary considerably in spatial and temporal scales (Diez *et al.* 2016), implying the availability of phytoplankton in frontal zones might favor the expression of *gregaria* s. str. ecotype.

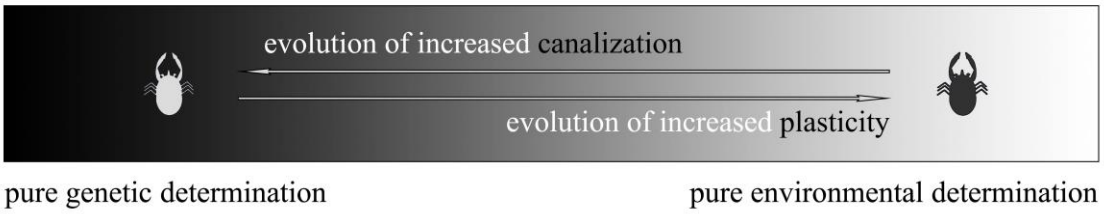


Fig. 7.1. A schematic spectrum depicting an evolutionary continuum of genetic and environmental interactions on phenotypes. Left end of the spectrum shows an extreme where genetic assimilation has completely taken over the effect of environmental factor in determining phenotypic variation, whereas the right end demonstrates phenotypic variation is sheer plasticity of genotype, purely induced by environmental variations. Most (perhaps all) traits, however, lie between these two extremes. Moreover, a trait's position may shift along this continuum over evolutionary time. The evolution of canalization would reduce environmental sensitivity, whereas the evolution of plasticity would increase environmental sensitivity. Such shifts can have profound impacts on diversification and speciation.

Even though phenotypic plasticity is the reaction of the same genotype to variable environmental selection, conversely, phenotypic plasticity can also promote the accumulation and release of cryptic genetic changes in the genotype (i.e. variation that is only expressed under certain environmental or genetic conditions) (Gibson & Dworkin 2004; Le Rouzic & Carlborg 2008). In fact, the observed plasticity of phenotypic traits is a result of an interplay between genetic and environmental factors involving different stages along the evolutionary continuum of speciation (Fig. 7.1). In one extreme, changes in phenotypes are purely induced by environmental factors. Plasticity may evolve to become canalized (which refers to a measure of the ability of a population to produce the same phenotype regardless of variability of its environment or genotype, Debat & David 2001) against environmental perturbations, via selection acting on different populations. The increase of canalization (i.e. the decrease of plasticity) is achieved by the process of genetic assimilation, which was first termed by Waddington (1961) and is a type of genetic accommodation (*sensu* Crispo 2007). The phenotypic traits, which initially are produced only in response to some environmental influence, through the persistence of selection, are gradually taken over by the genotype. Finally, even in the absence of environmental influence that needed at first to be necessary, plasticity of the phenotypic traits are still present (Waddington 1961). In this sense, it reaches the other extreme of genotype-environment interaction (Fig. 7.1), i.e., phenotypes are purely determined by different genes or genotypes, which implicates possibly the origin of new species (West-Eberhard 2005). With regard to *M. gregaria* s. l., the bimodal distributions of distinct ecotypes, rather than a broad continuous curve, implies each ecotype that resulted from

the genotype of *M. gregaria* s. l. may be buffered against environmental variation (environmentally insensitive), that is, genetic canalization might play a role at certain degree. Such assimilated genetic basis perhaps exists as tiny proportion of genome that are actually localized and responsible for the distinctness of the ecotypes. As discussed in detail above, this tiny proportion of genome, if exists, must involve a set of genes that are extremely selected, which might be uncovered unless complete genome is known.

Developmental variation (DV)

Given that *gregaria* s. str. and *subrugosa* ecotypes occur mostly in sympatry (Boschi *et al.* 1992; Tapella 2002), and the differences in morphology and life strategy is already discernable at the stage of megalopa (Rayner 1935; Roberts 1973), perhaps in their early developments environmental variation is absent in triggering the differentiation. In this event, other than genetic and environmental factors, developmental variation (DV), or developmental instability is interpreted as a third source of phenotypic determination (Graham *et al.* 2003) (but see Falconer & Mackay 1996 pp. 134-135, which considered DV to be a variation of environmental origin). Unlike phenotypic plasticity that is rather widely and well investigated, DV remained poorly touched and among the most causally opaque sources of phenotypic variation due to technical challenges in its measurement, i.e. the lack of an absolute standardization of experimental conditions and suitable laboratory model (Peaston & Whitelaw 2006). Nevertheless, as a basic biological principle, DV was documented in systematically distant taxa. For example, 30 yearlong analyse of different components of random variability in quantitative traits in standardized laboratory rats and mice (Gärtner 1990), population genetic calculations on human monozygotic twins (Falconer & Mackay 1996), resting egg formation in *Daphnia pulicaria* (Lajus & Alekseev 2004), polyembryonic armadillos (Loughry & McDonough 2002), inbred lines of *Drosophila*, guinea pigs (Astauroff 1930; Peaston & Whitelaw 2006; Wright & Chase 1936), and cloned mammals and plants (Archer *et al.* 2003a; Archer *et al.* 2003b; Jaenisch & Bird 2003; Scowcroft 1985) revealed that DV usually accounts for more than half of the total variation of a trait.

Heterochrony, which is generally defined as a developmental change in relation to size and shape in the timing or rate of ontogenic events (see review in Smith 2001), has been recognized as a major kind of developmental dissociation that increases morphological evolvability and is thus an important mode of evolution. Recent study showed that *M. gregaria* s. l. from the lower-latitudinal San Jorge Gulf has faster growth rate and earlier reproductive investment in

its early life history than the southern populations from Beagle Channel and Strait of Magellan (Varisco & Vinuesa 2015). Such differences in the timing of ontogeny may involve heterochrony. There are varying examples of heterochrony within crustacean species. For example, within the widespread squat lobster, *Pleuroncodes monodon*, along Pacific coast of South America, there is a smaller pelagic form in the north and a larger benthic form in the south. The morphological differences with lack of genetic differentiation were postulated to have undergone heterochronic process (Haye et al. 2010). Some other Crustacean species show variations in the timing of appearance and rates of character development, which occur at different stages in abbreviated larval development, such as euphausiids (Makarov & Maslennikov 1981), brachyurans (Clark 2005), and anomurans (Delgado & Defeo 2006). Especially for the anomuran mole crab, *Emerita brasiliensis*, neoteny was previously described as a characteristic of males (Efford 1967). As stated by Delgadoand Defeo (2006), ‘‘The principal benefit to early maturation is demographic: sexually mature neotenic males are most likely to succeed since they have less chance of dying before maturation.’’, the long ontogenetic pathways is probably maintaining the basal population, whereas an existing abbreviated pathway seems to be a response to population density and female biased sex ratio. Similarly, environmental variation across the broad distribution of *M. gregaria* s. l. may determine different geographical populations undergoing long or abbreviated ontogenetic pathways.

7.4 Gene flow across the EPB

Munida gregaria s. l. possesses a transpacific distribution on the continental shelf off southern South America and New Zealand (Baba *et al.* 2008). This apparent disjoint distribution hinted at the possibility of a cryptic speciation process between populations on both sides of the EPB. My results, i.e., the absence of different groups of haplotypes and measured gene flow constantly along evolutionary time, all suggest that populations on both sides of the EPB belong to the same species.

Although the uninterrupted 4,000-7,000 km stretch of deep water of the EPB had shown its potency in forming high levels of fauna affinities between the central and eastern Pacific (Briggs 1974; Castilla & Guiñez 2000; Cowman & Bellwood 2013; Glynn & Ault 2000; Macpherson *et al.* 2010), the question that whether the EPB serves as an absolute barrier to gene flow is not only answered by the case of *M. gregaria* s. l., but also witnessed by several other organisms. The onset of the EPB was studied to be ca. 65 Ma (Rosen & Smith 1988), if a previously widespread population was separated by this barrier and subsequently became vicariant, deep genetic divergence between separated populations should be evident. However,

Dana (1975) and later Glynnand Wellington (1983) and Cortés (1997) argued that the establishment of the eastern Pacific coral reef biota was more recent, possibly since Pleistocene low sea level stands, by dispersal from the other side of the EPB. Ongoing connectivity between central and eastern Pacific was also inferred in reef shore fishes (Lessios & Robertson 2006; Robertson *et al.* 2004; Rosenblatt & Waples 1986), sea urchins (Lessios *et al.* 2003) and sea stars (Nishida & Lucas 1988). Two aspects of difference make the case of *M. gregaria* s. l. particular to these examples.

Firstly, previous case studies have exclusively centered on tropical area, whereas in the higher latitude of southern Pacific, where the distance of open deep water is even broader without intermediate shallow water habitats and oceanographic regimes are different, there are few examples explicitly investigate genetic connectivity and gene flow over evolutionary time between central and eastern Pacific. Secondly, the advantage of my study is using fast-evolving microsatellites to provide convincing evidence of high level of gene flow. If the lack of specific divergence yielded from mitochondrial COI, e.g., shared haplotypes by NZ and SA, is interpreted as lack of resolution of marker itself (retention of shared ancestral polymorphisms), this alternative would be eliminated by the corroboration of microsatellites. In fact, my set of microsatellites has shown its advantage over COI in detecting the weak genetic structure between NZ and SA. Moreover, the combination of mitochondrial COI and nuclear microsatellites yielded a more detailed history of *Munida gregaria* s. l. along evolutionary time scale in demonstrating a consistent gene flow from west to east.

Maintenance of gene flow between NZ and SA are mainly ascribed to two factors: ocean current and life history traits. Gene flow between NZ and SA was suggested eastward, which is consistent with the direction of the ACC. Being the mightiest ocean current system, the ACC extends its influence north to ca. 45^{o} S as the Subtropical Front (STF). The ACC has been referred to as overwhelming transportation of pelagic larvae and thus responsible for the genetic connectivity. The lack of genetic differentiation at circum-subantarctic scale of southern bull kelp *Durvillaea antarctica* well exemplified the homogenizing effect of the ACC (Fraser *et al.* 2009). Even for benthic invertebrates with less dispersal ability, e.g. the brooding brittle Star *Astrotoma agassizii* (Hunter & Halanych 2008), the Antarctic brooding isopod *Septemserolis septemcarinata* (Leese *et al.* 2010), low genetic structure was realized with rafting on floating objects that were transported by the ACC.

Single factor of the ACC may not well enough explain the high level of connectivity for *Munida gregaria* s. l., whereby its life history traits play another important role. *M. gregaria* s. l. has a unique pelagic post-larval stage (pelagic larval duration, PLD) out of its family that lasts about 90-120 days (Tapella *et al.* 2002b). Even though adults settle benthic, they still perform migration through water column (Tapella 2002; Zeldis 1985). Further swarming bahaviour of both juveniles and adults may promote their dispersal in larger areas (Diez *et al.* 2012; Zeldis 1985). Even though some researchers argue that PLD may not be a good indicator to dispersal distance (Bowen *et al.* 2006; Selkoe & Toonen 2011), a majority of studies still support that PLD is a key factor in population connectivity (Cowen & Sponaugle 2009; Lester *et al.* 2007; Shanks 2009). Besides, the ability to raft with flotsam and broad environmental tolerance of *M. gregaria* s. l. can be instead accounted for as means to successive migration.

7.5 Outstanding diversity in small lakes vs the lack of that in the ocean

The high levels of gene flow maintained across the southern Pacific as well as between distinct ecotypes render *M. gregaria* a spectacular case that differs from the increasingly reported cryptic speciation in the marine regime (Cowen & Sponaugle 2009; Hellberg *et al.* 2002; Knowlton 2000; Palumbi 2003). In contrast to the comparatively evolutionary stasis of *M. gregaria*, the well-studied cichlid fish in African lakes, which is usually taken to exemplify the contentious sympatric speciation (as well as allopatric speciation), demonstrates the opposite extreme with high species richness and diversity in the following aspects. a) *Number of species*. Based on different delimiting criteria (e.g., morphology, genetics, physiology and behaviour), thousands of species are being identified or scientifically described, with ongoing new discoveries (Froese & Pauly 2012). For instance, it is estimated that there are at least 1 000 species in Lake Malawi (Kornfield & Smith 2000) and more than 500 phenotypically distinct putative species in Lake Victoria (Johnson *et al.* 2000; Turner *et al.* 2001). By contrast, my study confirms that *M. gregaria* s. l. is a single species. b) *Scales of geography*. Such enormous species richness of cichlids exist within tiny African Lakes (e.g. Tanganyika, Victoria, Malawi) compared to the span of thousands of kilometres in the southern Pacific for *M. gregaria* (Fig. 7. 2). Some crater lakes, which are only a few kilometres in diameter with shallow depth and completely isolated from neighbouring river-system. For example, Lakes Barombi Mbo, Bamin (Schliewen *et al.* 1994) and Ejagham (Schliewen *et al.* 2001) in Cameroon, Lake Apoyo in Nicaragua (Barluenga *et al.* 2006), provide unequivocal homogenous habitat evidencing sympatric speciation. c) *Solid genetic examination*. Like the comprehensive genetic investigation on the phenotypically different *M. gregaria* ecotypes, considerable genetic

differentiation with pervasive signatures of speciation for cichlids were confirmed by solid genetic examination. Genetic investigations were carried out using not only the standardly applied mitochondrial markers, but also genotypic markers with faster evolutionary rates, such as microsatellites (Barluenga *et al.* 2006; Takeda *et al.* 2013), AFLP (Barluenga *et al.* 2006; Bezault *et al.* 2011), SNPs (Loh *et al.* 2008), as well as next-generation sequencing of restriction-site-associated DNA (RAD tags) (Keller *et al.* 2013; Wagner *et al.* 2013). In particular, to distinguish speciation from intraspecific polymorphism, Bezault *et al.* (2011) performed AFLP genome scan with large number of putative species and sampling sizes for cichlids from Lake Victoria, which found considerable species-level genetic differentiation. In comparison, the genetic differentiation between NZ and SA for *M. gregaria* demonstrates intraspecific genetic polymorphism (Table 7.1). It is worth noting that the amount of genetic differentiation calculated in Bezault *et al.* (2011) is in the context of putative sympatric speciation, where occasional gene flow between related species offsets the actual level of differentiation in some degree. This explains why sympatric populations of related species can be more similar at neutral markers than allopatric populations of the same species (Konijnendijk *et al.* 2011). d) *Reproductive isolation*. This is of basic importance when following the BSC to corroborate the genetic evidence of speciation. Mate-choice experiments demonstrating that strong assortative mating (Baylis 1976) was in line with the specific status of genetic clusters (Barluenga *et al.* 2006). Work on pairs of phenotypically defined taxa demonstrated divergence from reproductive isolated sister species with strong phenotypic and significant neutral genetic differentiation (Magalhaes *et al.* 2009; Mzighani *et al.* 2010; Seehausen *et al.* 2008). e) *Rapid adaptive radiation*. The endemic cichlid fishes of African lakes are characterized by explosive speciation and adaptive radiation, where reproductive isolated lineages were found as young as ~ 10 000 yr (Barluenga *et al.* 2006; Schliewen *et al.* 2001; Won *et al.* 2005) and species flocks of the Lake Malawai and the Lake Victoria areas were shown to be monophyletic, indicating a single colonization event by an ancestral lineage (Salzburger & Meyer 2004). By contrast, divergence between NZ and SA dates back 0.254 Ma, and the populations still belong to a single species.

In summary, the well-studied African cichlids demonstrate an extreme of speciation coincided with ecological opportunity, intrinsic ecological versatility and genomic flexibility, behavioural mate choice and the amounts of standing genetic variation. In comparison, *M. gregaria* emerges as an opposite evolutionary model in which many fundamental questions in evolution and ecology still await to be tested and ultimately help enrich the knowledge of speciation.

The famous essay by Theodosius Dobzhansky (1973) "*Nothing in biology makes sense except in the light of evolution*" is often quoted to emphasize the importance of understanding evolution when asking questions about causal factors in biology. A corollary to this notion "*Nothing in evolution makes sense except in the light of ecology*" (Grant & Grant 2008) reminds us the notion of evolutionary consequences of ecological interactions. Molecular results helped us understand current patterns of biological diversity, but to fully understand why organisms today are the way they are, we need to comprehend how they have been shaped by their natural environment. Recent conceptual ideas of ′niche construction′ (Odling-Smee *et al.* 2003) demonstrate a framework for building a dynamic connection between niche and evolutionary process, which offers an interesting perspective for future studies such as the *M. gregaria* case.

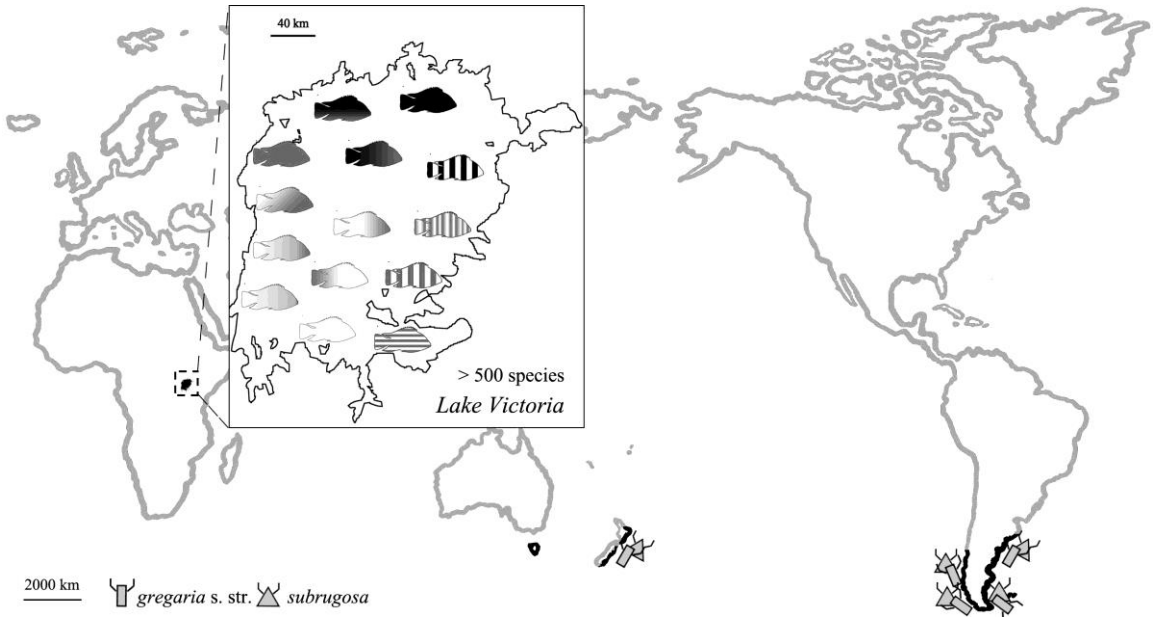


Fig. 7.2 A sketch demonstrating the contrast between highly diverged Cichlids in Lake Victoria and single truly transpacific species *M. gregaria*. Bold black lines indicate coasts along which *M. gregaria* s. l. occurs.

Table 7.1 Comparison of amount of genetic differentiation between cichlids in Lake Victoria and *M. gregaria.*

	No. of species/populations	Fixation Index (F_{ST})	Percentage of total genetic variance (%)
Cichlids in Lake Victoria*	72	0.235	19
Transpacific *M. gregaria*	2	0.088	8.8

* Data from Bezault *et al.* 2011 excluded satellite lakes of Lake Victoria.

7.6 Concluding remarks

In the present thesis genetic analyses performed with newly developed nuclear microsatellite markers and new sets of DNA barcoding, as well as morphological analysis, were carried out to reconstruct the evolutionary history of the transoceanic squat lobster *M. gregaria*. The superordinate aim was to test if phenotypic differences are reflected in underlying genetic differences, meanwhile whether great marine geographical barrier leads to cryptic speciation in widespread marine organisms.

The overall results highlighted that unlike the ubiquitous cryptic speciation among marine populations, there are high levels of gene flow homogenizing the genetic background of different ecotypes as well as distant populations, so that *M. gregaria* is maintained as a single species out of its global distribution.

1) Based on a sampling scope of the South American distribution, genetic analyses using multi-locus nuclear microsatellites and the mitochondrial COI fragment found that discrete ecotypes of *M. gregaria* share the same genotype, i.e. the phenotypic differentiation is not produced by genetic differences. The application of independent polymorphic nuclear microsatellites helped exclude alternative explanations when relying on a single mitochondrial marker, such as incomplete lineage sorting, introgressive hybridization or insufficient evolutionary rate. Morphological analyses affirmed the boundaries between the two ecotypes were not blurred with continental-scale geographic sampling, instead remain stable despite an ontogenetic dimension in the data.
2) The geographic separation by the more than 7,000 km open Pacific water as well as the landmass of Patagonia did not translate into diminished or further vanished gene flow for *M. gregaria* in its global disjunct distribution. The only detectable genetic structure is a differentiation across the Pacific but many shared alleles as well as testing in a Bayesian framework confirmed gene flow at different timescales across the EPB. This divergence between NZ and SA dated back 0.254 Ma, corresponding to late Pleistocene, indicating the Pleistocene climate fluctuation, rather than the EPB, was likely to be responsible for the divergence. The high level of gene flow that was consistently suggested eastward by mitochondrial and microsatellite data indicated that migration was realized in the venue of the ACC.

In summary, the frequently asked questions whether or not *gregaria* s. str. and *subrugosa* ecotypes, as well as disjunct populations on both sides of the EPB represent a truly single species are affirmatively answered in this thesis. The evolution of *M. gregaria* is a prominent example in contrast to the commonly expected allopatric speciation resulted from geographical separation or selection-led sympatric speciation. The insights provided by this thesis will motivate species delimitation by using multiple molecular markers with sufficient evolutionary rates from both nuclear and mitochondrial genomes. Meanwhile, present molecular investigation motivates to connect current knowledge of basic principles of evolution to insights from ecology, in which the causal factors of *M. gregaria* case should be sought.

7.7 Future perspectives

There are stimulating questions and ideas arising from the presented findings, which are outlined for future research to gain a more comprehensive understanding of evolutionary processes shaped by the interactions of genetic and environmental factors from the example of *M. gregaria*.

1) The caveat that neutral molecular markers do not allow predictions on adaptive genetic variation accumulated at certain areas of genome encourages further test to find out the existence of more pronounced difference at loci affected by selection, which might support the differentiation in the phenotypic traits and/or adaptation to environmental heterogeneity. The growing utilization of cost-reducing genome-wide sequencing approaches, i.e. next generation sequencing, provides the opportunity to investigate the whole genomes of *M. gregaria*. This offers the possibility to identify all regions of the genomes that are actually under divergent selection and responsible for the phenotypic or developmental variations. These additional insights would help me learn the necessary quantity of genes that build and maintain a genetic divergence based on selection. Furthermore the functions of those genes could help reveal relevant environmental factors that are playing the roles in adaptive divergence.
2) In current morphological analyses, a third cluster formed by juvenile *gregaria* s. str. from Northern Chilean Patagonia (NCP) emerged discrete from the known cluster represents *gregaria* s. str. adults. One hypothesis is the gap between them is natural irrespective sampling composition, i.e., the ontogenetic transition from juveniles to adults of *gregaria* s. str. is more pronounced than that in *subruguosa*. In future study, the inclusion of samples of juvenile *gregaria* s. str. from other Patagonia regions except

NCP will help test whether this hypothesis is true, or alternatively, this gap would be filled and then *gregaria* s. str. ecotype would have a broader spectrum of phenotypic plasticity.

3) Although there is no evidence of behavioural prezygotic barriers to gene flow (Perez-Barros *et al.* 2011), studies investigating the existence of intrinsic postzygotic isolation are crucial in order to draw the conclusion of no reproductive isolation between *gregaria* s. str. and *subrugosa*. Mating experiments are necessary to collect hatched larvae from homo- and heterotypic mating couples, whereby the set of microsatellites at hand provides the opportunity to perform parentage analysis for these larvae.
4) The causes of nearly 400 whales' death in the area of Penas Gulf are still mysterious, which spotlighted the ecological significance of *M. gregaria* in the food web due to its large abundance and its direct link to the top predators such as whales and primary producers. The presence of biotoxins generated in harmful algae blooms (HABs) in the stomach content of two whales and their prey (including *M. gregaria*) has been confirmed in the latest report of Patagonia Projects: Golfo de Penas (https://expedition2016.wordpress.com/), but more solid and direct evidences are needed to confirm HABs as the killer. In future research, long-term observation and measurements are necessary to monitor realize the degree of accumulation of toxins such as paralytic shellfish poisoning (PSP) in *M. gregaria* and study seasonal population dynamics in relation to the outbreak of harmful phytoplankton.

References

Abdelkrim J, Robertson B, Stanton JA, Gemmell N (2009) Fast, cost-effective development of species-specific microsatellite markers by genomic sequencing. *Biotechniques* **46**, 185-192.

Ajzenberg D, Collinet F, Mercier A, Vignoles P, Dardé M-L (2010) Genotyping of Toxoplasma gondii Isolates with 15 Microsatellite Markers in a Single Multiplex PCR Assay. *Journal of Clinical Microbiology* **48**, 4641-4645.

Allendorf FW, Hohenlohe PA, Luikart G (2010) Genomics and the future of conservation genetics. *Nature Reviews Genetics* **11**, 697-709.

Andersson L (1990) The Driving Force: Species Concepts and Ecology. *Taxon* **39**, 375-382.

Antao T, Lopes A, Lopes R, Beja-Pereira A, Luikart G (2008) LOSITAN: a workbench to detect molecular adaptation based on a F_{st}-outlier method. *Bmc Bioinformatics* **9**, 323.

Archer GS, Dindot S, Friend TH*, et al.* (2003a) Hierarchical Phenotypic and Epigenetic Variation in Cloned Swine. *Biology of Reproduction* **69**, 430-436.

Archer GS, Friend TH, Piedrahita J, Nevill CH, Walker S (2003b) Behavioral variation among cloned pigs. *Applied Animal Behaviour Science* **81**, 321-331.

Arntz W, Gorny M (1996) Cruise report of the Joint Chilean-German-Italian Magellan "Victor Hensen" Campaign in 1994. *Ber. Polarforsch* **190**, 1-113.

Arntz WE, Gorny M, Soto R*, et al.* (1999) Species composition and distribution of decapod crustaceans in the waters off Patagonia and Tierra del Fuego, South America. *Scientia Marina* **63**, 303-314.

Ashton GV, Willis KJ, Burrows MT, Cook EJ (2007) Environmental tolerance of *Caprella mutica*: Implications for its distribution as a marine non-native species. *Marine Environmental Research* **64**, 305-312.

Astauroff BL (1930) Analyse der erblichen Störungsfälle der bilateralen Symmetrie. *Zeitschrift für Induktive Abstammungs- und Vererbungslehre* **55**, 183-262.

Avise JC (2004) *Molecular markers, natural history and evolution (Second edition)* Sinauer Associates, Inc., Sunderland, MA.

Baba K, Macpherson E, Poore GC*, et al.* (2008) Catalogue of squat lobsters of the world (Crustacea: Decapoda: Anomura-families Chirostylidae, Galatheidae and Kiwaidae). *Zootaxa* **1905**, 1-220.

Baird HP, Miller KJ, Stark JS (2011) Evidence of hidden biodiversity, ongoing speciation and diverse patterns of genetic structure in giant Antarctic amphipods. *Molecular Ecology* **20**, 3439-3454.

Ballard JWO, Whitlock MC (2004) The incomplete natural history of mitochondria. *Molecular Ecology* **13**, 729-744.

Baras E, Prignon C, Gohoungo G, Méalard C (2000) Phenotypic sex differentiation of blue tilapia under constant and fluctuating thermal regimes and its adaptive and evolutionary implications. *Journal of Fish Biology* **57**, 210-223.

References

Barber PH, Palumbi SR, Erdmann MV, Moosa MK (2000) Biogeography: A marine Wallace's line? *Nature* **406**, 692-693.

Barluenga M, Stölting KN, Salzburger W, Muschick M, Meyer A (2006) Sympatric speciation in Nicaraguan crater lake cichlid fish. *Nature* **439**, 719-723.

Baums IB, Boulay JN, Polato NR, Hellberg ME (2012) No gene flow across the Eastern Pacific Barrier in the reef-building coral Porites lobata. *Molecular Ecology* **21**, 5418-5433.

Baylis JR (1976) A Quantitative Study of Long-Term Courtship: I. Ethological Isolation Between Sympatric Populations of the Midas Cichlid, *Cichlasoma Citrinellum*, and the Arrow Cichlid, *C. Zaliosum*. *Behaviour* **59**, 59-69.

Beerli P, Felsenstein J (2001) Maximum likelihood estimation of a migration matrix and effective population sizes in n subpopulations by using a coalescent approach. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 4563-4568.

Beerli P, Palczewski M (2010) Unified Framework to Evaluate Panmixia and Migration Direction Among Multiple Sampling Locations. *Genetics* **185**, 313-326.

Bezault E, Mwaiko S, Seehausen O (2011) Population Genomic Tests of Models of Adaptive Radiation in Lake Victoria Region Cichlid Fish. *Evolution* **65**, 3381-3397.

Bickford D, Lohman DJ, Sodhi NS*, et al.* (2007) Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution* **22**, 148-155.

Boschi E, Fischbach C, Iorio M (1992) Catálogo ilustrado de los crustáceos estomatópodos y decápodos marinos de Argentina [Illustrated catalogue of stomatopod and decapod crustraceans of the marine littoral of Argentina]. *Frente Marítimo* **10, sec. A**, 7-94.

Bouckaert R, Heled J, Kühnert D*, et al.* (2014) BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. *Plos Computational Biology* **10**, e1003537.

Bowen BW, Bass AL, Muss A, Carlin J, Robertson DR (2006) Phylogeography of two Atlantic squirrelfishes (Family Holocentridae): exploring links between pelagic larval duration and population connectivity. *Marine Biology* **149**, 899-913.

Brandt A, Gooday AJ, Brandao SN*, et al.* (2007) First insights into the biodiversity and biogeography of the Southern Ocean deep sea. *Nature* **447**, 307-311.

Brey T, Dahm C, Gorny M*, et al.* (1996) Do Antarctic benthic invertebrates show an extended level of eurybathy? *Antarctic Science* **8**, 3-6.

Briggs JC (1974) *Marine zoogeography* McGraw Hill, New York.

Brown WM, George M, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences* **76**, 1967-1971.

Bull J, Vogt R (1979) Temperature-dependent sex determination in turtles. *Science* **206**, 1186-1188.

Butlin RK, Galindo J, Grahame JW (2008) Sympatric, parapatric or allopatric: the most important way to classify speciation? *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **363**, 2997-3007.

References

Caley MJ, Carr MH, Hixon MA*, et al.* (1996) Recruitment and the Local Dynamics of Open Marine Populations. *Annual Review of Ecology and Systematics* **27**, 477-500.

Carstens BC, Pelletier TA, Reid NM, Satler JD (2013) How to fail at species delimitation. *Molecular Ecology* **22**, 4369-4383.

Castilla JC, Guiñez R (2000) Disjoint geographical distribution of intertidal and nearshore benthic invertebrates in the Southern Hemisphere. *Revista Chilena De Historia Natural* **73**, 1-23.

Chilton C (1909) Report on the Amphipoda obtained by the FIS "Endeavour" in Australian seas. *Biological Results of the Fishing Experiments carried on by the FIS "Endeavour"* **14**, 2.

Chow S, Jeffs A, Miyake Y*, et al.* (2011) Genetic Isolation between the Western and Eastern Pacific Populations of Pronghorn Spiny Lobster *Panulirus penicillatus*. *Plos One* **6**, e29280.

Clark PF (2005) The evolutionary significance of heterochrony in the abbreviated zoeal development of pilumnine crabs (Crustacea : Brachyura : Xanthoidea). *Zoological Journal of the Linnean Society* **143**, 417-446.

Clarke A, Johnston NM (2003) Antarctic marine benthic diversity. *Oceanography and Marine Biology* **41**, 47-114.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**, 1657-1659.

Cline TW (1976) A sex-specific, temperature-sensitive maternal effect of the daughterless mutation of *Drosophila melanogaster*. *Genetics* **84**, 723-742.

Cohen SN (1993) Bacterial plasmids: their extraordinary contribution to molecular genetics. *Gene* **135**, 67-76.

Colbourne JK, Pfrender ME, Gilbert D*, et al.* (2011) The ecoresponsive genome of *Daphnia pulex*. *Science* **331**, 555-561.

Collin R (2003) Phylogenetic relationships among calyptraeid gastropods and their implications for the biogeography of marine speciation. *Systematic Biology* **52**, 618-640.

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* **144**, 2001-2014.

Cortés J (1997) Biology and geology of eastern Pacific coral reefs. *Coral Reefs* **16**, S39-S46.

Cowen RK, Lwiza KMM, Sponaugle S, Paris CB, Olson DB (2000) Connectivity of Marine Populations: Open or Closed? *Science* **287**, 857-859.

Cowen RK, Sponaugle S (2009) Larval Dispersal and Marine Population Connectivity. *Annual Review of Marine Science* **1**, 443-466.

Cowman PF, Bellwood DR (2013) Vicariance across major marine biogeographic barriers: temporal concordance and the relative intensity of hard versus soft barriers. *Proceedings of the Royal Society of London B: Biological Sciences* **280**.

Coyne JA, Orr HA (2004) *Speciation* Sinauer Associates Sunderland, MA, Sunderland, MA.

References

Craig MT, Eble JA, Robertson DR, Bowen BW (2007) High genetic connectivity across the Indian and Pacific Oceans in the reef fish *Myripristis berndti* (Holocentridae). *Marine Ecology Progress Series* **334**, 245–254.

Crandall ED, Frey MA, Grosberg RK, Barber PH (2008) Contrasting demographic history and phylogeographical patterns in two Indo-Pacific gastropods. *Molecular Ecology* **17**, 611-626.

Crandall ED, Sbrocco EJ, DeBoer TS, Barber PH, Carpenter KE (2012) Expansion dating: calibrating molecular clocks in marine species from expansions onto the sunda shelf following the last glacial maximum. *Molecular Biology and Evolution* **29**, 707-719.

Crawford DL, Oleksiak MF (2007) The biological importance of measuring individual variation. *Journal of Experimental Biology* **210**, 1613-1621.

Crispo E (2007) The baldwin effect and genetic assimilation: revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. *Evolution* **61**, 2469-2479.

Cronquist A (1978) *Once again, what is a species?* Allanheld and Osmun, Montclair, NJ.

Cussac V, Ortubay S, Iglesias G*, et al.* (2004) The distribution of South American galaxiid fishes: the role of biological traits and post-glacial history. *Journal of Biogeography* **31**, 103-121.

Dana TF (1975) Development of contemporary Eastern Pacific coral reefs. *Marine Biology* **33**, 355-374.

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9**, 772-772.

Darwin C (1872) *The origin of species by means of natural selection or the preservation of favored races in the struggle for life*, six edn. John Murry, London.

Dasmahapatra KK, Mallet J (2006) DNA barcodes: recent successes and future prospects. *Heredity* **97**, 254-255.

Dawson MN, Barber PH, González-Guzmán LI*, et al.* (2011) Phylogeography of *Emerita analoga* (Crustacea, Decapoda, Hippidae), an eastern Pacific Ocean sand crab with long-lived pelagic larvae. *Journal of Biogeography* **38**, 1600-1612.

Dayton pk, Mordida bJ, Bacon f (1994) Polar Marine Communities. *American Zoologist* **34**, 90-99.

de Queiroz K (2007) Species Concepts and Species Delimitation. *Systematic Biology* **56**, 879-886.

Debat V, David P (2001) Mapping phenotypes: canalization, plasticity and developmental stability. *Trends in Ecology & Evolution* **16**.

Delgado E, Defeo O (2006) A complex sexual cycle in sandy beaches: the reproductive strategy of *Emerita brasiliensis* (Decapoda: Anomura). *Journal of the Marine Biological Association of the United Kingdom* **86**, 361-368.

Dieckmann U, Doebeli M (1999) On the origin of species by sympatric speciation. *Nature* **400**, 354-357.

References

Dietz L, Arango CP, Dömel JS*, et al.* (2015) Regional differentiation and extensive hybridization between mitochondrial clades of the Southern Ocean giant sea spider *Colossendeis megalonyx*. *Royal Society Open Science* **2**.

Diez MJ, Cabreira AG, Madirolas A, Lovrich GA (2016) Hydroacoustical evidence of the expansion of pelagic swarms of *Munida gregaria* (Decapoda, Munididae) in the Beagle Channel and the Argentine Patagonian Shelf, and its relationship with habitat features. *Journal of Sea Research*.

Diez MJ, Perez-Barros P, Romero MC*, et al.* (2012) Pelagic swarms and beach strandings of the squat lobster *Munida gregaria* (Anomura: Munididae) in the Beagle Channel, Tierra del Fuego. *Polar Biology* **35**, 973-983.

Dobzhansky T (1951) *Genetics and the Origin of Species*, 3rd edn. Columbia University Press, New York.

Dobzhansky T (1973) Nothing in biology makes sense except in the light of evolution. *American Biology Teacher* **35**, 125-129.

Dombrovsky A, Arthaud L, Ledger TN, Tares S, Robichon A (2009) Profiling the repertoire of phenotypes influenced by environmental cues that occur during asexual reproduction. *Genome Research* **19**, 2052-2063.

Donoghue MJ (1985) A Critique of the Biological Species Concept and Recommendations for a Phylogenetic Alternative. *The Bryologist* **88**, 172-181.

Drummond A, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *Bmc Evolutionary Biology* **7**, 214.

Earl D, von Holdt B (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources* **4**, 359-361.

Ebach MC, Holdrege C (2005) DNA barcoding is no substitute for taxonomy. *Nature* **434**, 697-697.

Efford IE (1967) Neoteny in Sand Crabs of the Genus *Emerita* (Anomura, Hippidae). *Crustaceana* **13**, 81-93.

Ekman S (1953) *Zoogeography of the sea* Sidgwick and Jackson, London.

Ellegren H (2000) Microsatellite mutations in the germline: implications for evolutionary inference. *Trends in Genetics* **16**, 551-558.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**, 2611-2620.

Ewers RM, Didham RK (2006) Confounding factors in the detection of species responses to habitat fragmentation. *Biological Reviews* **81**, 117-142.

Excoffier L, Lischer HE (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**, 564-567.

References

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**, 479-491.

Falconer DS, Mackay TFC (1996) *Introduction to quantitative genetics* Longman, Burnt Mill, England.

Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes* **7**, 574-578.

Feldmann RM, Schweitzer CE (2006) Paleobiogeography of southern hemisphere decapod crustacea. *Journal of Paleontology* **80**, 83-103.

Fell H (1967) Cretaceous and Tertiary surface currents of the oceans. *Oceanography and Marine Biology Annual Reviews* **5**, 317-341.

Fell HB (1962) West-wind-drift dispersal of echinoderms in the southern hemisphere. *Nature* **193**, 759-761.

Fitzpatrick BM, Fordyce JA, Gavrilets S (2008) What, if anything, is sympatric speciation? *Journal of Evolutionary Biology* **21**, 1452-1459.

Fitzpatrick BM, Fordyce JA, Gavrilets S (2009) Pattern, process and geographic modes of speciation. *Journal of Evolutionary Biology* **22**, 2342-2347.

Fitzpatrick JM, Carlon DB, Lippe C, Robertson DR (2011) The West Pacific diversity hotspot as a source or sink for new species? Population genetic insights from the Indo-Pacific parrotfish Scarus rubroviolaceus. *Molecular Ecology* **20**, 219-234.

Fleming CA (1979) *The geological history of New Zealand and its life* Auckland University Press, Auckland.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular marine biology and biotechnology* **3**, 294-299.

Fowler-Walker MJ, Wernberg T, Connell SD (2006) Differences in kelp morphology between wave sheltered and exposed localities: morphologically plastic or fixed traits? *Marine Biology* **148**, 755-767.

Fraser C, Thiel M, Spencer H, Waters J (2010) Contemporary habitat discontinuity and historic glacial ice drive genetic divergence in Chilean kelp. *Bmc Evolutionary Biology* **10**, 203.

Fraser CI, Nikula R, Spencer HG, Waters JM (2009) Kelp genes reveal effects of subantarctic sea ice during the Last Glacial Maximum. *Proceedings of the National Academy of Sciences* **106**, 3249-3253.

Frézal L, Leblois R (2008) Four years of DNA barcoding: Current advances and prospects. *Infection, Genetics and Evolution* **8**, 727-736.

Froese R, Pauly D (2012) List of Nominal Species of Cichlidae, in Froese, Rainer, and Daniel Pauly, eds. *FishBase*.

References

Fu Y-X (1997) Statistical Tests of Neutrality of Mutations Against Population Growth, Hitchhiking and Background Selection. *Genetics* **147**, 915-925.

Galtier N, Nabholz B, Glémin S, Hurst GDD (2009) Mitochondrial DNA as a marker of molecular diversity: a reappraisal. *Molecular Ecology* **18**, 4541-4550.

Gärtner K (1990) A third component causing random variability beside environment and genotype. A reason for the limited success of a 30 year long effort to standardize laboratory animals? *Laboratory Animals* **24**, 71-77.

Gavrilets S (2014) Models of Speciation: Where Are We Now? *Journal of Heredity* **105**, 743-755.

Gavrilets S, Vose A (2005) Dynamic patterns of adaptive radiation. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 18040-18045.

Gibson G, Dworkin I (2004) Uncovering cryptic genetic variation. *Nat Rev Genet* **5**, 681-690.

Gilbert DA, Lehman N, O'Brien SJ, Wayne RK (1990) Genetic fingerprinting reflects population differentiation in the California Channel Island fox. *Nature* **344**, 764-767.

Glynn PW, Ault JS (2000) A biogeographic analysis and review of the far eastern Pacific coral reef region. *Coral Reefs* **19**, 1-23.

Glynn PW, Wellington GM (1983) *Corals and coral reefs of the Galápagos Islands* Univ of California Press, Berkeley, CA.

Goldstein DB, Ruiz Linares A, Cavalli-Sforza LL, Feldman MW (1995) Genetic absolute dating based on microsatellites and the origin of modern humans. *Proceedings of the National Academy of Sciences* **92**, 6723-6727.

Gómez A, Wright PJ, Lunt DH*, et al.* (2007) Mating trials validate the use of DNA barcoding to reveal cryptic speciation of a marine bryozoan taxon. *Proceedings of the Royal Society B: Biological Sciences* **274**, 199-207.

González-Wevar CA, Nakano T, Cañete JI, Poulin E (2010) Molecular phylogeny and historical biogeography of *Nacella* (Patellogastropoda: Nacellidae) in the Southern Ocean. *Molecular Phylogenetics and Evolution* **56**, 115-124.

Goudet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Lausanne University, Lausanne, Switzerland.

Graham JH, Emlen JM, Freeman DC (2003) Nonlinear dynamics and developmental instability. In: *Developmental Instability. Causes and Consequences* (ed. Polak M). New York, Oxford Univ Press.

Grant PR, Grant BR (2008) *How and why species multiply: the radiation of Darwin's finches* Princeton University Press, Princeton, NJ.

Green J (1967) The distribution and variation of *Daphnia lumholtzi* (Crustacea: Cladocera) in relation to fish predation in Lake Albert, East Africa. *Journal of Zoology* **151**, 181-197.

References

Griffiths AM, Sims DW, Cotterell SP*, et al.* (2010) Molecular markers reveal spatially segregated cryptic species in a critically endangered fish, the common skate (*Dipturus batis*). *Proceedings of the Royal Society of London B: Biological Sciences* **277**, 1497-1503.

Griffiths HJ (2010) Antarctic marine biodiversity ? What do we know about the distribution of life in the Southern Ocean? *Plos One* **5**, e11683.

Griffiths HJ, Barnes DKA, Linse K (2009) Towards a generalized biogeography of the Southern Ocean benthos. *Journal of Biogeography* **36**, 162-177.

Guichoux E, Garnier-Géré P, Lagache L*, et al.* (2013) Outlier loci highlight the direction of introgression in oaks. *Molecular Ecology* **22**, 450-462.

Guichoux E, Lagache L, Wagner S*, et al.* (2011) Current trends in microsatellite genotyping. *Molecular Ecology Resources* **11**, 591-611.

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**, 696-704.

Guisan A, Thuiller W (2005) Predicting species distribution: offering more than simple habitat models. *Ecology Letters* **8**, 993-1009.

Gutt J, Helsen E, Arntz WE, Buschmann A (1999) Biodiversity and community structure of the mega-epibenthos in the Magellan region (South America). *Scientia Marina* **63**, 155-170.

Gutt J, Sirenko BI, Smirnov IS, Arntz WE (2004) How many macrozoobenthic species might inhabit the Antarctic shelf? *Antarctic Science* **16**, 11-16.

Hajibabaei M, Singer GA, Hebert PD, Hickey DA (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in genetics : TIG* **23**, 167-172.

Hammer Ø, Harper D, Ryan P (2001) PAST: Paleontological Statistics Software Package for education and data analysis. **4**, 9.

Harr B (2006) Genomic islands of differentiation between house mouse subspecies. *Genome Research* **16**, 730-737.

Harris KDM, Bartlett NJ, Lloyd VK (2012) *Daphnia* as an Emerging Epigenetic Model Organism. *Genetics Research International* **2012**, 8.

Hart MW, Sunday J (2007) Things fall apart: biological species form unconnected parsimony networks. *Biology Letters* **3**, 509-512.

Hasegawa M, Kishino H, Yano T-a (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22**, 160-174.

Hausdorf B (2011) Progress toward a general species concept. *Evolution* **65**, 923-931.

Hausdorf B, Hennig C (2010) Species Delimitation Using Dominant and Codominant Multilocus Markers. *Systematic Biology* **59**, 491-503.

References

Hausdorf B, Wilkens H, Strecker U (2011) Population genetic patterns revealed by microsatellite data challenge the mitochondrial DNA based taxonomy of Astyanax in Mexico (Characidae, Teleostei). *Molecular Phylogenetics and Evolution* **60**, 89-97.

Hayden MJ, Nguyen TM, Waterman A, Chalmers KJ (2008) Multiplex-Ready PCR: A new method for multiplexed SSR and SNP genotyping. *Bmc Genomics* **9**, 1-12.

Haye P, Salinas P, Acuna E, Poulin E (2010) Heterochronic phenotypic plasticity with lack of genetic differentiation in the southeastern pacific squat lobster *pleuroncodes monodon*. *Evolution & Development* **12**, 627 - 633.

Hebert PDN, Cywinska A, Ball SL, DeWaard JR (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society B-Biological Sciences* **270**, 313-321.

Hebert PDN, Gregory TR (2005) The Promise of DNA Barcoding for Taxonomy. *Systematic Biology* **54**, 852-859.

Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 14812-14817.

Hedrick PW (1999) Perspective: highly variable loci and their interpretation in evolution and conservation. *Evolution* **53**, 313-318.

Hedrick PW (2005) A standardized genetic differentiation measure. *Evolution* **59**, 1633-1638.

Held C (2003) Molecular evidence for cryptic speciation within the widespread Antarctic crustacean *Ceratoserolis trilobitoides* (Crustacea, Isopoda). *Antarctic biology in a global context*, 135–139.

Held C, Leese F (2007) The utility of fast evolving molecular markers for studying speciation in the Antarctic benthos. *Polar Biology* **30**, 513-521.

Hellberg ME, Burton RS, Neigel JE, Palumbi SR (2002) Genetic assessment of connectivity among marine populations. *Bulletin of Marine Science* **70**, 273-290.

Hemery LG, Eléaume M, Roussel V*, et al.* (2012) Comprehensive sampling reveals circumpolarity and sympatry in seven mitochondrial lineages of the Southern Ocean crinoid species *Promachocrinus kerguelensis* (Echinodermata). *Molecular Ecology* **21**, 2502-2518.

Hemmer-Hansen J, Nielsen EE, Frydenberg J, Loeschcke V (2007) Adaptive divergence in a high gene flow environment: Hsc70 variation in the European flounder (*Platichthys flesus* L.). *Heredity* **99**, 592-600.

Hendrickx ME (2003) The temperate species of the genus *Munida Leach* (Crustacea, Decapoda, Galatheidae) in the east Pacific, with the description of a new species and additional records for tropical-subtropical species. *Bulletin de l'Institut royal des Sciences naturelles de Belgique* **73**, 115-136.

References

Hendry AP, Bolnick DI, Berner D, Peichel CL (2009) Along the speciation continuum in sticklebacks. *Journal of Fish Biology* **75**, 2000-2036.

Huelsenbeck JP, Andolfatto P (2007) Inference of population structure under a Dirichlet process model. *Genetics* **175**, 1787-1802.

Hughes TP, Bellwood DR, Connolly SR (2002) Biodiversity hotspots, centres of endemicity, and the conservation of coral reefs. *Ecology Letters* **5**, 775-784.

Hunter RL, Halanych KM (2008) Evaluating Connectivity in the Brooding Brittle Star *Astrotoma agassizii* across the Drake Passage in the Southern Ocean. *Journal of Heredity* **99**, 137-148.

Hurlbert SH (1971) The nonconcept of species diversity: a critique and alternative parameters. *Ecology* **52**, 577-586.

Huxley TH (1868) On the classification and distribution of the Alectoromorphae and Heteromorphae. *Proceedings of the Zoological Society of London*, 294-319.

Ilves KL, Taylor EB (2008) Evolutionary and biogeographical patterns within the smelt genus *Hypomesus* in the North Pacific Ocean. *Journal of Biogeography* **35**, 48-64.

Irwin DE, Bensch S, Irwin JH, Price TD (2005) Speciation by distance in a ring species. *Science* **307**, 414-416.

Jaenisch R, Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet*.

Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* **23**, 1801-1806.

Jansen RPS (2000) Germline passage of mitochondria: quantitative considerations and possible embryological sequelae. *Human Reproduction* **15**, 112-128.

Jarrett JN (2008) Inter-population variation in shell morphology of the barnacle. *Journal of Crustacean Biology* **28**, 16-20.

Jarrett JN (2009) Predator-induced defense in the barnacle. *Journal of Crustacean Biology* **29**, 329-333.

Jensen J, Bohonak A, Kelley S (2005) Isolation by distance, web service. *Bmc Genetics* **6**, 13.

Johannsen W (1911) The genotype conception of heredity. *The American Naturalist* **45**, 129-159.

Johnson TC, Kelts K, Odada E (2000) The Holocene history of Lake Victoria. *AMBIO: A Journal of the Human Environment* **29**, 2-11.

Kass RE, Raftery AE (1995) Bayes factors. *Journal of the American Statistical Association* **90**, 773-795.

Keller I, Wagner CE, Greuter L*, et al.* (2013) Population genomic signatures of divergent adaptation, gene flow and hybrid speciation in the rapid radiation of Lake Victoria cichlid fishes. *Molecular Ecology* **22**, 2848-2863.

Klass M, Wolf N, Hirsh D (1976) Development of the male reproductive system and sexual transformation in the nematode *Caenorhabditis elegans*. *Developmental Biology* **52**, 1-18.

References

Knowles LL, Carstens BC (2007) Delimiting species without monophyletic gene trees. *Systematic Biology* **56**, 887-895.

Knowlton N (2000) Molecular genetic analyses of species boundaries in the sea. *Hydrobiologia* **420**, 73-90.

Knox GA (2006) *Biology of the Southern Ocean* CRC Press, Boca Raton, FL.

Konijnendijk N, Joyce DA, Mrosso HDJ, Egas M, Seehausen O (2011) Community genetics reveal elevated levels of sympatric gene flow among morphologically similar but not among morphologically dissimilar species of Lake Victoria cichlid fish. *International Journal of Evolutionary Biology* **2011**, 12.

Kornfield I, Smith PF (2000) African cichlid fishes: Model systems for evolutionary biology. *Annual Review of Ecology and Systematics* **31**, 163-196.

Koumoundouros G, Pavlidis M, Anezaki L*, et al.* (2002) Temperature sex determination in the European sea bass, *Dicentrarchus labrax* (L., 1758) (Teleostei, Perciformes, Moronidae): Critical sensitive ontogenetic phase. *Journal of Experimental Zoology* **292**, 573-579.

Krabbe K, Leese F, Mayer C, Tollrian R, Held C (2009) Cryptic mitochondrial lineages in the widespread pycnogonid *Colossendeis megalonyx* Hoek, 1881 from Antarctic and Subantarctic waters. *Polar Biology* **33**, 281-292.

Kraemer L, Beszteri B, G bler-Schwarz S*, et al.* (2009) STAMP: Extensions to the STADEN sequence analysis package for high throughput interactive microsatellite marker design. *Bmc Bioinformatics* **10**, 41.

Lagerberg T (1906) Anomura und Brachyura der Schwedischen Südpolar Expedition. *Wissenschaftliche Ergebnisse der Schwedischen Südpolar Expedition 1901-1903* **5**, 6-10.

Lajus DL, Alekseev VR (2004) Phenotypic variation and developmental instability of life-history traits: a theory and a case study on within-population variation of resting eggs formation in *Daphnia*. *2004*, 8.

Landry P-A, Koskinen MT, Primmer CR (2002) Deriving evolutionary relationships among populations using microsatellites and $(\delta\mu)^2$: All loci are equal, but some are more equal than others …. *Genetics* **161**, 1339-1347.

Lang JW, Andrews HV (1994) Temperature-dependent sex determination in crocodilians. *Journal of Experimental Zoology* **270**, 28-44.

Larmuseau MHD, Huyse T, Vancampenhout K, Van Houdt JKJ, Volckaert FAM (2010) High molecular diversity in the rhodopsin gene in closely related goby fishes: A role for visual pigments in adaptive speciation? *Molecular Phylogenetics and Evolution* **55**, 689-698.

Latinne A, Waengsothorn S, Rojanadilok P*, et al.* (2012) Combined mitochondrial and nuclear markers revealed a deep vicariant history for *Leopoldamys neilli*, a cave-dwelling rodent of Thailand. *Plos One* **7**, e47670.

References

Le Rouzic A, Carlborg Ö (2008) Evolutionary potential of hidden genetic variation. *Trends in Ecology & Evolution* **23**, 33-37.

Lee CW, Song J-H, Min G-S, Kim S (2016) The complete mitochondrial genome of squat lobster, *Munida gregaria* (Anomura, Galatheoidea, Munididae). *Mitochondrial DNA Part B*, 1-3.

Leese F, Agrawal S, Held C (2010) Long-distance island hopping without dispersal stages: transportation across major zoogeographic barriers in a Southern Ocean isopod. *Naturwissenschaften* **97**, 583-594.

Leese F, Held C (2008) Identification and characterization of microsatellites from the Antarctic isopod *Ceratoserolis trilobitoides*: nuclear evidence for cryptic species. *Conservation Genetics* **9**, 1369-1372.

Leese F, Mayer C, Held C (2008) Isolation of microsatellites from unknown genomes using known genomes as enrichment templates. *Limnology & Oceanography: Methods* **6**, 412-426.

Leese FM, C; Held, C (2008) Isolation of microsatellites from unknown genomes using known genomes as enrichment templates.

Lefébure T, Douady CJ, Gouy M, Gibert J (2006) Relationship between morphological taxonomy and molecular divergence within Crustacea: proposal of a molecular threshold to help species delimitation. *Molecular Phylogenetics and Evolution* **40**, 435-447.

Lessios HA, Kane J, Robertson DR (2003) Phylogeography of the pantropical sea urchin tripneustes: contrasting patterns of population structure between oceans. *Evolution* **57**, 2026-2036.

Lessios HA, Robertson DR (2006) Crossing the impassable: genetic connections in 20 reef fishes across the eastern Pacific barrier. *Proceedings of the Royal Society of London B: Biological Sciences* **273**, 2201-2208.

Lester SE, Ruttenberg BI, Gaines SD, Kinlan BP (2007) The relationship between dispersal ability and geographic range size. *Ecology Letters* **10**, 745-758.

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451-1452.

Liebers D, De Knijff P, Helbig AJ (2004) The herring gull complex is not a ring species. *Proceedings of the Royal Society of London B: Biological Sciences* **271**, 893-901.

Litt M, Luty JA (1989) A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *American Journal of Human Genetics* **44**, 397-401.

Loh Y-HE, Katz LS, Mims MC*, et al.* (2008) Comparative analysis reveals signatures of differentiation amid genomic polymorphism in Lake Malawi cichlids. *Genome Biology* **9**, 1-12.

Lohman DJ, de Bruyn M, Page T*, et al.* (2011) Biogeography of the Indo-Australian Archipelago. *Annual Review of Ecology, Evolution, and Systematics* **42**, 205-226.

Loughry WJ, McDonough CM (2002) Phenotypic variablity within and between litters of nine-banded armadillos. *Southeastern Naturalist* **1**, 287-298.

References

Lovrich G, Casalinuovo M, Molina S, Cárcamo C, Pierotti R (1998) Las langostillas *Munida subrugosa* y *M. gregaria* (Decapoda, Anomura) como potencial recurso economico Patagonico.[The galatheid crabs *Munida subrugosa* and *M. gregaria* (Decapoda, Anomura) as Patagonian potential economic resource]. *Naturalia Patagónica. Ciencias Biológicas* **89**, 92.

Luiz OJ, Madin JS, Robertson DR*, et al.* (2012) Ecological traits influencing range expansion across large oceanic dispersal barriers: insights from tropical Atlantic reef fishes. *Proceedings of the Royal Society of London B: Biological Sciences* **279**, 1033-1040.

MacKenzie AL, Harwood T (2014) Grazing on a toxic Alexandrium catenella bloom by the lobster krill *Munida gregaria* (Decapoda: Galatheoidea: Munididae). *Harmful Algae* **39**, 161-164.

Macpherson E, Richer de Forges B, Schnabel K*, et al.* (2010) Biogeography of the deep-sea galatheid squat lobsters of the Pacific Ocean. *Deep Sea Research Part I: Oceanographic Research Papers* **57**, 228-238.

Magalhaes IS, Mwaiko S, Schneider MV, Seehausen O (2009) Divergent selection and phenotypic plasticity during incipient speciation in Lake Victoria cichlid fish. *Journal of Evolutionary Biology* **22**, 260-274.

Makarov RR, Maslennikov VV (1981) Ecology of larval development of the crustacean *Euphausia superba*. Change in dominant larval forms as a function of environmental conditions. *Marine Ecology Progress Series* **4**, 265-271.

Malausa T, Gilles A, MeglÉCz E*, et al.* (2011) High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. *Molecular Ecology Resources* **11**, 638-644.

Mallet J (1995) A species definition for the modern synthesis. *Trends in Ecology & Evolution* **10**, 294-299.

Mallet J (2005) Hybridization as an invasion of the genome. *Trends in Ecology & Evolution* **20**, 229 - 237.

Mallet J (2008) Hybridization, ecological races and the nature of species: empirical evidence for the ease of speciation. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **363**, 2971-2986.

Marchinko KB (2003) Dramatic phenotypic plasticity in barnacle legs (*Balanus glandula darwin*): magnitude, age dependence, and speed of response. *Evolution* **57**, 1281-1290.

Matthews LH (1932) Lobster-krill. *Discovery Reports* **5**, 467-484.

Maynard Smith J (1962) Disruptive selection, polymorphism and sympatric speciation. *Nature* **195**, 60-62.

Maynard Smith J (1966) Sympatric speciation. *American Naturalist*, 637-650.

Mayr E (1942) *Systematics and the origin of species, from the viewpoint of a zoologist.* Columbia University Press, New York

Mayr E (1947) Ecological Factors in Speciation. *Evolution* **1**, 263-288.

References

Mayr E (1954) Change of genetic environment and evolution. *In: Evolution as a Process (eds Huxley J, Hardy AC, Ford EB)*, 157-180.

Mayr E (1963) *Animal species and evolution* Belknap Press of Harvard University Press Cambridge.

Mazaud A, Michel E, Dewilde F, Turon JL (2010) Variations of the Antarctic Circumpolar Current intensity during the past 500 ka. *Geochemistry, Geophysics, Geosystems* **11**, Q08007.

McCartney MA, Keller G, Lessios HA (2000) Dispersal barriers in tropical oceans and speciation in Atlantic and eastern Pacific sea urchins of the genus *Echinometra*. *Molecular Ecology* **9**, 1391-1400.

McClatchie S, Jillett JB, Gerring P (1991) Observations of gulls foraging on beach-stranded plankton in Otago Harbor, New Zealand. *Limnology and Oceanography* **36**, 1195-1200.

McDowall RM (1970) *The galaxiid fishes of New Zealand* Museum of Comparative Zoology, Harvard University, Cambridge, MA.

Mcdowall RM (1978) Generalized tracks and dispersal in biogeography. *Systematic Zoology* **27**, 88-104.

Meirmans PG (2006) Using the AMOVA framework to estimate a standardized genetic differentiation measure. *Evolution* **60**, 2399-2402.

Meyer CP, Paulay G (2005) DNA barcoding: Error rates based on comprehensive sampling. *Plos Biology* **3**, e422.

Mishler BD (1985) The Morphological, Developmental, and Phylogenetic Basis of Species Concepts in Bryophytes. *The Bryologist* **88**, 207-214.

Mobley KB, Lussetti D, Johansson F, Englund G, Bokma F (2011) Morphological and genetic divergence in Swedish postglacial stickleback (*Pungitius pungitius*) populations. *Bmc Evolutionary Biology* **11**, 1-18.

Moritz C, Cicero C (2004) DNA barcoding: Promise and pitfalls. *Plos Biology* **2**, e354.

Müller CB, Williams IS, Hardie J (2001) The role of nutrition, crowding and interspecific interactions in the development of winged aphids. *Ecological Entomology* **26**, 330-340.

Mullis K, Faloona F, Scharf S*, et al.* (1986) Specific Enzymatic Amplification of DNA In Vitro: The Polymerase Chain Reaction. *Cold Spring Harbor Symposia on Quantitative Biology* **51**, 263-273.

Mzighani SI, Nikaido M, Takeda M*, et al.* (2010) Genetic variation and demographic history of the *Haplochromis laparogramma* group of Lake Victoria—An analysis based on SINEs and mitochondrial DNA. *Gene* **450**, 39-47.

Nance HA, Klimley P, Galván-Magaña F, Martínez-Ortíz J, Marko PB (2011) Demographic processes underlying subtle patterns of population structure in the scalloped hammerhead shark, *sphyrna lewini*. *Plos One* **6**, e21459.

Nei M (1987) Molecular evolutionary genetics. *Columbia University Press, New York*.

Nishida M, Lucas JS (1988) Genetic differences between geographic populations of the Crown-of-thorns starfish throughout the Pacific region. *Marine Biology* **98**, 359-368.

Nolte AW, Stemshorn KC, Tautz D (2005) Direct cloning of microsatellite loci from *Cottus gobio* through a simplified enrichment procedure. *Molecular Ecology Notes* **5**, 628-636.

Nosil P (2012) *Ecological speciation* Oxford University Press, Oxford.

Nosil P, Funk DJ, Ortiz-Barrientos D (2009) Divergent selection and heterogeneous genomic divergence. *Molecular Ecology* **18**, 375-402.

Odling-Smee FJ, Laland KN, Feldman MW (2003) *Niche construction: the neglected process in evolution* Princeton University Press, Princeton, NJ.

Padilla DK (1998) Inducible phenotypic plasticity of the radula in *Lacuna* (Gastropoda: Littorinidae). *Veliger* **41**, 201-204.

Paetkau D, Slade R, Burden M, Estoup A (2004) Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. *Molecular Ecology* **13**, 55-65.

Page TJ, Hughes JM (2010) Comparing the performance of multiple mitochondrial genes in the analysis of Australian freshwater fishes. *Journal of Fish Biology* **77**, 2093-2122.

Palumbi SR (1994) Genetic Divergence, Reproductive Isolation, and Marine Speciation. *Annual Review of Ecology and Systematics* **25**, 547-572.

Palumbi SR (2003) Population genetics, demographic connectivity, and the design of marine reserves *Ecological Applications* **13**, 146-158.

Parejko K, Dodson SI (1991) The evolutionary ecology of an antipredator reaction norm: *Daphnia pulex* and *Chaoborus americanus*. *Evolution* **45**, 1665-1674.

Parsons KJ, Robinson BW (2007) Foraging performance of diet-induced morphotypes in pumpkinseed sunfish (*Lepomis gibbosus*) favours resource polymorphism. *Journal of Evolutionary Biology* **20**, 673-684.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**, 288-295.

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics* **28**, 2537-2539.

Peaston AE, Whitelaw E (2006) Epigenetics and phenotypic variation in mammals. *Mammalian Genome* **17**, 365-374.

Perez-Barros P, Calcagno JA, Lovrich GA (2011) Absence of a prezygotic behavioural barrier to gene flow between the two sympatric morphs of the squat lobster *Munida gregaria* (Fabricius, 1793) (Decapoda: Anomura: Galatheidae). *Helgoland Marine Research* **65**, 513-523.

Pérez-Barros P, D'Amato ME, Guzmán NV, Lovrich GA (2008) Taxonomic status of two South American sympatric squat lobsters, *Munida gregaria* and *Munida subrugosa* (Crustacea:

Decapoda: Galatheidae), challenged by DNA sequence information. *Biological Journal of the Linnean Society* **94**, 421-434.

Perez Barros P, Tapella F, Romero M, Calagno JA, Lovrich GA (2004) Benthic decapod crustaceans associated with captures of *Munida spp.*(Decapoda: Anomura) in the Beagle Channel, Argentina. *Scientia Marina* **68**, 237-246.

Petit RJ, El Mousadik A, Pons O (1998) Identifying populations for conservation on the basis of genetic markers. *Conservation biology* **12**, 844-855.

Petit RJ, Excoffier L (2009) Gene flow and species delimitation. *Trends in Ecology & Evolution* **24**, 386-393.

Pigliucci M, Murren CJ, Schlichting CD (2006) Phenotypic plasticity and evolution by genetic assimilation. *Journal of Experimental Biology* **209**, 2362-2367.

Piry S, Alapetite A, Cornuet J-M*, et al.* (2004) GENECLASS2: A software for genetic assignment and first-generation migrant detection. *Journal of Heredity* **95**, 536-539.

Pons JM, Sonsthagen S, Dove C, Crochet PA (2014) Extensive mitochondrial introgression in North American Great Black-backed Gulls (*Larus marinus*) from the American Herring Gull (*Larus smithsonianus*) with little nuclear DNA impact. *Heredity* **112**, 226-239.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-959.

Prospero S, Grünwald NJ, Winton LM, Hansen EM (2009) Migration patterns of the emerging plant pathogen *Phytophthora ramorum* on the west coast of the United States of America. *Phytopathology* **99**, 739-749.

R Core Team (2015) R: A language and environment for statistical computing. *R Foundation for Statistical Computing, Vienna, Austria*.

Raeymaekers JAM, Maes GE, Audenaert E, Volckaert FAM (2005) Detecting Holocene divergence in the anadromous–freshwater three-spined stickleback (*Gasterosteus aculeatus*) system. *Molecular Ecology* **14**, 1001-1014.

Ramírez-Soriano A, Ramos-Onsins SE, Rozas J, Calafell F, Navarro A (2008) Statistical power analysis of neutrality tests under demographic expansions, contractions and bottlenecks with recombination. *Genetics* **179**, 555-567.

Ramos-Onsins SE, Rozas J (2002) Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution* **19**, 2092-2100.

Raxworthy CJ, Ingram CM, Rabibisoa N, Pearson RG (2007) Applications of ecological niche modeling for species delimitation: A review and empirical evaluation using day geckos (*Phelsuma*) from Madagascar. *Systematic Biology* **56**, 907-923.

Raymond M, Rousset F (1995) An exact test for population differentiation. *Evolution* **49**, 1280-1283.

Rayner GW (1935) *The Falkland species of the crustacean genus Munida* Discovery Report.

References

Reed DH, Frankham R (2001) How closely correlated are molecular and quantitative measures of genetic variation? A meta-analysis. *Evolution* **55**, 1095-1103.

Retamal MA (1981) Catálogo ilustrado de los crustáceos decápodos de Chile. *Gayana Zoología* **44**, 1-110.

Retamal MA (2000) *Decápodos de Chile* World Biodiversity Database CD-ROM Series.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution* **43**, 223-225.

Rieseberg LH (2001) Chromosomal rearrangements and speciation. *Trends in Ecology & Evolution* **16**, 351-358.

Roberts PE (1973) Larvae of *Munida subrugosa* (White), 1847, from Perseverance Harbour, Campbell Island. *Journal of the Royal Society of New Zealand* **3**, 393-408.

Robertson DR, Grove JS, McCosker JE (2004) Tropical transpacific shore fishes. *Pacific Science* **58**, 507-565.

Rogers A, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution* **9**, 552 - 569.

Römer U, Beisenherz W (1996) Environmental determination of sex in *Apistogrammai* (Cichlidae) and two other freshwater fishes (Teleostei). *Journal of Fish Biology* **48**, 714-725.

Romero MC, Lovrich GA, Tapella F, Thatje S (2004) Feeding ecology of the crab *Munida subrugosa* (Decapoda: Anomura: Galatheidae) in the Beagle Channel, Argentina. *Journal of the Marine Biological Association of the UK* **84**, 359-365.

Rosen BR, Smith AB (1988) Tectonics from fossils? Analysis of reef-coral and sea-urchin distributions from late Cretaceous to Recent, using a new method. *Geological Society, London, Special Publications* **37**, 275-306.

Rosenberg NA (2004) DISTRUCT: a program for the graphical display of population structure. *Molecular Ecology Notes* **4**, 137-138.

Rosenblatt RH, Waples RS (1986) A genetic comparison of allopatric populations of shore fish species from the eastern and central pacific ocean: Dispersal or vicariance? *Copeia* **1986**, 275-284.

Roughgarden J, Iwasa Y, Baxter C (1985) Demographic theory for an open marine population with space-limited recruitment. *Ecology* **66**, 54-67.

Rousset F (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics* **145**, 1219-1228.

Rubinoff D, Cameron S, Will K (2006) A genomic perspective on the shortcomings of mitochondrial DNA for "barcoding" identification. *Journal of Heredity* **97**, 581-594.

Rundle HD, Nosil P (2005) Ecological speciation. *Ecology Letters* **8**, 336-352.

Ryman N, Palm S (2006) POWSIM: a computer program for assessing statistical power when testing for genetic differentiation. *Molecular Ecology Notes* **6**, 600-602.

Saez AG, Lozano E (2005) Body doubles. *Nature* **433**, 111-111.

Salzburger W, Meyer A (2004) The species flocks of East African cichlid fishes: recent advances in molecular phylogenetics and population genetics. *Naturwissenschaften* **91**, 277-290.

Sands CJ, Lancaster ML, Austin JJ, Sunnucks P (2009) Single copy nuclear DNA markers for the onychophoran *Phallocephale tallagandensis*. *Conservation Genetics Resources* **1**, 17-19.

Sanford E, Kelly MW (2011) Local adaptation in marine invertebrates. *Annual Review of Marine Science* **3**, 509-535.

Santana QC, Coetzee MPA, Steenkamp ET*, et al.* (2009) Microsatellite discovery by deep sequencing of enriched genomic libraries. *Biotechniques* **46**, 217-223.

Schliewen U, Rassmann K, Markmann M*, et al.* (2001) Genetic and ecological divergence of a monophyletic cichlid species pair under fully sympatric conditions in Lake Ejagham, Cameroon. *Mol Ecol* **10**, 1471-1488.

Schliewen UK, Tautz D, Paabo S (1994) Sympatric speciation suggested by monophyly of crater lake cichlids. *Nature* **368**, 629-632.

Schlötterer C (2004) The evolution of molecular markers—just a matter of fashion? *Nature Reviews Genetics* **5**, 63-69.

Schlötterer C, Wiehe T (1999) Microsatellites, a neutral marker to infer selective sweeps. In: *Microsatellites* (eds. Goldstein DB, Schlötterer C), pp. 238-248. Oxford University Press, Oxford.

Schluter D (2001) Ecology and the origin of species. *Trends in Ecology & Evolution* **16**, 372-380.

Schluter D, Conte GL (2009) Genetics and ecological speciation. *Proceedings of the National Academy of Sciences* **106**, 9955-9962.

Schnabel KE, Ahyong ST, Maas EW (2011) Galatheoidea are not monophyletic – Molecular and morphological phylogeny of the squat lobsters (Decapoda: Anomura) with recognition of a new superfamily. *Molecular Phylogenetics and Evolution* **58**, 157-168.

Schubart CD, Diesel R, Hedges SB (1998) Rapid evolution to terrestrial life in Jamaican crabs. *Nature* **393**, 363-365.

Scowcroft WR (1985) Somaclonal variation: the myth of clonal uniformity. In: *Genetic flux in plants* (eds. Hohn B, Dennis ES), pp. 217-245. Springer, Wien.

Seehausen O, Terai Y, Magalhaes IS*, et al.* (2008) Speciation through sensory drive in cichlid fish. *Nature* **455**, 620-626.

Selkoe K, Toonen RJ (2011) Marine connectivity: a new look at pelagic larval duration and genetic metrics of dispersal. *Marine Ecology Progress Series* **436**, 291-305.

Selkoe KA, Toonen RJ (2006a) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters* **9**, 615-629.

Selkoe KA, Toonen RJ (2006b) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters* **9**, 615-629.

Shaffer HB, Thomson RC (2007) Delimiting species in recent radiations. *Systematic Biology* **56**, 896-906.

Shanks AL (2009) Pelagic larval duration and dispersal distance revisited. *The Biological Bulletin* **216**, 373-385.

Shearer TL, Coffroth MA (2008) DNA barcoding: Barcoding corals: limited by interspecific divergence, not intraspecific variation. *Molecular Ecology Resources* **8**, 247-255.

Sites JW, Marshall JC (2004) Operational criteria for delimiting species. *Annual Review of Ecology Evolution and Systematics* **35**, 199-227.

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics* **139**, 457-462.

Smith KK (2001) Heterochrony revisited: the evolution of developmental sequences. *Biological Journal of the Linnean Society* **73**, 169-186.

Song H, Buhay JE, Whiting MF, Crandall KA (2008) Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences* **105**, 13486-13491.

Sorte CJB, Williams SL, Carlton JT (2010) Marine range shifts and species introductions: comparative spread rates and community impacts. *Global Ecology and Biogeography* **19**, 303-316.

Spanier KI, Leese F, Mayer C*, et al.* (2010) Predator-induced defences in *Daphnia pulex*: Selection and evaluation of internal reference genes for gene expression studies with real-time PCR. *Bmc Molecular Biology* **11**, 1-11.

Stibor H, Lüning J (1994) Predator-induced phenotypic variation in the pattern of growth and reproduction in *Daphnia hyalina* (Crustacea: Cladocera). *Functional Ecology* **8**, 97-101.

Szpiech ZA, Jakobsson M, Rosenberg NA (2008) ADZE: a rarefaction approach for counting alleles private to combinations of populations. *Bioinformatics* **24**, 2498-2504.

Tajima F (1989) The effect of change in population size on DNA polymorphism. *Genetics* **123**, 597-601.

Takeda M, Kusumi J, Mizoiri S*, et al.* (2013) Genetic structure of pelagic and littoral cichlid fishes from Lake Victoria. *Plos One* **8**, e74088.

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**, 512-526.

Tamura K, Peterson D, Peterson N*, et al.* (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731-2739.

Tapella F (2002) Reproducción, crecimiento, distribución y abundancia de la langostilla *Munida subrugosa* (Anomura: Galatheidae) del Canal Beagle, Tierra del Fuego, Argentina. *DPhil Thesis, Universidad Nacional de Córdoba*.

Tapella F, Lovrich GA (2006) Morphological differences between '*subrugosa*' and '*gregaria*' morphs of adult *Munida* (Decapoda: Anomura: Galatheidae) from the Beagle Channel, southern South America. *Journal of the Marine Biological Association of the United Kingdom* **86**, 1149-1155.

Tapella F, Lovrich GA, Romero MC, Thatje S (2002a) Reproductive biology of the crab *Munida subrugosa* (Decapoda: Anomura: Galatheidae) in the Beagle Channel, Argentina. *Journal of the Marine Biological Association of the UK* **82**, 589-595.

Tapella F, Romero MC, Lovrich GA, Chizzini A (2002b) Life history of the galatheid crab *Munida subrugosa* in subantarctic waters of the Beagle Channel, Argentina. *Crabs in cold waters regions: biology, management, and economics. University of Alaska Sea Grant, AK-SG-02-01, Fairbanks*, 115-134.

Tautz D (1993) Notes on the definition and nomenclature of tandemly repetitive DNA sequences. In: *DNA Fingerprinting: State of the Science* (eds. Pena SDJ, Chakraborty R, Epplen JT, Jeffreys AJ), pp. 21-28. Birkhäuser Basel, Basel.

Tittensor DP, Mora C, Jetz W*, et al.* (2010) Global patterns and predictors of marine biodiversity across taxa. *Nature* **466**, 1098-1101.

Toews DPL, Brelsford A (2012) The biogeography of mitochondrial and nuclear discordance in animals. *Molecular Ecology* **21**, 3907-3930.

Tollrian R, Harvell CD (1999) *The ecology and evolution of inducible defenses* Princeton University Press.

Tollrian R, Leese F (2010) Ecological genomics: steps towards unraveling the genetic basis of inducible defenses in *Daphnia*. *Bmc Biology* **8**, 1-4.

Tóth G, Gáspári Z, Jurka J (2000) Microsatellites in different eukaryotic genomes: Survey and analysis. *Genome Research* **10**, 967-981.

Trussell GC (1996) Phenotypic plasticity in an intertidal snail: the role of a common crab predator. *Evolution* **50**, 448-454.

Turelli M, Barton NH, Coyne JA (2001) Theory and speciation. *Trends in Ecology & Evolution* **16**, 330-343.

Turner GF, Seehausen O, Knight ME, Allender CJ, Robinson RL (2001) How many species of cichlid fishes are there in African lakes? *Molecular Ecology* **10**, 793-806.

Turner TL, Hahn MW, Nuzhdin SV (2005) Genomic islands of speciation in *Anopheles gambiae*. *Plos Biology* **3**, e285.

Valentini A, Pompanon F, Taberlet P (2009) DNA barcoding for ecologists. *Trends in Ecology & Evolution* **24**, 110-117.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* **4**, 535-538.

Van Valen L (1976) Ecological species, multispecies, and oaks. *Taxon* **25**, 233-239.

References

Vanelslander B, Créach V, Vanormelingen P*, et al.* (2009) Ecological differentiation between sympatric pseudocryptic species in the estuarine benthic diatom *Navicula phyllepta* (bacillariophyceae). *Journal of Phycology* **45**, 1278-1289.

Vanhaecke D, de Leaniz CG, Gajardo G*, et al.* (2012) DNA barcoding and microsatellites help species delimitation and hybrid identification in endangered galaxiid fishes. *Plos One* **7**, e32939.

Varisco M, Vinuesa J (2010) Occurrence of pelagic juveniles of *Munida gregaria* (fabricius, 1793) (anomura, galatheidae) in San Jorge Gulf, Argentina. *Crustaceana* **83**, 1147-1151.

Varisco M, Vinuesa JH (2015) Growth and reproduction investment of the young of the year of the squat lobster *Munida gregaria* (Crustacea: Anomura) in the Patagonian coast. *Scientia Marina* **79**, 345-353.

Varisco M, Vinuesa JH, Góngora M (2015) Bycatch of the squat lobster *Munida gregaria* in bottom trawl fisheries in San Jorge Gulf, Argentina. *Revista De Biologia Marina Y Oceanografia* **50**, 249-259.

Vences M, Thomas M, Bonett RM, Vieites DR (2005) Deciphering amphibian diversity through DNA barcoding: chances and challenges. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **360**, 1859-1868.

Vermeij GJ (1978) *Biogeography and adaptation: patterns of marine life* Harvard University Press, Cambridge, MA.

Vermeij GJ (2005) From Europe to America: Pliocene to Recent trans-Atlantic expansion of cold-water North Atlantic molluscs. *Proceedings of the Royal Society of London B: Biological Sciences* **272**, 2545-2550.

Vinuesa JH (1977) Aportes al conocimiento de los crustáceos decápodos de Tierra del Fuego, con algunas observaciones zoogeográficas. *Physis, Sec. A* **36**, 9-19.

Vinuesa JH, Varisco M (2007) Trophic ecology of the lobster krill *Munida gregaria* in San Jorge Gulf, Argentina. *Investigaciones marinas* **35**, 25-34.

Vogt G, Huber M, Thiemann M*, et al.* (2008) Production of different phenotypes from the same genotype in the same environment by developmental variation. *Journal of Experimental Biology* **211**, 510-523.

Wada E, Terazaki M, Kabaya Y, Nemoto T (1987) 15N and 13C abundances in the Antartic Ocean with emphasis on the biogeochemical structure of the food web. *Deep Sea Research Part A. Oceanographic Research Papers* **34**, 829-841.

Waddington CH (1961) Genetic Assimilation. In: *Advances in Genetics* (eds. Caspari EW, Thoday JM), pp. 257-293. Academic Press.

Wagner CE, Keller I, Wittwer S*, et al.* (2013) Genome-wide RAD sequence data provide unprecedented resolution of species boundaries and relationships in the Lake Victoria cichlid adaptive radiation. *Molecular Ecology* **22**, 787-798.

Wang C, Held C (2015) Isolation and characterization of 11 microsatellite markers from the squat lobster *Munida gregaria* (Decapoda: Galatheidae) around the Falkland Islands/Islas Malvinas. *Conservation Genetics Resources* **7**, 147-149.

Wares JP (2010) Natural distributions of mitochondrial sequence diversity support new null hypotheses. *Evolution* **64**, 1136-1142.

Wares JP, Cunningham CW (2001) Phylogeography and historical ecology of the north Atlantic intertidal. *Evolution* **55**, 2455-2469.

Watson JD, Crick FH (1953) Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* **171**, 737-738.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution* **38**, 1358-1370.

West-Eberhard MJ (2003) *Developmental plasticity and evolution* Oxford University Press, Oxford.

West-Eberhard MJ (2005) Developmental plasticity and the origin of species differences. *Proceedings of the National Academy of Sciences* **102**, 6543-6549.

Whitlock MC, McCauley DE (1999) Indirect measures of gene flow and migration: FST[ne]1/(4Nm+1). *Heredity* **82**, 117-125.

Whitman DW, Agrawal AA (2009) What is phenotypic plasticity and why is it important? In: *Phenotypic Plasticity of Insects* (eds. Whitman DW, Ananthakrishnan TN), pp. 1-63. CRC Press, Boca Raton, FL.

Whitmore TC (1981) *Wallace's line and plate tectonics* Clarendon Press, Oxford.

Whitworth T (1988) The Antarctic circumpolar current. *Oceanus* **31**, 53-58.

Whitworth T, Dawson R, Magalon H, Baudry E (2007) DNA barcoding cannot reliably identify species of the blowfly genus *Protocalliphora* (Diptera: Calliphoridae). *Proceedings of the Royal Society B: Biological Sciences* **274**, 1731-1739.

Wiemers M, Fiedler K (2007) Does the DNA barcoding gap exist?–a case study in blue butterflies (Lepidoptera: Lycaenidae). *Frontiers in Zoology* **4**, 1-16.

Williams BG (1973) The effect of the environment on the morphology of *Munida gregaria* (Fabricius)(Decapoda, Anomura). *Crustaceana*, 197-210.

Williams BG (1980) The pelagic and benthic phases of post-metamorphic *Munida gregaria* (Fabricius) (Decapoda, Anomura). *Journal of Experimental Marine Biology and Ecology* **42**, 125-141.

Wilson GA, Rannala B (2003) Bayesian inference of recent migration rates using multilocus genotypes. *Genetics* **163**, 1177-1191.

Wilson NG, Maschek JA, Baker BJ (2013) A species flock driven by predation? Secondary metabolites support diversification of slugs in Antarctica. *Plos One* **8**, e80277.

Won Y-J, Sivasundar A, Wang Y, Hey J (2005) On the origin of Lake Malawi cichlid species: A population genetic analysis of divergence. *Proceedings of the National Academy of Sciences* **102**, 6581-6586.

References

WoRMS Editorial Board (2016) World Register of Marine Species. Society for the Management of Electronic Biodiversity Data (SMEBD).

Wright S (1965) The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution*, 395-420.

Wright S, Chase HB (1936) On the Genetics of the Spotted Pattern of the Guinea Pig. *Genetics* **21**, 758-787.

Wu C-I, Ting C-T (2004) Genes and speciation. *Nature Reviews Genetics* **5**, 114-122.

Zakas C, Wares JP (2012) Consequences of a poecilogonous life history for genetic structure in coastal populations of the polychaete *Streblospio benedicti*. *Molecular Ecology* **21**, 5447-5460.

Zeldis JR (1985) Ecology of *Munida gregaria* (Decapoda, Anomura): Distribution and abundance, population dynamics and fisheries. *Marine Ecology Progress Series* **22**, 77-99.

Zeldis JR (1989) *A fishery for Munida gregaria in New Zealand: ecological considerations* N.Z. Fish. Tech. Rep.

Zhang D-X (2004) Lepidopteran microsatellite DNA: redundant but promising. *Trends in Ecology & Evolution* **19**, 507-509.

Zupo V (2000) Effect of microalgal food on the sex reversal of *Hippolyte inermis* (Crustacea: Decapoda). *Marine Ecology Progress Series* **201**, 251-259.