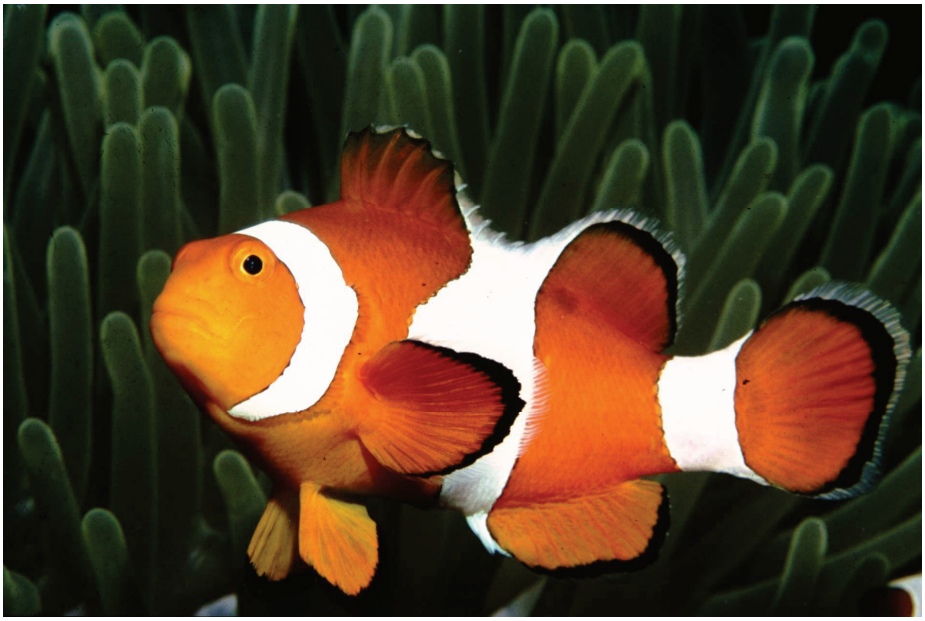
Molecular ecology and evolution of anemonefishes (*Amphiprion* spp) in the Indo-Malay Archipelago



„Amphiprion ocellaris“; Photography by Marc Kochzius

Janne Timm

Dissertation submitted as a partial fulfilment of the requirements for the degree

Doctor of Natural Sciences (*Dr. rer. Nat.*)

Faculty of Biology and Chemistry

University of Bremen

Bremen, October 2008

Examination Committee:

First Examiner: Prof. Dr. Dietmar Blohm
Bremen University

Second Examiner: Dr. Christoph Held
Alfred-Wegener Institut, Bremerhaven

Additional Examiner I: Prof. Dr. Claudio Richter
Alfred-Wegener Institut, Bremerhaven

Additional Examiner II: Dr. Marc Kochzius
Bremen University

Student Member I: Dipl. Biol. Tina Dohna
PhD student, Bremen University

Student Member II: Nicole Fuchs
Diploma student, Bremen University

Date of public defence: Thursday, 27th November 2008

Table of Contents

Acknowledgements

I would like to thank Prof. Dietmar Blohm for being my doctoral adviser, letting me accomplish my thesis within the working group “Biotechnology and Molecular Genetics” independently, but giving me impulses on the large frame of my work. As well a warm thank you goes to Dr. Christoph Held for agreeing to be reviewer for my thesis and additionally for help with methodological as well as the content concerning questions.

I want to appreciate the “all around” supervision and good teamwork of Marc Kochzius, starting with sampling in Indonesia, lab work, data analysis, countless corrections and comments on manuscripts and talks, as well as the many discussions about the data and its interpretations. He supported me in every step of the thesis, even when time was running short. Thanks Marc, the work would not have been finished without your guidance and patience. I would also like to express my thanks to Claudio Richter for taking me „on board“ of the project and being on the committee of my thesis defence.

To Tina Dohna goes a thank you for being in the committee, but even more I’m grateful that she joined our working group and the project, often bringing in a new point of view. Talking to her always gave new ideas on possible interpretations and especially a new friendship for me. Nike Fuchs helped me in many ways, throughout my time at the UFT and in Bremen generally. Thank you for helping me with lab work when you were still „HiWi“ at the AG Blohm, and for your friendship.

Many thanks also go to Agus Nuryanto, my companion through most of the project. It was a pleasure to do the fieldwork together - we surely would have been lost without you! We had a great teamwork while planning the sampling trips, travelling, and in the lab, appeasing the „lab-ghost“. I want to thank as well the whole working group “Biotechnology and Molecular Genetics”, as everyone was always there for help, suggestions, or just for a chat. Especially Reinhard Zelm was always up to dealing with problems, regardless of his own tight time schedule and I want to thank him for helping me a lot with the formatting of the thesis. I’m also very grateful to Christian Seidel, for support with computational problems and Frank Meyerjürgens who was never annoyed by the many questions about reagents or machines in the lab, especially at the beginning. All the students, HiWis and master or diploma students that worked directly or indirectly for my part of the project as well deserve a great thank you.

„Terima kasih“ to Dr. Jamaluddin Jompa and the whole staff of the Centre for Coral Reef Research at the UNHAS in Makassar for all their help in the project, especially during our cruises in Spermonde Archipelago.

A large appreciation and thanks goes to Serge Planes for supporting me in coming to Perpignan and start with the microsatellite analysis there. A warm „merci“ goes as well to his working group at the Centre de Biologie et d'Écologie Tropicale et Méditerranée, Université Perpignan, especially to Caroline Hervet for her help in the lab, and to Elisabeth Rochel and Suzie Miller for support, company, and even „a roof over my head“.

A great thank you goes to Uwe John, who supported me with advices in lab work, analysis, and interpretation of data. He helped me with structural problems of the thesis and I appreciate very much, that I could use the equipment of his working group at the Alfred-Wegener Institute in Bremerhaven on various occasions, often when time was running short. I would also like to thank Tilman Alpermann for always having an „open ear” and suggestions for my questions about microsatellites or other topics of my work, and the help in the lab at the AWI. As well I want to thank Annegret Müller for her help using the sequencer at the AWI.

Esther Borell and Leyla Knittweis I want to thank so much for all their help with my writing, for corrections and input. I was very glad we were together in this project, although they were in Indonesia for such a long time. They made the cruises in Spermonde a fun time and I was happy to have them as friends as well here in Bremen.

Wiebke Krämer I want to thank for being my friend, giving me great support during the „final days and hours“. She was amazingly helping me through the last minutes – thank you so much! Another thank you goes to Silke Laakmann who provided great help and comfort, especially during the final spurt.

Sandra Damm I would like to thank for the long talks on the phone about work and life. You helped me through my diploma thesis, I used a lot of what I learned during that time for my PhD thesis, and you were always understanding and comforting during difficult times. I want to thank as well all the friends that made life worthwhile, they always gave strength when the mood was low.

I also want to thank my sister Mareile for various talk rehearsals and for being there for me with support and comfort whenever it was necessary.

A great appreciation goes to my father Reinhart. I cannot express my thanks for everything he has done for me. He made everything in my life possible and supported me in whatever I’d chosen to do. He gave me the feeling that I could achieve every aim, backing me up, as well through passes of financial shortage.

A special thank you goes to Sascha Klöpper for his support and love through the last years. That he is always there for me makes life easier, happier and whole. Without that backup work would have been much more difficult.

Finally, I want to thank the German Federal Ministry of Education and Research (BMBF, grant no. 03F0390B) for funding the project „Molecular Genetics as a Tool for the Management of Marine Ornamentals in Sulawesi (Indonesia)" in the framework of the SPICE project (Science for the Protection of Indonesian Coastal Marine Ecosystems), within which my PhD thesis was realised, and to the DFG that gave financial support for the participation in the Indo Pacific Fish Conference in Taiwan in 2005.

Summary

The Indo-Malay Archipelago, located between the Pacific and Indian Ocean comprises the world's richest marine shallow water biodiversity. The development of the high biodiversity in this region is discussed on the base of three different theories, the (1) "centre of accumulation", (2) "centre of overlap", and (3) "centre of origin" theory. The area consists of thousands of islands and peninsulas of different size, shape, and geological origin resulting in a high complexity. Furthermore, it has large shallow shelf areas, offering potentially large shallow water habitat to evolve, e.g. coral reefs. In times of sea level low stands during glacial epochs, these areas were above sea level, leading to the loss and fragmentation of habitat for shallow water communities. This separation of ocean basins triggered differentiation in marine organisms, even leading to speciation.

In this thesis, genetic differentiation processes on different levels were studied in the genus *Amphiprion*. This genus comprises anemonefish species, living in close symbiosis with sea anemones, on which they depend for protection.

As many coral reef fishes, the anemonefishes are distinguished mainly by morphology and colour patterns, although several species have quite similar colouration and some overlapping morphological characters, making differentiation difficult. Two species complexes of the subgenera *Actinicola* (*A. ocellaris*, and *A. percula*) and *Phalerebus* (*A. akallopisos, A. sandaracinos, A. perideraion*) were investigated to reveal species boundaries and evaluate speciation processes in the Indo-Malay Archipelago. The phylogenetic analyses were based on mitochondrial cytochrome *b* (cyt *b*) and control region (CR) sequences.

The two studied species complexes showed differences in their patterns of species boundaries despite the fact of belonging to the same genus as well as having a similar biology. Within the subgenus Actinicola, five clades were found, representing different geographical regions. Two major divergences indicated three instead of two deep evolutionary lineages, evolved in the Pliocene. That time period was characterised by oscillations in climate with various small glacial epochs, leading to fluctuations of the sea level. These intermediate disturbances for shallow water communities might have provoked conditions for enhanced diversification in the Indo-Malay Archipelago. Within the subgenus *Phalerebus*, three clades were revealed, not correlated to certain geographical regions, but concordant to the morphological species classification. Based on molecular clock calculations, these species separated in the time range bordering the Pliocene and Pleistocene, when increased glacier formation led to further

sea level lowering, separating the Indian from the Pacific Ocean and possibly other sea areas within the archipelago.

To investigate differentiation patterns on the intra-specific level, analyses of the genetic population structure of the species *A. ocellaris* were conducted. *A. ocellaris* has a high potential of population structuring, because it has demersal eggs, a short larval stage of only 8-12 days, and a very site-attached behaviour of the adults. Additionally, it is a very popular marine aquarium fish, caught in high numbers from coral reefs, and therefore of commercial value. For the analyses on population level, sequences of *A. ocellaris* from different locations across the Indo-Malay Archipelago of a CR fragment were used as well as six microsatellite loci.

Both marker systems revealed strong population structuring in *A. ocellaris*, showing similar but not the same differentiation patterns. Four major genetic lineages with eight distinct clades were revealed with CR, while the microsatellite dataset discovered three major lineages and increased lineage mixing. Possibly, these two marker systems resolve different time ranges. While the mitochondrial DNA still contains traces of historical separation processes the microsatellites reveal the more recent genetic situation. Both markers show the influences of geological history and distance between sites, as well as connectivity through the major surface currents on the population structure of *A. ocellaris*. The information about connectivity patterns among populations of the ornamental fish species *A. ocellaris* along major surface currents and restrictions of gene flow in remote areas, e.g. east coast of Sulawesi, can be used for the spacing of marine protected areas in Indonesia that effectively secure replenishment in exploited areas. In addition, the different genetic lineages should be managed as separate stocks for the sustainable use and conservation of genetic diversity.

Furthermore, the phylogenetic analysis uncovered cryptic diversity within the genus *Amphiprion* that should be considered when assessing diversity of the region and the classification of conservation units.

Altogether, the differentiation patterns found in the two species complexes and within the species *A. ocellaris* indicate favourable conditions for diversification within the Indo-Malay Archipelago through the location, dynamic geological history and complexity of the area. This would support the “centre of origin theory”, although the influence of other processes might as well increase the biodiversity of the region.

Zusammenfassung

Das Indo-Malayische Archipel liegt zwischen dem Indischen und Pazifischen Ozean und weist weltweit die größte marine Biodiversität im Flachwasser auf. Es gibt verschiedene Theorien, die diese enorme Biodiversität erklären sollen. So wird dikutiert, ob die hohe Biodiversität aus der Akkumulation von Arten resultiert, die in peripheren Bereichen entstanden sind („centre of acculumation"), aus einer Überlappung der Faunen des Indischen und Pazifischen Ozeans entstanden ist („centre of overlap"), oder ob das Archipel tatsächlich das Zentrum der Diversifikation darstellt („centre of origin").

Das Indo-Malayische Archipel ist geprägt durch eine hohe Komplexität, bestehend aus Tausenden von Inseln und Halbinseln verschiedenster Größe, Form und geologischen Ursprungs, sowie ein entsprechend kompliziertes Strömungsmuster. Große Bereiche des Archipels werden von flachen Schelfmeeren dominiert, die riesige Flachwasserhabitate darstellen, z.B. Korallenriffe. Durch Schwankungen des Meeresspiegels, wie sie während der Eiszeiten auftraten, wurden insbesondere Lebensgemeinschaften dieser Flachwasserhabitate beeinflusst. Durch das Trockenfallen großer Gebiete der Schelfmeere bei Meeresspiegelsenkungen von bis zu 120 m (z.B. während des glazialen Maximums im Pleistozän) kam es zum einen zu einem starken Habitatverlust dieser Lebensgemeinschaften, zum anderen aber auch zur Trennung zwischen den Ozeanen und zu einer Fragmentierung in separate Seebecken. Daraus resultierte auch die Trennung von Populationen mariner Organismen, die sich dadurch unterschiedlich entwickeln und sogar neue Arten bilden konnten.

In der vorliegenden Arbeit wurden genetische Differenzierungen in der Gattung *Amphiprion* auf verschiedenen Ebenen untersucht. Diese Gattung umfasst ausschließlich Anemonenfischarten, welche in enger Symbiose mit verschiedenen Seeanemonen leben, die ihnen Schutz vor Raubfischen bieten. Wie viele Korallenriff-Fische werden auch Anemonenfischarten hauptsächlich anhand morphologischer Merkmale und ihrer Farbgebung unterschieden. Einige Arten sind allerdings aufgrund ähnlicher Färbungen und teilweise überlappender Morphologie schwer zu differenzieren.

Um Artbildungsprozessen in der Gattung *Amphiprion* zu ergründen und Artgrenzen aufzuklären wurden zwei Artkomplexe der Gattung mit sehr ähnlicher Biologie phylogenetisch untersucht. Dafür wurden die mitochondrialen Sequenzmarker Cytochrom *b* und die Kontrollregion analysiert.

Innerhalb des ersten Artkomplexes, der Untergattung *Actinicola* (*A. ocellaris* und *A. percula*), wurden fünf unterschiedliche Kladen gefunden, welche verschiedenen geographischen Regionen zugeordnet werden konnten. Es zeigten sich drei stark divergente genetische Linien statt der zwei, die nach der klassischen Artbeschreibung zu erwarten wären. Die Aufspaltung dieser genetischen Linien konnte mit Hilfe der molekularen Uhr dem Pliozän zugeordnet werden. In dieser Ära waren oszillierende klimatische Bedingungen vorherrschend, die zu Schwankungen des Meeresspiegels geführt haben. Diese intermediären Störungen der Flachwassergemeinschaften könnten Diversifikationsprozesse stimuliert haben.

In dem zweiten Artkomplex, der Untergattung Phalerebus *A. sandaracinos, A. akallopisos* und *A. perideraion*), wurden drei Kladen detektiert, die nicht bestimmten geographischen Regionen zugeordnet werden konnten, aber mit der morphologischen Klassifikation übereinstimmten. Die Differenzierung dieser Arten wurde auf den Übergang zwischen dem Pliozän und dem darauf folgenden Pleistozän datiert. In dieser Periode kam es zunehmend zu großen Vereisungen und damit zu einer starken Meeresspiegelsenkung. Seebecken wurden zunehmend isoliert und somit eine allopatrische Artbildung forciert.

Die genetische Populationsstruktur des Anemonenfischs *A. ocellaris* wurde analysiert, um genetische Differenzierungen auf intraspezifischer Ebene zu beurteilen. Diese Art wird für den Handel mit marinen Zierorganismen in großem Umfang in Korallenriffen gefischt, vor allem in dem größten Exportland Indonesien. Außerdem lässt die Biologie von *A. ocellaris* ein eingeschränktes Ausbreitungspotenzial erwarten: sie legen demersale Eier, haben eine sehr kurze Larvenphase von nur 8-12 Tagen und die adulten Tiere bleiben ihrer Wirtsanemone treu.

Die genetische Populationsstruktur von *A. ocellaris* im Indo-Malayischen Archipel wurde mit Hilfe der mitochondrialen Kontrollregion sowie sechs Mikrosatelliten analysiert. Beide Markersysteme zeigten ähnliche Populationsstrukturen, wenn auch nicht identische. Die Kontrollregion detektierte vier genetische Hauptlinien mit acht verschiedenen Kladen. Die Mikrosatellitenanalyse hingegen deckte drei genetische Gruppen sowie eine stärkere Durchmischung der Linien im zentralen Bereich des Archipels auf als es in der mitochondrialen Kontrollregion der Fall war. Möglicherweise weist die untersuchte mitochondriale DNA noch mehr Spuren einer stärkeren Trennung der genetischen Linien aus der jüngsten geologischen Vergangenheit auf, während die Mikrosatelliten eher rezente genetische Strukturen aufzeigen. Die Ergebnisse beider Markersysteme unterstreichen jedoch den Einfluss historischer geologischer Prozesse, der geographischen Distanz und der Konnektivität bestimmter Orte durch dominante Meesresströmungen auf die

Populationsstruktur von *A. ocellaris*. Durch den Vergleich der Marker konnten Ergebnisse teilweise unterstützt werden und weiterführende Erkenntnisse über Differenzierungs- und Vermischungsprozesse gesammelt werden.

Informationen über die Konnektivität zwischen Populationen der Zierfischart *A. ocellaris* können nützlich sein, um effektive Entfernungen zwischen marinen Schutzgebieten zu ermitteln, die den genetischen Austausch und damit eine stabile Population auch in befischten Gebieten gewährleisten. Des Weiteren sollten die verschiedenen genetischen Linien als getrennte Bestände für die Fischerei und als Ressourcen für die Erhaltung der Diversität innerhalb der Art behandelt werden.

Durch die phylogenetischen Analysen konnte eine kryptische Diversität aufgedeckt werden, die bei der Beurteilung der Biodiversität und ihrem Schutz beachtet werden sollte.

Die in dieser Arbeit sowohl auf Art- als auch auf Populationsebene gefundene Differenzierung unterstützt die Theorie, dass das Indo-Malayische Archipel das Zentrum der Diversifikation darstellt („centre of origin“). Allerdings ist es möglich, dass das Zusammenwirken von Akkumulation, Überlappung und Ursprung die besonders hohe Biodiversität bewirkt haben könnte.

Abbreviations

π	nucleotide diversity
µl	microlitre
µmol	micromol
A	Adenine
AMOVA	Analysis of Molecular Variance
Ann. Temp.	annealing temperature
bp	basepairs
C	Cytosine
cDNA	copy DNA (from mRNA reverse transcribed DNA)
COI	cytochrome oxidase subunit I
CR	control region
cyt *b*	cytochrome *b*
ddH2O	double distilled Water
DNA	deoxyribonucleic acid
dNTPs	deoxinucleotidetriphosphates
EMBL	European molecular Biology Laboratory
FAM	Carboxyfluorescein
G	Guanine
GBR	Great Barrier Reef
Gene Div.	mean gene diversities over all loci
GTR	general time reversible model
H	haplotype diversity
Het. Def.	heterozygote deficit
HEX	Hexachlorofluorescein phosphoramidite
HRI	Harpending's raggedness index
HWE	Hardy Weinberg Equilibrium
ITF	Indonesian Through Flow
k	number of clusters
m	metre
Mg^{2+}	$Magnesium^{2+}$
min	minutes
ML	Maximum Likelihood
MP	Maximum Parsimony
MPA	Marine Protected Area
mRNA	messenger ribonucleic acid
mt	mitochondrial
mtDNA	mitochondrial DNA
MYA	millions years ago
$N_{Alleles}$	number of alleles
nc	nuclear
NECC	North Equatorial Countercurrent
ng	nanogram
N_{Haplo}	number of individuals
N_{haplo}/N_{ind}	ratio of N_{haplo} to N_{ind}
N_{Ind}	number of haplotypes
NJ	Neighbour Joining
nt	nucleotide

PCR	polymerase chain reaction
pmol	picomol
RAPD	random amplified polymorphic DNA
RFLP	restriction length polymorphism
s	seconds
SEC	South Equatorial Current
SNP	single nucleotide polymorphism
SSD	sum of squared deviation
T	Thymine
Tm	melting temperature of complementary nucleic acids
tRNA-Phe	transfer ribonucleic acid phenylalanine
tRNA-Pro	transfer ribonucleic acid praline
Ts/Tv	ratio of transitions to tranversions
TVM	total variation metric model
U	Unit

List of Figures

List of Tables

List of publications

Publication 1

Title: Contrasting patterns in species boundaries and evolution of anemonefishes (Amphiprioninae, Pomacentridae) in the centre of marine biodiversity
Authors: Janne Timm, Malgozata Figiel, Marc Kochzius
Journal: Molecular Phylogenetics and Evolution

The idea to this study was developed by Janne Timm and Marc Kochzius. Most of the sampling was done by Janne Timm together with Marc Kochzius and Agus Nuryanto. The analysis in the laboratory was carried out independently by Janne Timm and Malgozata Figiel in the UFT Bremen. The computer analysis was as well carried out by Janne Timm and Malgozata Figiel. The manuscript was written by Janne Timm with revisions and improvements by Marc Kochzius.

Publication 2

Title: Geological history and Oceanography of the Indo-Malay Archipelago shape the genetic population structure of the False Clown Anemonefish (Amphiprion ocellaris)
Authors: Janne Timm, Marc Kochzius
Journal: Molecular Ecology

The idea to this study was developed by Marc Kochzius and Janne Timm. All sampling was done by Janne Timm together with Marc Kochzius and Agus Nuryanto. The analysis in the laboratory was carried out independently by Janne Timm in the UFT Bremen. The computer analysis was as well carried out by Janne Timm. The manuscript was written by Janne Timm with revisions and improvements by Marc Kochzius.

Publication 3

Title: Microsatellite analysis revealed population patterns consistent with findings of mitochondrial DNA analysis in the false clown anemonefish (Amphiprion ocellaris, Pomacentridae)

Authors: Janne Timm, Serge Planes, Marc Kochzius

Journal: anticipated submittion to Heredity

The idea to this study was developed by Janne Timm and Marc Kochzius. All sampling was done by Janne Timm together with Marc Kochzius and Agus Nuryanto. The laboratory analysis was carried out by Janne Timm partly in Perpignan, France, with the help of Serge Planes and partly in the UFT Bremen. The computer analysis was carried out independently by Janne Timm. The manuscript was written by Janne Timm with revisions and improvements by Marc Kochzius and Serge Planes.

Thesis Overview

1. Thesis Overview

1.1. General Introduction

1.1.1. Biodiversity

Biodiversity as defined by the Convention on Biological Diversity refers to "the variability among living organisms from all sources, including, 'inter alia', terrestrial, marine, and other aquatic ecosystems, and the ecological complexes of which they are part: this includes diversity within species, between species and of ecosystems" (Convention on Biological Diversity, Article 2; http://www.cbd.int/convention/convention.shtml). A shorter and easier definition was formulated by Gaston & Spicer (2004) summarising biodiversity as the "variation of life on all levels of biological organization" pertaining to diversity within and among species, and among ecosystems.

The basic processes for the evolution of biodiversity comprise the genetic variability due to mutations within a species, recombination, isolation, inheritance, and natural selection. Microevolution (Storch et al. 2001), namely the diversification on population level, is an important paradigm in order to understand the processes of speciation and evolution as a whole. The connectivity and possible isolations of populations result in the genetic population structure. Often, barriers that prevent the exchange of populations play a major role in diversification processes (Mayr 2001). The isolated populations evolve independently, so that new mutations lead to the formation of different alleles and/or haplotypes which may become fixed. Without barriers, new mutations would usually spread to other populations through gene flow resulting in similar allelic patterns. Barriers are often of geological or other physical origin and include land or sea barriers, salinity breaks, or temperature gradients/changes, leading to vicariance. If the separation between populations is long enough, their differentiation might become very strong resulting in a genetic break, which may finally culminate in allopatric speciation (Campbell 1997, Storch et al. 2001). If restrictions to random reproduction among individuals within species are not due to physical barriers, but rather a result of intra-specific differentiation in behaviour or adaptation to ecological niches, it may lead to sympatric speciation (Campbell 1997, Storch et al. 2001).

Through these possible processes, leading from differentiations on the population level to the evolution of species, the fields of population genetics and evolutionary biology are linked to each other. Genetic indications for speciation events are sometimes already evident on the

population level even though different species are not detectable by other characters yet (e.g. morphological characters).

1.1.2. The Indo-Malay region

The Indo-Malay Archipelago is located between the Pacific and Indian Ocean, consisting of more than 25,000 islands of different sizes, shapes and geological origin (Hall 1996). Complex current patterns exist (Kuhnt et al. 2004), where some are seasonal, i.e. changing direction with the monsoon seasons while others being unidirectional. One of the strongest surface current of the region is the unidirectional Indonesian Throughflow (ITF), which features velocities of more than 1 m per second (Wyrtki 1961) and a transport capacity of 8 to 20 million m^3 water per second (Godfrey 1996, Gordon & Fine 1996, Susanto & Gordon 2005). It is a constant water flow connecting the Pacific Ocean with the Indian Ocean. It goes through the Indo-Malay Archipelago by passing the Sulawesi Sea, Makassar Strait, the Flores Sea and turning towards the Indian Ocean in the Banda Sea (Wyrtki 1961).

Typical features of the diverse landscape are numerous different bays and ocean basins. Some of these are shallow shelf seas, with depths of less than 100 m. During the last glacial periods, beginning by the end of the Pliocene and increasingly in the Pleistocene, large amounts of water were bound and therefore the sea level dropped by up to 120 m worldwide (Voris 2000). This change in sea level had a great influence on shallow water communities such as coral reefs, because of the loss of habitat. In the Indo-Malay Archipelago, large areas of the Sunda, Sahul, and Arafura shelf became land area (Voris 2000). Despite the loss of shallow water habitats, this caused the disconnection of different ocean basins and subsequent changes in the current regime of the area. The Indonesian Throughflow for example was generally reduced and influenced by South Pacific waters during the last glacial maximum in the Pleistocene instead of North Pacific influences nowadays (Kuhnt et al. 2004). Before that time, at the end of the Miocene and during the Pliocene, the area experienced dynamic geological changes, featured by the forming of new land areas, mountains, and ocean basins (Hall 1996, Kuhnt et al. 2004).

With all the islands, peninsulas and shallow shelf seas, the region contains one of the highest number and diversity of coral reefs world wide, and is therefore referred to as the “Coral Triangle”. Other diverse coastal and shallow water habitats are abundant as well, so the area is described to contain the worlds’ highest marine shallow water biodiversity (Allen & Werner 2002, Briggs 2005, Hoeksema 2007). The question of why this high biodiversity occurs in

this region remains controversial. The “centre of overlap” theory suggests an overlap of the Pacific and Indian Ocean marine faunas (Woodland 1983), so both faunas contribute to the total biodiversity of the “Coral Triangle”. The “centre of accumulation” theory proposes that the high biodiversity is the result of an accumulation of species that evolved at the peripheries of the area (in the Pacific and Indian Ocean), migrating into the archipelago where they find favourable conditions and persist, while sometimes they disappear at their site of origin (Jokiel & Martinelli 1992). The third theory is based on the notion that the Indo-Malay Archipelago constitutes the “centre of origin” (Briggs 2000, 2005) as the complexity of the area and its geological history are assumed to provide exceptionally good conditions for the evolution of high diversity.

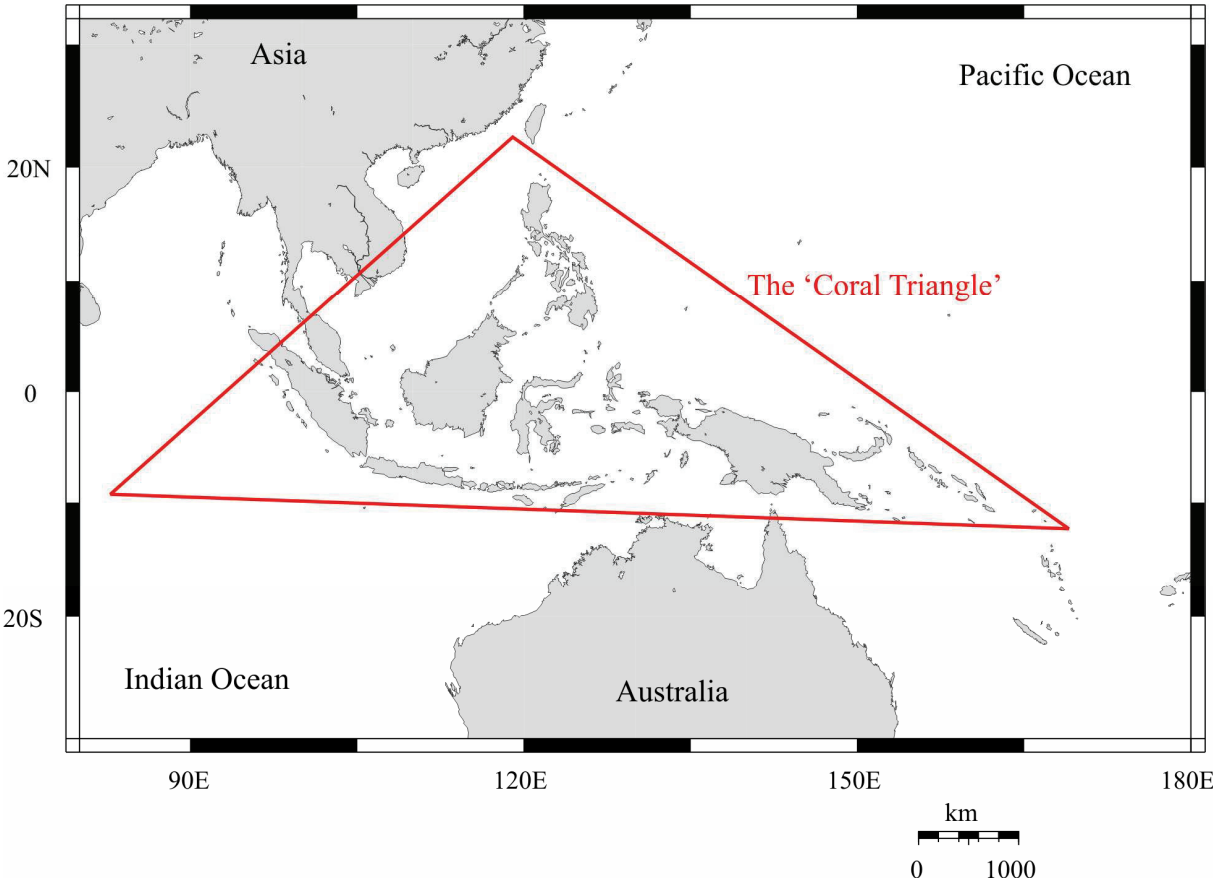


Fig. 1: Map of the Indo-West Pacific region, including the „Coral Triangle“ (after Briggs 2005).

1.1.3. Indonesia and the exploitation of coral reefs

Indonesia comprises over 17,000 islands and large coral reef areas, and is centred in the “Coral Triangle”. It is a large country with 222 million inhabitants (Indonesian Central

Statistics Bureau 2006) of which the majority is either directly or indirectly dependent on the sea and in particular the resources that are provided by coral reefs (Wabnitz et al. 2003).
Extensive exploitation of coral reefs and the settlements along coastal areas give rise to many problems associated with the productivity of resources, maintenance of species richness, and the ecological entity of the ecosystem. Waste due to human settelment and sewage can influence and damage the reefs. Destruction of coral reefs by extensive fishing for food and ornamental organisms employing destructive fishing methods (e.g. dynamite fishing techniques, extraction and breaking off corals, fishing with cyanide), and a general over-exploitation are likely to lead to the depletion of species abundance, local extinctions, shifting of community structure, and the loss of diversity in whole areas (Wood 2001). This is not only negative in terms of conservation issues, but could also lead to less success for the local fishery and the need for increases in fishing expenses (Randall 1987). Marine ornamental organisms are those marine vertebrate or invertebrate species that are attractive for the aquarium trade. Usually they exhibit a colourful appearance or special behavioural and/or biological features. If managed properly, especially the ornamental trade could secure the livelihood of many people, with a low volume and high value correlation (Wabnitz et al. 2003). With the right fishing techniques (e.g. no usage of toxics, no over-exploitation, no destruction of coral habitat for fish extraction), enough source areas for replenishment of the targeted populations (e.g. MPAs) and better transport condition as well as fair pricing for these valuable resources (Wood 2001), the ornamental trade would have less negative impact on reefs. Fishing for seafood is of lower value (regarding selling prices) and often associated with even more destruction, and much lower profit/yield is gained for extracting corals as limestone or construction material (Wabnitz et al. 2003). Due to its large coral reef area, Indonesia is one of the worlds' top exporters for marine ornamentals (Wood 2001, Wabnitz et al. 2003).

1.1.4. Anemonefishes

Among the coral reef fish species, collected for the aquarium trade, are the anemonefishes (subfamily Amphiprioninae) living in close symbioses with certain sea anemones. Most of the approximately 28 species are targeted in the different areas, but there is a particularly high demand for the clown anemonefish (Wood 2001, Wabnitz et al. 2003), that was described as two different species: the clown anemonefish or eastern clown anemonefish (*A. percula*), distributed from the east coast of New Guinea down to the Great Barrier reef, and the false

clown anemonefish or western clown anemonefish (*A. ocellaris*), with a distribution area covering the whole Indo-Malay Archipelago until the west coast of New Guinea, the north-western coast of Australia and in the North up to Okinawa, Japan (Fautin & Allen 1994). Indonesia is one of the top exporting countries and close scrutiny of both exporters and importers data reveals, that *A. ocellaris* is among the three most traded marine ornamental fish species. Although incomplete, the number of traded individuals of *A. ocellaris* recorded only between 1997 and 2002 ranged between 145,015 and 166,119 according to exporter and importer information, respectively. This data represents an average of only 20 companies in Indonesia and four wholesalers in the USA. In those years *A. ocellaris* was as well the most imported marine ornamental fish to the European Union (Wabnitz et al. 2003).

1.1.5. Impacts of ornamental fishery and efforts for conservation

It is difficult to proof impacts of high fishing pressure on the populations of target species (Wood 2001), but local depletions could be observed in areas of intensive collections for the aquarium trade (e.g. Pulau Seribu and Eastern Java, Soegiarto & Polunin 1982). Our own observations in the area of Pulau Seribu in 2005 indicated high collection pressure as well. Many potential host anemones were not occupied by anemonefish or were inhabited by only one individual, which was never the case in any other regions of our study area.

Next to a direct effect on the population size, there might be a change in community structure of the reef as a whole, when mainly valuable species are collected. Decreased fecundity of a population might be provoked by targeting the largest reproducing adults, only one sex (e.g. in the popular mandarine fish), or even juveniles before they reproduce (Molloy et al. 2007). In the case of anemonefish species, some host anemone species (e.g. *Heteractis magnifica*) are collected and traded as well, which prevent that new recruits of the fish can re-colonise the potential habitat. A decrease in anemone abundance would therefore have a great influence on the possibility of anemonefish larvae to find free space to settle. In Australia, a sustainable collecting practise was implemented, that prohibits the extraction of all fish from one anemone and the host itself, to maintain the population (QFMA 1999, cited in Wood 2001 and Shuman et al. 2005). In exploited areas in both the Philippines and the Great Barrier Reef, significantly lower abundances of anemonefish and anemones were found relative to areas that are closed to the ornamental fishery. In addition, the present specimens of fish and host anemones were smaller in size, probably resulting in lower fitness (Shuman et al. 2005, Jones et al. 2008). For isolated and small populations and even more in regionally restricted

endemic species, an exploitation that seems to maintain the population size, could already decrease the genetic diversity within the population. A low genetic diversity might have the effect of decreased adaptation capabilities and the risk of the fixation of deleterious recessive alleles (inbreeding depression, Frankham 1995).

For a sustainable use and the conservation of coral reef resources, various efforts such as quotas and size regulations, licensing, the prohibition of destructive fishing methods, and the setting up of marine protected areas (MPAs) have been implemented, (Wood 2001, Wabnitz et al. 2003). These regulations are important initiatives but are often difficult to manage and control under the local conditions, e.g. in Indonesia (Wood 2001). Furthermore, the applied strategies might not be effective for all targeted organisms (Knittweis 2008) since therefore profound knowledge about the biology of affected organisms is necessary, which to date however is not sufficiently available. Genetic studies can provide information about the genetic diversity of populations, the connectivity among locations, possible source and sink relationships of areas, and dispersal distances of larvae. This information is important for the effective management of marine recourses and the conservation of species, e.g. the location and sizing of MPAs or MPA networks.

1.2. Thesis Aims

The two main objectives of this thesis were as follows:

1) Investigation of possible evolutionary processes leading to the high marine biodiversity found in the Indo-Malay region with focus on the genus *Amphiprion*. The genus is presented by several species in the Indo-Malay Archipelago, closely associated to the coral reef habitat. Within this genus, species complexes with high similarities in biology and morphology provide indication of dynamic evolutionary processes. By revealing species boundaries among species complexes within the genus *Amphiprion*, cryptic diversity might be discovered and the definition of conservation units is possible.

2) Analysing the intra-specific diversity and population structure of the species *A. ocellaris* with the aim to trace genetic diversification processes on the population level in the Indo-Malay region, as well as connectivity patterns and the historical demography of the species. This information could aid in conservation efforts and the sustainable use of coral reef resources.

1.3. Priciples of applied methods

1.3.1. Species boundaries and evolution in *Amphiprion*

For the identification of species boundaries among two different species complexes of the genus *Amphiprion* phylogenetic analyses were conducted.

Sequences of the five species included in this study, were obtained from the mitochondrial genome, a fragment of the control region (CR) and the cytochrome *b* gene (cyt *b*). The control region is located between the tRNA-Pro and the tRNA-Phe gene and is non-coding. Its function is not fully understood yet, but a central conserved region is suggested to be under functional constraints, probably critical for the mitochondrial metabolism (Lee et al. 1995). Next to the conserved parts it contains a hypervariable region (the so called "d-loop"; Alvarado et al. 1995), which was amplified and sequenced for this work. The mitochondrial control region is widely used for population genetic analyses of vertebrates because of its high variability (Lee et al. 1995) as well as for some phylogenetic studies up to the family level (Lee e al. 1995, Santini & Polacco 2006). Cyt *b* is coding for the cytochrome *b* protein, which is an important component of the respiratory chain complex III (Esposti et al. 1993). Because of its function, it is usually conserved at the first and second, but highly variable at the third codon position (Cantatore et al. 1994, Zhu et al. 1994). It is therefore suitable to resolve phylogenetic relationships on genus (Casey et al. 2004, Santini & Polacco 2006) and family level (Concheiro Pérez et al. 2007, Kuriiwa et al. 2007), and was even used for population genetic approaches (Nelson et al. 2000, Chenoweth et al. 2002, Lourie et al. 2005).

For the phylogenetic analysis, the traditional tree building algorithms Neighbour joining (NJ), Maximum parsimony (MP), and Maximum likelihood (ML) were utilised (Paup*, ver. 4.0b10; Swofford 1998). NJ analysis is a distance based method, were closest characters are sharing branches (Saitou & Nei 1987). In the case of genetic phylogenies, the genetic distances between taxa define the topology of the tree.

The principle of MP is based on topologies inferred by the least number of evolutionary steps (Felsenstein 1983). The word "parsimony" is derived from the latin word "parcere" which means "to spare". This principle underlies the assumption that the probability of a result being caused by a complicated process evolved once is higher than that it evolved several times. Therefore it is more probable that the same characters found in two taxa are the result of relationship rather than parallel evolution (Wägele 2000). With the MP algorithm phylogenies as well as haplotype networks can be reconstructed. ML incorporates specific evolutionary models in order to resemble more realistic natural conditions of evolution (Felsenstein 1981,

Lewis 1998). The models vary in the weighting of substitutions, their number of invariable sites, and the gamma shape distribution of the given data. The best-fit evolutionary model for the studied dataset is chosen by the highest likelihood value, tested by the programme Modeltest (Posada & Crandall 1998) and incorporated in the estimations of the phylogeny.
The times of lineage separation that probably led to the evolution of the studied species, were estimated by the genetic distances assuming the molecular clock. Molecular clock estimations are based on the observation that the mutation rate of a certain gene or genetic region is relatively constant within taxa but may vary between between taxa. Therefore the „speed“ or scaling of the studied taxon must be considered when dating divergences. Usually this is done by the help of fossil data or phylogeography of the organism itself or closely related taxa giving clear separation dates (Bromham & Penny 2003). In this thesis, scaling was done according to Messmer et al. (2005), who used the same DNA fragment of the mitochondrial control region for reef fishes. Various mutation rates found in literature for cyt *b* in fish, resulted in time ranges for the divergence of species. Haplotype networks were drawn for both DNA regions, compared and related to geographical regions if possible. Haplotype networks usually define population gene genealogies, where descendant genes might coexist with ancestral genes producing multifurcating, instead of bifurcating relationships leading to network structures rather than directed tree topologies (Posada & Crandall 2001). Networks are sometimes also inferred for genealogies of close related species/species complexes (Chiari et al. 2004).

1.3.2. Population dynamics of *A. ocellaris*

The first approach for the identification of diversity and population structures in *A. ocellaris* was the analysis of sequences of the CR. As in the phylogenetic analysis (see above), the same fragment including the hypervariable region was used.
The diversity (haplotype and nucleotide diversity) for the different populations of *A. ocellaris* were calculated and compared in order to find locations of high and low diversity. The overall population structure was revealed by an Analysis of Molecular Variance (AMOVA) with the programme Arlequin (ver.3.1, Excoffier et al. 2005), and the detailed picture was calculated by pairwise comparison as well as a hierarchical AMOVA. The AMOVA for sequence data estimates the variances within and among populations, considering haplotype frequencies as well as nucleotide substitutions between haplotypes, which give rise to the fixation index Φ_{ST}. In the hierarchical AMOVA another level is included, given by groupings of the populations

(Excoffier et al. 2005). The demographic history was studied by neutrality tests and mismatch distributions for the whole dataset and each subset, in order to see if a population bottleneck and/or population expansion or other factors (e.g. lineage mixing) were responsible for the present genetic population structures in *A. ocellaris*. The factor "gographic distance" was also taken into consideration by an "isolation-by-distance" analysis testing for a significant correlation between geographic and genetic distance.

The second approach, analysing nearly the same datasets as for the mitochondrial DNA analysis, was the study of microsatellites in *A. ocellaris*. Microsatellites are tandemly repeated short sequence motives of two to six bases that are spread over the eukaryote genome (Tautz 1989). The major mode of mutation is the increase or decrease of one or sometimes more repeats by polymerase slippage during DNA replication, causing a change of length (Levinson & Gutman 1987). The number of repeats is usually < 50, whereat the length itself has an influence on the probability of further increase or decrease of the number of repeats at the locus (Ellegren 2000). Microsatellite sequences are prone to mutation due to this typical mode. Additionally they are usually non-coding and not under selection, resulting in high mutation rates (Weber & Wong 1993). As the flanking regions of microsatellites are often also variable, most microsatellite primers are species specific, or at least only usable for closely related species. In this study, over 13 different microsatellite loci isolated for its sibling species *A. percula*, were tested for *A. ocellaris*, as well as 11 loci isolated for the saddleback anemonefish species, *A. polymnus*. Out of the tested loci, three of each set of loci were finally chosen for the analysis of diversity and population structure in *A. ocellaris*. The other tested loci were either not amplifiable, monomorphic, or showed stutterbands that were not clearly readable. For the analysis of microsatellites, amplification with fluorescence labelled primers is conducted. The resulting labelled fragments, usually of sizes between 100 to 500 basepairs, are separated by electrophoresis either in a high percentage polyacrylamid gel or a capillary sequencer to ensure the clear separation of alleles.

Microsatellites are co-dominant markers, as different alleles can be detected within an individual. Thus genotypes can be recognised and heterozygosity rates calculated, distinguishing the analyses from those of mitochondrial DNA markers. In microsatellite analysis, genetic distances are calculated by the allele frequencies and distributions and the genetic diversity of the populations is expressed as allelic richness and gene diversity. Whereas in sequence data analysis, the differences due to point mutations between sequences are used to calculate nucleotide diversity and genetic distances. Isolation-by-distance, overall AMOVA, hierarchical AMOVA, as well as pairwise F-Statistics were conducted in order to

find population structures, similar to the analysis of the mitochondrial sequence marker, resulting in the fixation index F_{ST} and calculated without the information of the nucleotide differences between sequences. Additionally, Bayesian analyses were performed to find probabilities of certain clusters in the datasets (STRUCTURE; vers. 2.2., Pritchard et al. 2000a). The Bayesian analysis estimates probabilities for the truth of a tested hypothesis (Goloboff & Pol 2005). It is based on Monte Carlo Markow chain calculations (Larget & Simon 1999). This method was used to address the hypothesis that the dataset is subdivided into a certain number of clusters (*k*).

The used loci were also tested for the presence of null-alleles and linkage disequilibrium among loci in the populations and clusters. Null-alleles are alleles that contain mutations in the primer binding site, preventing the amplification. While homozygotes for this allele would not be amplified, but in heterozygotes only the one allele would be amplified, which leads to unusually high frequencies of homozygotes in a population where the null-allele is fixed (Van Oosterhout 2004). If significant linkage disequilibrium among loci is detected, these loci cannot be considered independent. If the linkage is not consistent among the populations though, other factors might have caused the observed patterns, like small and stable population sizes, or lineage mixing (Laan & Pääbo 1997, Zavattari et al. 2000, Barrière & Félix 2007).

1.4. Outline

1.4.1. Contrasting patterns in species boundaries and evolution of anemonefishes (Amphiprioninae, Pomacentridae) in the centre of marine biodiversity

Among the anemonefishes (subfamily Amphiprioninae) are complexes composed of species with very similar morphology and colouration patterns. In some cases their morphological characters are even overlapping. For the correct measurement of biodiversity and the definition of conservation units, an extensive knowledge about species boundaries and integrities is important. Therefore, the phylogenetic relationships within two different species complexes of the genus *Amphiprion* were studied in order to clarify species boundaries on a genetic level. The first complex contains close related species of the subgenus *Phalerebus* (Allen 1991) that exhibits great morphological similarities and only slight differences in their colouration patterns. In the second complex we find the sibling species *A. ocellaris* and *A. percula* that show very similar colouration and overlapping morphological characters. The

phylogenetic analysis gives an insight into evolutionary processes, leading to diversification and speciation of a group of coral reef associated fish in the Indo-Malay Archipelago, an area with extraordinary high biodiversity.

1.4.2. Geological history and oceanography of the Indo-Malay Archipelago shape the genetic population structure of the false clown anemonefish (*Amphiprion ocellaris*)

After defining diversification on the species level, a more detailed study of intra-specific diversity and differentiation patterns was initiated. The species *A. ocellaris* is a popular aquarium fish and extracted in high numbers from the wild. It is a host specialist depending on the symbiosis with one of three possible anemone species. Due to its localised biology, it has been suggested to have low dispersal capabilities. Therefore it might be suffering genetic reduction due to high exploitation pressure and low exchange with other populations in some collecting areas. To ensure population replenishment and sufficient genetic diversity for adaptation to environmental changes in fished populations, the establishment of adequately sized and spaced MPAs could be one possible option. The genetic population structure of *A. ocellaris* was studied by analysing a fragment of the mitochondrial control region (CR). The genetic connectivity among locations and the genetic status of populations are helpful information for conservation efforts. Not only the factors leading to the specific population structure of *A. ocellaris* are interpreted but also possible influences on the evolution and loss of diversity in the Indo-Malay Archipelago on population level are discussed.

1.4.3. Microsatellite analysis revealed population patterns similar to findings of mitochondrial control region analysis in the false clown anemonefish (*Amphiprion ocellaris*)

This chapter concentrates on the comparison of results found with two different genetic marker systems, mitochondrial DNA sequence marker and microsatellite loci. The use of mitochondrial markers for population genetic analysis is critically discussed. In order to debilitate possible errors in the identification of population structure and diversity with the CR, nearly the same set of samples as used in the CR analysis (see above) was used for microsatellite analysis. Microsatellites are suggested to have high resolution power on population level, especially for small scale studies, and are used as markers, giving a picture of the nuclear genome inheritance and distribution, including both parental lineages. The

combined information of both marker systems can shed light onto possible differences of resolution of time scales, mode of inheritance and evolution.

1.5. Synoptic Discussion

1.5.1. Species boundaries in species complexes of *Amphiprion*

Speciation and the underlying evolutionary processes were studied in two different species complexes of the anemonefish genus *Amphiprion*.

The phylogenetic analyses supported the morphological species classification and the close relatedness within the first species complex, containing *A. akallopisos, A. sandaracinos* and *A. perideraion*. The first speciation event in this complex took place with the separation of populations of their common ancestor in the Indian Ocean and the other side of the Indo-Malay Archipelago during sea level low stands in the time range around the Pliocene-Pleistocene border (1.1-4.8 MYA). The species *A. akallopisos* evolved in the Indian Ocean and the ancestor of the other two species must have gone through another allopatric or even sympatric speciation on the Pacific side of the Archipelago. An allopatric speciation might have been triggered by the decrease of sea level in the Pleistocene (0.5-1.5 MYA), the time when the speciation between *A. sandaracinos* and *A. perideraion* was dated by using the molecular clock. A separation of other sea areas within the Archipelago, like the Sulu Sea and the South China Sea, followed the decrease of sea level (Voris 2000), leading to vicariance. Although this allopatric speciation is most probable, a sympatric speciation could not be totally rejected, regarding the similar distribution pattern of *A. sandaracinos* and *A. perideraion*, with a larger distribution range in the latter species (Fautin & Allen 1994). The two species show different degrees of host specialisation, with *A. perideraion* being rather generalised using four different anemone species and *A. sandaracinos* being a specialist occurring only into two anemone species.

In the second species complex, including *A. ocellaris* and *A. percula*, we find a different picture, although the biology is similar for all anemonefish. The whole dataset was divided into five clades, where two showed levels of strong genetic differentiation, but still on population level, with divergence times dating back to the Pleistocene. Three clades showed divergences on species level, with separation times dating back to the Pliocene (1.4-7.2 MYA). During this era, multiple sea level changes due to unstable climatic conditions, generated disturbances that might have led to differentiation to species level. All clades found

in the haplotype networks could be correlated to distinct geographic regions in the archipelago suggesting that vicariance is the factor responsible for the observed divergences.

1.5.2. Population structure of *A. ocellaris*

Strong differentiations among regions could be observed in both used marker systems. CR detected four major groups. Among these major groups, eight sub-divisions were found reflecting a picture of separated ocean basins within the Indo-Malay Archipelago during sea level low stands in glacial epochs. The historical isolation of lineages was still apparent in the genetic structure of the species, which might be caused by the localised biology of the species. In addition, the archipelago itself exhibits complex current patterns, restricting exchange to populations in areas off the major currents, e.g. east coast of Sulawesi, but facilitates dispersal along the major current pathways (e.g. ITF). Lineage mixing was shown mainly in the central region of the archipelago, where the strongest currents of the region cross and interact (Wyrtki 1961).

The results of the microsatellite analyses supported strong population differentiation in some areas of the Indo-Malay Archipelago, although an increased lineage mixing was observed compared to the structure found in the CR analysis. The genetic separation of previously isolated regions (e.g. West coast of New Guinea and Misool) was already more alleviated than in the CR, indicating higher present-day connectivity to those regions. Obviously, traces of the separation in recent history during times of sea level low stands were more pronounced in the mitochondrial lineages than in the fast evolving nuclear microsatellite loci.

Although microsatellites are supposed to show best resolution power, a population structure on the small scale could not be detected, which might be due to low sample sizes. The inclusion of more highly polymorphic loci could enhance the resolution.

1.5.3. Diversification in the “Coral Triangle”

In the genus *Amphiprion,* recent speciation processes were discovered, to some part triggered by barriers within the Indo-Malay Archipelago. Within the subgenus *Phalerebus*, land barriers during sea level low stands led at least to the separation between *A. akallopisos* and the ancestor of *A. sandaracinos* and *A. perideraion* on the other side, but may have also led to a separate evolution of the latter two species. The earlier split among species/clades of the

Actinicola subgenus was also triggered by changing geographic conditions and probably current regimes during the dynamic Pliocene in the "Coral Triangle".

Regional diversification in the Indo-Malay Archipelago was shown for *A. ocellaris* on the population level. A strongly diverged clade evolved in the Indian Ocean but also those populations of temporarily isolated sea areas within the archipelago showed differentiated genetic lineages. This phenomenon was observed in other marine organisms, like sea horses of the genus Hippocampus (Lourie et al. 2005), the mantis shrimp *Haptosquilla pulchella* (Barber et al. 2002), the mushroom coral *Heliofungia actiniformes* (Knittweis et al. 2008), and the boring giant clam *Tridacna crocea* (Kochzius & Nuryanto 2008). In these species, the separation periods among populations were not long enough for the evolution of reproductive barriers. In *A. ocellaris* recent increasing lineage mixing prevented further speciation processes.

Differentiation on the species and intra-specific level within the "Coral Triangle" support the idea, that this area possesses conditions enhancing biodiversity, due to the dynamic geological history (Hall 1996) and oceanography (Kuhnt et al. 2004). Even today, the Indo-Malay Archipelago experiences unstable geologic conditions with high volcanic activity due to its location at the border of tectonic plates (UNEP 2005). Additionally, the complexity of the area with countless islands, peninsulas and bays, and the resulting complex current regimes would favour patterns of high diversity. The observation of diverged clades at adjacent areas underlines that developments at the periphery might even increase biodiversity in the "Coral Triangle".

1.5.4. Implications for conservation

In order to conserve biodiversity, an ambitious task given current species extinction rates, it must first be evaluated. Therefore, the definition of species boundaries, with consideration of cryptic diversity, and a following classification of "conservation units" should be enforced.

With the findings of more than one species boundary in the *A. ocellaris/A. percula* complex and different genetic lineages within the species *A. ocellaris*, these distinct genetic groups should be considered separate "conservation units", providing "genetic stocks" for biodiversity. Possible management plans should consider these as different resources.

The management for a sustainable use and conservation of *A. ocellaris*, targeted by the aquarium trade and species with similar biology should incorporate the information found by the genetic population structure analyses. The size and spacing of MPAs for example, need to

encounter requirements of exploited coral reef organisms (e.g. *A. ocellaris*). In *A. ocellaris*, higher connectivity was found among populations along major currents which contrasts areas remote of these major water pathways. Such differences must be accounted for when spacing MPAs. Longer distances can be tolerated when higher connectivity is measured but spacing should be closer and sizes of reserves large enough in remote areas as these populations depend largely on self-recruitment. Isolated populations are more endangered to over-exploitation as a result of shortage of external larval input. When these populations become diminished, effects like genetic drift and inbreeding depression can have further negative impacts on the populations health and recovery capability (Lande & Barrowclough 1987). A reduced gene pool might also lead to less adaptability to changing environmental conditions, e.g. climate change. Even in *A. ocellaris*, a species with a widespread distribution within the Indo-Malay Achipelago, one population probably recovering high fishing pressure during the last years, showed a pronounced reduction of genetic diversity.

The dispersal capability of the larvae per generation would be helpful information for the correct sizing of MPAs to ensure self-recruitment. Unfortunately, the analyses used in this study could not sufficiently resolve the dispersal capabilities of *A. ocellaris*. This question merits further investigation with advanced analytical tools and resolution power.

1.6. Conclusions

Different diversification patterns in the two studied species complexes within *Amphiprion* were identified by phylogenetic analyses despite their similar biology. Within the subgenus *Actinicola*, five clades were found, corresponding to certain geographical regions. Two strong genetic breaks indicate three instead of four deep evolutionary lineages. Within the subgenus *Phalerebus* three clades were found, not subdivided into geographical clades but following the morphological species classification.

Traces of former isolation of lineages were still present in *A. ocellaris* populations due to the species biology that prevent high dispersal between regions. More than two major genetic lineages were found indicating that populations were isolated in different ocean basins during times of sea level low stands.

Mitochondrial and microsatellite markers revealed a similar population structure in *A. ocellaris*, although these markers are of different origin, show a different mode of mutation, and mutation rate. Data can be verified through the comparison of different marker systems.

The lineage mixing in *A. ocellaris* was more pronounced in the microsatellite than in the mitochondrial control region analysis, indicating that the resolution of these markers might be focussed on different time ranges.
The diversification on species and intra-specific level found in this study support the "centre of origin" theory for high biodiversity found in the "Coral Triangle" but additional increases of diversity through accumulation and overlap is very probable.

1.7. Future perspectives

The grouping of sequences of *A. percula* from New Britain and the Solomon Islands indicates, that there might be a higher connectivity from New Britain to the South than to the strongly differentiated clade in Biak and the Tomini Bay. To support this indication, populations from Southeastern New Guinea and the Great Barrier Reef could be sampled and included into the analysis. Possibly, the clade status could be confirmed, revealing effectively three instead of two species in the *A. ocellaris/A. percula* complex. These analyses should be accompanied by other approaches, e.g. the detailed analysis of morphological characters. As well the molecular genetic analysis should be extended to include nuclear markers.
As we revealed inconsistencies in the formerly described distribution of A. ocellaris and A. percula, a comprehensive study on the geographical borders of the species distribution and possible hybrid zones would be interesting, especially considering the question if the different genetic lineages hybridise in nature, or would do so under unnatural conditions.
An advances small scale genetic population structure analysis of *A. ocellaris* in the Spermonde Archipelago would be necessary to entangle the precise dispersal limits of the species and possible source-sink relationships among areas. This information is important for conservation efforts and could not satisfyingly be obtained. For this approach, a larger number of polymorphic markers would be necessary as well as larger sample sizes.
One of the most interesting characters of the anemonefishes is probably the symbiotic relationship with its host, and the question if we can find similar genetic structures and patterns of dispersal in these anemone species. This topic may be addressed by analysing the host anemones of the anemonefish.

1.8. References

Allen GR, Werner TB (2002) Coral reef fish assessment in the "coral triangle" of southeastern Asia. *Environmental Biology of Fishes*, 65, 209-214.

Alvarado, B.J.R., Baker, A.J., Mejuto, J., 1995. Mitochondrial DNA control region sequences indicate extensive mixing of swordfish (*Xiphias gladius*) populations in the Atlantic Ocean. *Canadian Journal of Fisheries and Aquatatic Sciences*, 52, 1720-1732.

Barber PH, Palumbi SR, Erdmann MV, Moosa MK (2002) Sharp genetic breaks among populations of *Haptosqulla pulchella* (Stomatopoda) indicate limits to larval transport: patterns, causes and consequences. *Molecular Ecology*, 11, 659-674.

Briggs JC (2000) Centrifugal speciation and centres of origin. *Journal of Biogeography*, 27, 1183-1188.

Briggs JC (2005) The marine East Indies: diversity and speciation. *Journal of Biogeography*, 32, 1517-1522.

Barrière A, Félix M (2007) Temporal dynamics and linkage disequilibrium in natural Caenorhabditis elegans populations. *Genetics*, 176, 999-1011.

Bromham L, Penny D (2003) The modern molecular clock. *Nature*, 4, 216-224.

Campbell NA (1997) *Biologie*. Spektrum Akademischer Verlag GmbH, Heidelberg, Berlin, Oxford, pp. 1440.

Cantatore P, Roberti M, Pesole G, Ludovico A, Milella F, Gadaleta MN, Saccone C (1994) Evolutionary analysis of cytochrome b sequences in some Perciformes: Evidence for a slower rate of evolution than in mammals. *Journal of Molecular Evolution*, 39, 589-597.

Casey, SP., Hall, H.J., Stanley, H.F., Vincent, A.C.J., 2004. The origin and evolution of seahorses (genus *Hippocampus*): a phylogenetic study using the cytochrome *b* gene of mitochondrial DNA. Mol. Phylogenet. Evol. 30, 261-272.

Chenoweth SF, Hughes JM, Connolly RC (2002) Phylogeography of the pipefish, *Urocampus carinirostris*, suggests secondary intergradation of ancient lineages. *Marine Biology*, 141, 541-547.

Chiari Y, Vences M, Vieites DR, Rabemananjara F, Bora P, Ramilijaona O, Ravoahangimalala, Meyer A (2004) New evidence for parallel evolution of color patterns in Madagasy poison frogs (*Mantella*). *Molecular Ecology*, 13, 3763-3774.

Concheiro Pérez GA, Říčan O, Ortí G, Bermingham E, Doadrio I, Zardoya R (2007) Phylogeny and biogeography of 91 species of heroine cichlids (Teleostei: Cichlidae)

based on sequences of the cytochrome *b* gene. Molecular *Phylogenetics and Evolution*, 43, 91-110.

Convention on Biological Diversity (1992) http://www.cbd.int/convention/convention.shtml.

Ellegren H (2000) Heterogeneous mutation processes in human microsatellite DNA sequences. *Nature*, 24, 400-402.

Esposti MD, De Vries S, Crimi M, Ghelli A, Tomaso P, Meyer A (1993) Mitochondrial cytochrome b: evolution and structure of the protein. *Biochimica et Biophysica Acta (BBA)-Energetics*, 11, 243-271.

Excoffier L, Laval LG, Schneider S (2005) Arlequin vers. 3.1: an integrated software package for population genetic data analysis. *Evolutionary Bioinformatics online*, 1, 47-50.

Frankham R (1995) Conservation Genetics. *Annual Reviews of Genetics*, 29, 305-327.

Fautin DG, Allen GR (1994) *Anemonenfische und Ihre Wirte*. Tetra-Verlag, Melle, Germany.

Felsenstein J (1981) Evolutionary trees from DNA sequences: A maximum likelihood approach. *Journal of Molecular Evolution***,** 17(6), 368-376.

Felsenstein J (1983) Parsimony and systematics: biological and statistical issues. *Annual Reviews of Ecology and Systematics*, 14, 313-333.

Gaston KJ, Spicer JI (2004) Biodiversity: An introduction. Blackwell Publishing, pp. 208.

Godfrey JS (1996) The effect of the Indonesian Throughflow on ocean circulation and heat exchange with the atmosphere: a review. *Journal of Geophysics Research*, 101, 12217-12237.

Goloboff PA, Pol D (2005) Parsimony and Bayesian phylogenetics. In: Albert VA (ed) *Parsimony Phylogeny and Genomics*. Oxford University Press, Oxford, 148-159.

Gordon AL, Fine RA (1996) Pathways of water between the Pacific and Indian oceans in the Indonesian seas. *Nature*, 379, 146-149.

Hall R (1996) Reconstructing Cenozoic SE Asia. In: Hall R, Blundell D (eds) Tectonic Evolution of Southeast Asia. Geological Society of London Special Publication No. 106, pp. 153-184.

Hoeksema BW (2007) Delineation of the Indo-Malayan centre of maximum biodiversity: the coral triangle. In: *Biogeography, Time, and Place: Distributions, Barriers, and Islands* (ed. W Renema), pp. 117-178. Springer, The Netherlands.

Jokiel P, Martinelli FJ (1992) The vortex model of coral reef biogeography. *Journal of Biogeography*, 19, 449-458.

Jones AM, Gardner S, Sinclair W (2008) Losing „Nemo“: bleaching and collection appear to reduce inshore populations of anemonefishes. *Journal of Fish Biology*, 73, 753-761.

Knittweis L (2008) Population demographics and life history characteristics of Heliofungia actiniformis: A fungiid coral species exploited for the live coral aquarium trade in the Spermonde Archipelago, Indonesia. PhD Thesis, University of Bremen.

Knittweis L, Krämer WE, Timm J, Kochzius M (2008) Genetic structure of *Heliofungia actiniformis* (Scleractinia: Fungiidae) populations in the Indo-Malay Archipelago: implications for live coral trade management efforts. *Conservation Genetics*, doi: 10.1007/s10592-008-9566-5.

Kochzius M, Nuryanto A (2008) Strong genetic population structure in the boring giant clam *Tridacna crocea* across the Indo-Malay Archipelago: implications related to evolutionary processes and connectivity. *Molecular Ecology*, doi: 10.1111/j.1365-294X.2008.03803.x.

Kuhnt W, Holbourn A, Hall R, Zuvela M, Käse R (2004) Neogene history of the Indonesian Throughflow. *American Geophysical Union*, 1-22.

Kuriiwa K, Hanzawa N, Yoshino T, Kimura S, Nishida M (2007) Phylogenetic relationships and natural hybridization in rabbitfishes (Teleostei: Siganidae) inferres from mitochondrial and nuclear DNA analyses. *Molecular Phylogenetics and Evolution*, 5, 69-80.

Laan M, Pääbo S (1997) Demographic history and linkage disequilibrium in human populations. *Nature Genetics*, 17, 435-438.

Lande R, Barrowclough GF (1987) Effective population size, genetic variation, and their use in population management. In: Soulé ME (ed) *Viable populations for conservation*. Cambridge University Press, Cambridge, 87-124.

Larget B, Simon DL (1999) Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution*, 16, 750-759.

Lee WJ, Howell WH, Kocher TD (1995) Structure and evolution of teleost mitochondrial control regions. *Journal of Molecular Evolution*, 41, 54-66.

Levinson G, Gutman GA (1987) Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Molecular Biology and Evolution*, 4, 203-221.

Lewis PO (1998) A genetic algorithm for maximum-likelihood phylogeny inference using nucleotide sequence data. *Molecular Biology and Evolution*, 15, 277-283.

Lourie SA, Green DM, Vincent ACJ (2005) Dispersal, habitat differences, and comparative phylogeography of Southeast Asia seahorses (Syngnathidae: *Hippocampus*). *Molecular Ecology*, 14, 1073-1094.

Mayr E (2001) *What Evolution Is*. Basic Books, New York.

Messmer V, Van Herwerden L, Munday PL, Jones GP (2005) Phylogeography of colour polymorphism in the coral reef fish *Pseudochromis fuscus*, from Papua New Guinea and the Great Barrier Reef. *Coral Reefs*, 24, 392-402.

Molloy PP, Goodwin NB, Côte IM, Gage MJG,Reynolds JD (2007) Predicting effects of exploitation on male-first sex-changig fish. *Animal Conservation*, 10, 30-38.

Nelson JS, Hoddel RJ, Chou LM, Chan WK, Phang VPE (2000). Phylogeographic structure of false clownfish, Amphiprion ocellaris, explained by sea level changes on the Sunda shelf. *Marine Biology*, 137, 722-736.

Posada D, Crandall K (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics,* 14, 817-818.

Posada D, Crandall KA (2001) Intraspecific genealogies: trees grafting into networks. *Trends in Ecology and Evolution*, 16, 37-45.

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

QFMA (1999) Discussion paper 10. Queensland marine fish and coral collection fisheries. Prepared for the Queensland Fisheries Management Authority and Harvest MAC by the Aquarium Fish and Coral Fisheries Working Group. Queensland.

Randall JE (1987) *Collecting reef fishes for aquaria*. In: Salvat B (ed) Antenne Museum E.P.H.E., French Polynesia, 29-39.

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylognetic trees. *Molecular Biology and Evolution*, 4, 406-425.

Santini S & Polacco G (2006). Finding Nemo: Molecular phylogeny and evolution of the unususal life style of anemonefish. *Gene* 385, 19-27.

Shuman CS, Hodgson G, Ambrose AF (2005) Populations impacts of collecting sea anemones and anemonefish for the marine aquarium trade in the Philippines. *Coral Reefs*, 24, 564-573.

Soegiarto A, Polunin NVC (1982) The marine environment of Indonesia. Report for the government of Indonesia under Sponsorship of IUCN and WWF.

Sokal RR, Rohlf FJ (1969) *Biometry: The principles and pratice of statistics in biological research.* W.H. Freeman, San Francisco, pp. 776.

Storch V, Welsch U, Wink M (2001) Evolutionsbiologie. Springer-Verlag, Berlin, Heidelberg, pp. 449.

Susanto RD, Gordon AL (2005) Velocity and transport of the Makassar Strait Throughflow. *Journal of Geophysical Research*, 110, C01005, doi:10.1029/2004JC002425.

Swofford DL (1998). PAUP*. Phylogenetic Analysissing Parsimony (*and Other Methods). Sunderland, MA: Sinauer.

Tautz D (1989) Hypervariabflity of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research*, 17, 6463-6471.

UNEP (2005) Vantier L, Wilkinson C, Lawrence D, Souter D (eds.) Indonesian Seas GIWA Regional assessment 57. University of Kalmar, Kalmar, Sweden.

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.

Voris HK (2000) Maps of Pleistocene sea levels in Southeast Asia: shorelines, river systems and durations. *Journal of Biogeography*, 27, 1153-1167.

Wabnitz C, Taylor M, Green E, Razak T (2003) *From Ocean to Aquarium*. UNEP-WCMC, Cambridge, UK.

Wägele J (2000) *Grundlagen der Phylogenetischen Systematik*. Verlag Dr. Friedrich Pfeil, München, pp. 315.

Weber JL, Wong C (1993) Mutation of short tandem repeats. *Human Molecular Genetics*, 2, 1123-1128.

Wood E (2001) *Collection of Coral Reef Fish for aquaria: Global Trade, Conservation Issues and Management Strategies.* Marine Conservation Society, Ross-on-Wye,UK.

Woodland DJ (1983) Zoogeography of the Siganidae (Pisces): an interpretation of distribution and richness patterns. *Bulletin of Marine Sciences*, 33, 713-717.

Wyrtki K (1961) *Physical Oceanography of the Southeast Asian Waters*. NAGA Report Vol. 2, The University of California, Scripps Institution of Oceanography, La Jolla, California.

Zavattari P, Deidda E, Whalen M, Lampis R, Mulargia A, Loddo M, Eaves L, Mastio G, Todd, JA, Cucca F (2000) Major factors influencing linkage disequilibrium by analysis of different chromosome regions in distinct populations: demography, chromosome recombination frequency and selection. *Human Molecular Genetics*, 9, 2947-2957.

Zhu D, Jamieson BGM, Hugall A, Moritz C (1994) Sequence evolution and phylogenetic signal in control-region and cytochrome b sequences of rainbow fishes (Melanotaeniidae). *Molecular Biology and Evolution*, 11(4), 672-683.

Contrasting patterns in species boundaries and evolution of anemonefishes (Amphiprioninae, Pomacentridae) in the centre of marine biodiversity

2. Contrasting patterns in species boundaries and evolution of anemonefishes (Amphiprioninae, Pomacentridae) in the centre of marine biodiversity

Molecular Phylogenetics and Evolution (2008) doi: 10.1016/j.ympev.2008.04.024

Janne Timm*, Malgorzata Figiel, Marc Kochzius

Biotechnology and Molecular Genetics, FB2-UFT, University of Bremen, Leobener Strasse UFT, 28359 Bremen, Germany

* Corresponding author.
E-mail addresses: janne.timm@uni-bremen.de, janneket@gmx.de (J. Timm).

2.1. Abstract

Many species of coral reef fishes are distinguished by their colour patterns, but genetic studies have shown these are not always good predictors of genetic isolation and species boundaries. The genus *Amphiprion* comprises several species that have very similar colouration. Additionally, morphological characters are so variable, that sibling species can show a considerable overlap, making it difficult to differentiate them. In this study, we investigated the species boundaries between the sibling species pair *A. ocellaris* and *A. percula* (Subgenus *Actinicola*) and three closely related species of the subgenus *Phalerebus* (*A. akallopisos*, *A. perideraion*, *A. sandaracinos*) by phylogenetic analysis of mitochondrial cytochrome *b* and control region sequences. These two subgenera show strong differences in their patterns of species boundaries. Within the *A. ocellaris/A. percula* complex, five clades were found representing different geographic regions. Two major divergences both with genetic distances of 4-7 % in cty *b* and 17-19 % in the d-loop region indicate the presence of three instead of two deep evolutionary lineages. The species of the subgenus *Phalerebus* show three monophyletic clades, independent of the geographical location of origin, but concordant to the morphological species classification. The genetic distances between the *Phalerebus* species were 2-5 % in cty *b* and 10-12 % in the control region.

Keywords: *Amphiprion*, molecular phylogeny, phylogeography, Southeast Asia, clownfishes

2.2. Introduction

The Indo-Malay Archipelago, also called the "Coral Triangle", contains the world's richest marine shallow water biodiversity (Briggs, 2005; Allen and Werner, 2002) and is therefore a well-suited region to study divergence and speciation processes. If the high diversity in this area is caused by an overlap of the Pacific and Indian Ocean faunas (Woodland, 1983), or the result of an accumulation of species that evolved at the periphery (Jokiel and Martinelli, 1992), or if it is actually the „center of origin" where species evolve (Briggs, 2000, 2005) is discussed controversially.

In the genus *Amphiprion*, the latter theory was supported by Santini and Polacco (2006), who found the „center of origin" in an area reaching from the Philippines to the Great Barrier Reef and from Sumatra to Melanesia, which does not exclude speciation in peripheral remote areas (e.g. *A. tricinctus*, Marshall Islands endemic). Furthermore, a rather recent radiation was indicated in the Indian Ocean, because derived and endemic species are dominant (Santini and Polacco, 2006).

In order to evaluate biodiversity correctly it is important to clarify species boundaries, integrities, and phylogenetic relationships (Frankham et al., 2002). Many species of coral reef fishes are distinguished by their colour patterns, but genetic studies have shown that these are not always sufficient indicators of genetic isolation and species boundaries (Bernardi et al., 2002). The genus *Amphiprion* comprises several species with very similar colouration. Additionally, morphological characters are so variable, that sibling species can show a considerable overlap, making it difficult to differentiate them.

There are closely related species that only show slight differences in their colour pattern in the subgenus *Phalerebus* (Allen, 1991), such as *A. akallopisos* and *A. sandaracinos*. *A. akallopisos* has a white caudal fin and an orange to pinkish body colour, whereas *A. sandaracinos* has an orange caudal fin and its body colour is usually clearly orange. The white stripe on the back is supposed to be slightly longer in *A. sandaracinos*, spanning from the upper lip to the caudal peduncle, whereas in *A. akallopisos* it begins more on the forehead. However, this character seems to vary especially in the latter species. Regarding our observations, the white stripe often started also at the upper lip in *A. akallopisos*, so it is an overlapping and therefore rather weak character for distinguishing these two species. A more stable character separating the two species is the differently shaped teeth, indicating slightly

different ecological adaptations (Fautin and Allen, 1994). *A. perideraion* has a similar body colour like *A. akallopisos*, but shows an additional white stripe between head and trunk. *A. perideraion* and *A. sandaracinos* show a sympatric distribution with the latter having a more restricted range and higher host specificity, accepting only two anemone species (*Heteractis crispa* and *Stichodactyla mertensii*) instead of four (*Heteractis crispa*, *H. magnifica*, *Stichodactyla gigantea*, and *Macrodactyla doreensis*) in *A. perideraion* (Fautin and Allen, 1994). *A. akallopisos* has a parapatric distribution with the former two species, overlapping around the upper Sunda Islands and is also associated with only two anemone species (*Heteractis magnifica* and *Stichodactyla mertensii*; Fautin and Allen, 1994; Fig. 2a).

The sibling species *A. ocellaris* and *A. percula* show more or less the same colour pattern, although *A. percula* is described to have larger black bands in its colouration (Fautin and Allen, 1994), which could not be confirmed by our observations. There is rather a large variation: some specimens showing no black bands in their colouration while others do. Morphologically, these two species are differentiated by the number of spines in the dorsal fin, but also this character is overlapping: *A. ocellaris* has 10–11 and *A. percula* 9–10 spines. The ecological requirements of both species seem to be identical; both of them prefer the same host anemone species (*Heteractis magnifica* and *Stichodactyla gigantea*). Regarding Fautin and Allen (1994) these siblings have an allopatric distribution (Fig. 2b), but Kuiter and Tonozuka (2004) reported both species in the Tomini Bay (Sulawesi), which indicates a parapatric distribution.

The high morphological similarity of the above mentioned species raises the question whether these species form distinct genetic clades within the subgenera. Additionally, their similar biology could lead to similar species boundaries patterns. This study aims to reveal (1) species boundaries within the anemonefish genus *Amphiprion* in the Indo-Malay Archipelago, and (2) speciation processes in the hotspot of marine shallow water biodiversity.

2.3. Materials and methods

2.3.1. Sampling

A total of 86 tissue samples of five coral reef associated fish species of the genus *Amphiprion* were collected at different locations in the Indo-Malay Archipelago (Table 1 and Fig. 2). The fishes were caught with two aquarium nets. A fin clip from the caudal fin was taken and the

fishes were released into their host anemones. It was therefore possible to obtain tissue samples without killing the animals. The samples were stored in 96% ethanol.

Table 1: Number of sampled individuals from 5 species of the genus *Amphiprion*, abbreviations used (Abbr.), accession numbers (EMBL), and the corresponding sample locations in the Indo-Malay Archipelago

Species	Location	Abbr.	No. CR	Accession no. CR	No. cyt *b*	Accession no. cyt *b*
A. ocellaris	Spermonde, Sulawesi	AoSp	5	AM747125-AM747129 AM941135-AM941137	3	AM942669-AM942671
	Banggi Islands, North Borneo	AoBI	5	AM747144-AM747148	-	
	Kota Kinabalu, Northwest Borneo	AoKK	6	AM941144-AM941149	6	AM942678-AM942683
	Sangalaki, Northeast Borneo	AoSa	6	AM941138-AM941143	6	AM942672-AM942677
	Padang, Sumatra	AoPa	6	AM747139-AM747143 AM941150-AM941152	6	AM942684-AM942689
A. percula	Tomini Bay, Sulawesi	AperTI	9	AM747130-AM747138 AM941155-AM941156	9	AM942690-AM942695
	Biak, New Guinea	AperBk	10	AM747149-AM747157 AM941153-AM941154	6	AM942696-AM942701
	New Britain, New Guinea	AperNB	4	AM745732-AM745734	4	AM942702-AM942705
	Solomon Islands	AperSolo	1	DQ343939	1	DQ343958
A. akallopisos	Padang, Sumatra	AaPa	5	AM747158-AM747162	2	AM942706-AM942707
	Karimun Jawa, Jawa Sea	AaKa	5	AM747163-AM747167	3	AM942708-AM942710
A. perideraion	Manado, Sulawesi	ApMa	5	AM747168-AM747171	3	AM942711-AM942713
	Karimun Jawa, Jawa Sea	ApKa	5	AM747172-AM747174	3	AM942714-AM942716
A. sandaracinos	Manado, Sulawesi	AsMa	5	AM747175-AM747178	3	AM942717-AM942719
	Kupang, Timor	AsKu	5	AM747179-AM747183	2	AM942720-AM942721

The sequence from the Solomon Islands was obtained from Genebank. CR, control region; cyt *b*, cytochrome *b*.

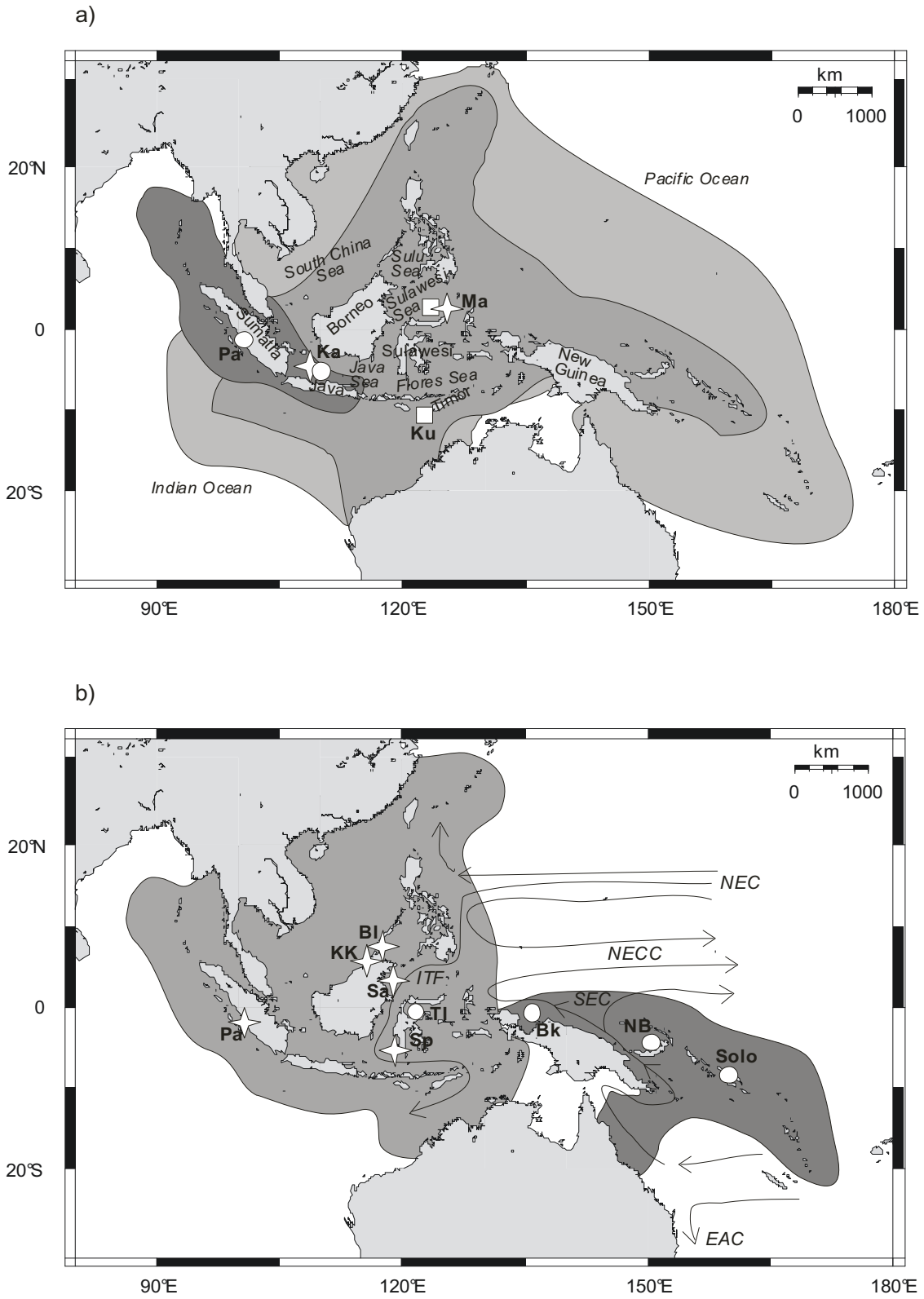


Fig. 2: (a) Distribution patterns and sample sites of the species *Amphiprion akallopisos* (dark grey, circles), *A. perideraion* (grey, stars) and *A. sandaracinos* (light grey, diamonds) in the Indo-Malay Archipelago (Fautin and Allen 1994). Names of the main ocean basins and islands are added. (b) Distribution patterns and sample sites of the species *Amphiprion ocellaris* (grey, stars) and *A. percula* (dark grey, circles) in the Indo-Malay Archipelago (Fautin and Allen 1994). Dominant currents are added (simplified after Godfrey 1996).

Thirty-two samples of *A. ocellaris* were obtained from five different locations over a range of 1,500 km. The sibling species *A. percula* was collected in Biak (New Guinea; 10 samples) and from New Britain (New Guinea; 4 samples). An additional number of 9 tissue samples have been collected from depths between 1 and 25 m from the Togian Islands (Tomini Bay, Sulawesi). Since *A. ocellaris* is distributed across the Indo-Malay Archipelago (Fautin and Allen, 1994), with its eastern border of distribution around the Molucca Islands and the most western tip of New Guinea, the samples from the Togian Islands have been classified as *A. ocellaris*. One sequence of *A. percula* from Solomon Islands was added for each marker from GenBank (Table 1).

Within the subgenus *Phalerebus*, 10 specimens from two locations in the Indo-Malay Archipelago were analysed for each of the species *A. akallopisos*, *A. sandaracinos*, and *A. perideraion*.

As outgroup for the phylogenetic analyses *Chromis viridis* was used, which is a member of the same family (Pomacentridae).

2.3.2. DNA extraction and amplification

Genomic DNA was extracted with filter column based extraction kits from Qiagen and Macherey-Nagel, following the manufacturers' protocols.

A fragment with a maximum length of 420 bp of the mitochondrial control region (CR) was amplified by PCR with the primers CR-A (TTC CAC CTC TAA CTC CCA AAG CTA G) and CR-E (CCT GAA GTA GGA ACC AGA TG) (Lee et al., 1995). PCR was performed in a Perkin Elmer and Eppendorf Ep S Mastercycler with the following thermo-profile: 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s. The Terminal elongation was at 72 °C for 2 min. 25 µl reactions contained 2.5 µl 10x PCR buffer, 0.075 µmol Mg^{2+}, 0.25 µmol dNTP mix, 10 pmol of each primer and 0.5 U Taq polymerase. Between 10 and 30 ng genomic DNA was used of each sample as template.

For a subset of samples of each species cytochrome *b* (cyt *b*) sequences were obtained (Table 1). This fragment of the mitochondrial genome is suitable for resolving phylogenetic patterns on intraspecific (Nelson et al., 2000) to intrageneric level (Kocher et al., 1989).

For the amplification of the cyt *b* fragment of around 400 bp length, the primers tRNAgluF (AAA ACC ACC GTT GTT ATT CAA CTA CA; Nelson et al., 2000) and H15149 (AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A; Kocher et al., 1989) were used.

PCR was performed in Eppendorf Ep Mastercyclers in 25 µl reaction mix, containing 2.5 µl 10x PCR buffer, 0.0625 µmol Mg^{2+}, 0.25 µmol dNTP mix, 10 pmol of each primer and 0.5 U Taq polymerase. Again 10-30 ng genomic DNA was used of each sample. The thermo-profile was 95 °C for 5 min, 35 cycles of 95 °C for 45 s, 58 °C for 45 s and 72 °C for 60 s. The final elongation was 72 °C for 5 min.

All PCR products were purified with the QIA-quick PCR Purification Kit (Qiagen). Sequencing of both strands was conducted with the PCR primers using the Big Dye Terminator Cycle Sequencing Kit (ver. 1.3 and ver. 3.1; Applied Bioscience) according to the manufacturer's recommendations and an ABI 310 and 3100 automated sequencer.

2.3.3. Phylogenetic analyses

Both strands were assembled and edited with help of the programme Seqman (ver. 4.05, DNASTAR). Multiple sequence alignment was done using Clustal W (Thompson et al., 1994) as implemented in the software Bioedit (ver. 7.0.0.1, Hall 1999).

The phylogenetic relationships of species were analysed with all sequences available for cyt *b* and a subset of sequences for the CR, adjusted to the cyt *b* dataset, by using maximum parsimony (MP), maximum likelihood (ML) and neighbour joining (NJ) algorithms with the software Paup* (ver. 4.0b10; Swofford 1998). The statistical confidences were evaluated by 1000 non-parametric bootstrap replicates for NJ and MP analyses and by 100 for ML analysis. In order to test if the molecular clock will be rejected, the ML analysis was done with and without molecular clock enforced. The Shimodaira–Hasegawa (Goldman et al. 2000; Shimodaira & Hasegawa 1999) and Kishino–Hasegawa (Kishino & Hasegawa 1989) tests were used to verify if the topologies of the two ML trees are significantly different.

Haplotype networks were drawn including the CR sequences of all samples and cyt *b* sequences from a subset of samples, based on the results obtained from Arlequin (Excoffier et al. 2005).

The programme Modeltest (ver. 3.06; Posada & Crandall 1998) was used to determine the best-fit model of DNA evolution for the two datasets.

Sequence divergences between individuals were calculated with Paup* (Swofford, 1998) and the average within and between each group was given, as well as the genetic distances after correcting for within population diversity ($d_A = d_{XY} - (\pi_X + \pi_Y)/2$, Nei 1987 as cited in Camptonet al. 2000).The corrected genetic distances were further used for comparison and molecular divergence time calculations, whereat the latter were done based on a mutation rate

of 6.41 % per million years for the CR, estimated in a study on the phylogeography of the coral reef fish *Pseudochromis fuscus* (Messmer et al. 2005). This species belongs to the same order and the same CR fragment was used, including a conserved and a hypervariable region (Alvarado et al. 1995). For cyt *b*, mutation rates of 1.0–2.8 % per million years were utilised, as assumed for different fish species (Ortí et al. 1994; Martin and Bermingham 1998; Perdices et al. 2002; Chenoweth et al. 2002; Banford et al. 2004; Casey et al. 2004).

2.4. Results

2.4.1. Phylogenetic trees

An alignment of 371 base pairs of the CR fragment, containing 55 sequences from five species, was obtained. The alignment included several gaps and the Ts/Tv ratio was 1.77. The best-fit model of evolution for the present dataset was the General Time Reversible model (GTR; Tavaré 1986) with a proportion of invariable sites of 0.17, and a gamma distribution shape parameter of 1.34.

The 55 sequences of the cyt *b* fragment resulted in an alignment of 357 base pair length. The sequences represent the same species as the CR sequences. The best-fit model of evolution for the cyt *b* dataset was the Total Variation Metric model (TVM; Pond 2007), with a proportion of invariable sites of 0.67. The latter model is a modified GTR model with equal substitution rates for A-G and C-T (Paraskeris et al. 2004). In the cyt *b* dataset the Ts/Tv ratio was 2.24. Of the observed 93 substitutions, 94 % were at third codon positions, not changing the aminoacid sequence of the fragment, whereas 4 % were at first and 2 % at second codon positions.

These evolutionary models were used for the NJ and ML analyses. The phylogenetic analysis of the CR dataset is presented as a NJ cladogram with bootstrap values of NJ, MP and ML analysis (Fig. 3a), showing a grouping of all species in two main clades, both of them supported by high bootstrap values.

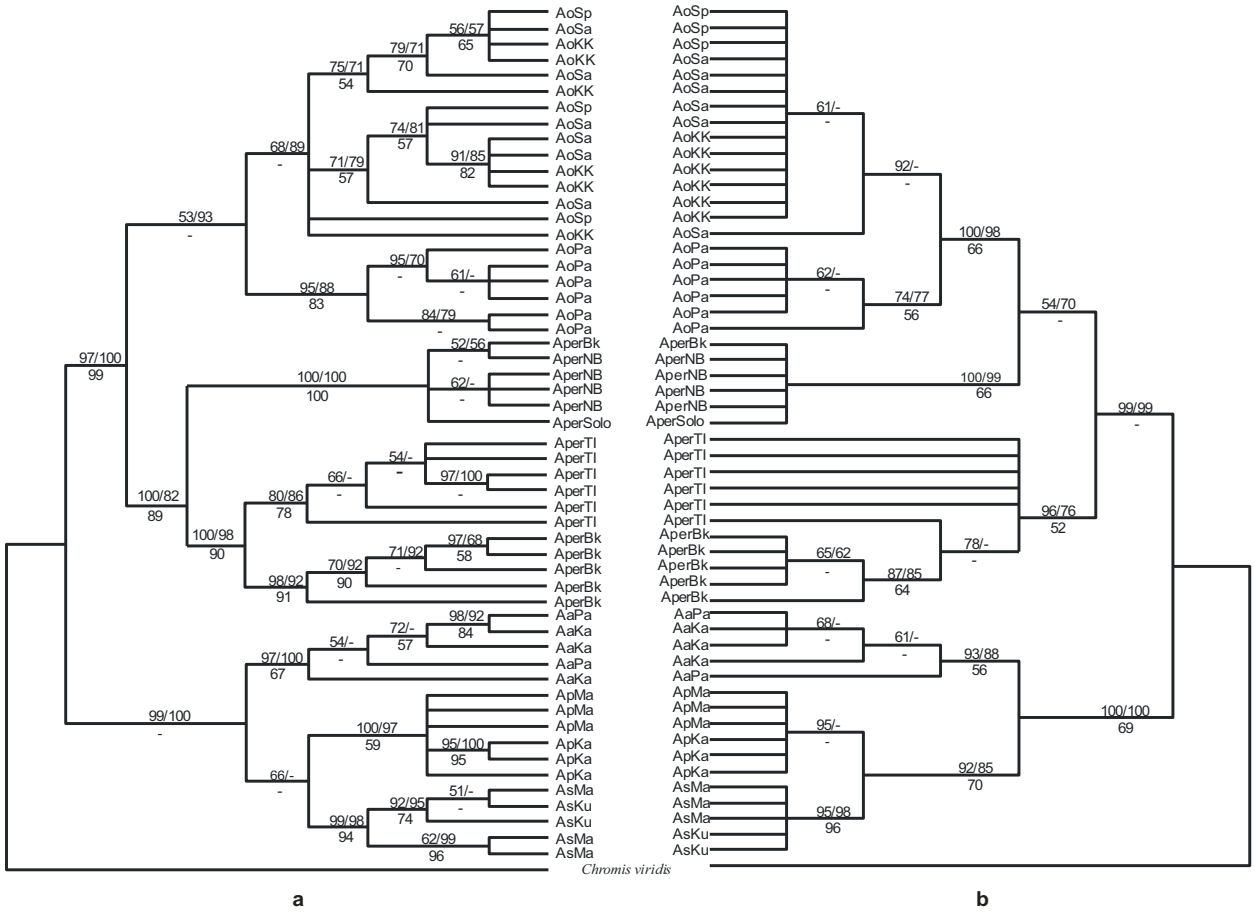


Fig. 3: Neighbour-Joining trees of CR (a) and cyt b (b) sequences of anemonefish species from the genus *Amphiprion* in the Indo-Malay Archipelago. Bootstrap values above branches are based on 1,000 replicates for the NJ/MP analyses and below branches on 100 replicates for the ML analysis. For abbr. see Table 1.

One clade contained the species *A. sandaracinos*, *A. akallopisos*, and *A. perideraion*, the other *A. ocellaris* and *A. percula*. The species of the subgenus *Phalerebus* (Allen 1991) form monophyletic subclades in all analyses, well supported by bootstrap values between 97 % and 100 % for the MP and NJ analysis. The ML analysis gave weaker bootstrap support for many branches. With a low bootstrap support of 66 % only in the NJ analysis *A. sandaracinos* and *A. perideraion* formed sister clades, and *A. akallopisos* was basal to them (Fig. 3a).

The species *A. ocellaris* and *A. percula* formed one well-supported monophyletic clade, in all analyses, with a clear subdivision into an *A. ocellaris* and an *A. percula* sub-clade. The specimens from Togian Islands (Tomini Bay) were found within the *A. percula* clade. This clade showed a subdivision into three branches, corresponding to the geographical regions of Tomini Bay, Biak, and New Britain. The sequence from the Solomon Islands was included into the New Britain group. One sample from Biak was as well clearly found within the New Britain subdivision. A phylogeographic structure regarding the population from Padang (Indian Ocean) was found in *A. ocellaris* (Fig. 3a).

The phylogenetic analyses done for the cyt *b* dataset is represented by a NJ tree as well. In this case, the grouping of the *A. ocellaris* and *A. percula* clades was not resolved in the ML analysis. All algorithms showed the same major groupings as the CR dataset, but in the NJ and MP analyses the branch of *A. percula* from New Britain and the Solomon Islands was associated to *A. ocellaris* instead of *A. percula* from Tomini Bay and Biak (Fig. 3b). This inconsistency was not well supported (Bootstrap values of 54 for NJ and 70 for MP) and not shown in the ML analysis, which did not resolve the relationships among the *A. ocellaris/A. percula* species complex. In the NJ tree one sequence of *A. percula* from Tomini Bay was grouped basal to the Biak clade, but this was not supported by the other algorithms and the other *A. percula* sequences from Tomini Bay were not resolved at all. The same sample of *A. percula* from Biak which appeared in the *A. percula* group from New Britain in the CR dataset was found in the New Britain clade in the cyt *b* dataset as well.

2.4.2. Haplotype networks

The parsimonious haplotype networks of the *A. akallopisos/perideraion/sandaracinos* complex revealed three clearly separated clades for both markers (Fig. 4). These clades were concordant to the morphologically defined species and no geographical pattern could be observed. For the CR network the numbers of mutational steps in the *A. akallopisos* clade were 6-13, and in the *A. perideraion* clade 1-21. Two sub-clades, separated by 19 mutations, were present in *A. sandaracinos*. Within these sub-clades the variation was between one and four steps. The mutational steps between species pairs were 59 (*A. akallopisos* - *A. perideraion*) and 53 (*A. perideraion* - *A. sandaracinos*).
In the cyt *b* network the number of mutational steps between *A. akallopisos* and *A. perideraion* was 10 and between *A. perideraion* and *A. sandaracinos* 5. Within the species clades, there was one dominant haplotype in each, and one or two other haplotypes separated by only one or two mutational steps.

The haplotype network of the *A. ocellaris/percula* species complex based on the CR dataset showed that *A. ocellaris* is separated from *A. percula* collected in Tomini Bay by 80 substitutions (Fig. 5a). The genetic break between *A. ocellaris* individuals from Padang and its conspecifics from other sample sites was determined by 31 substitutions. Two to 25 mutational steps separated the haplotypes within each clade of this species. The populations of *A. percula* in Tomini Bay and Biak were divided by 41 steps. The sequences of *A. percula*

from New Britain were strongly separated to those from Biak by 82 substitutions. Within each population of *A. percula* we found 2-26 mutational steps. Especially among haplotypes from Tomini Bay a high variability could be observed.

The cyt *b* network (Fig. 5b) showed the same division into five clades corresponding to geographical regions. The population of Padang was separated from the other *A. ocellaris* populations by three steps. The same separation was revealed between *A. percula* from Tomini Bay and Biak. The mutational steps between *A. ocellaris* and *A. percula* from Tomini Bay was with 15 noticeable higher. In this dataset, the clade of New Britain and Solomon Islands was connected to Tomini Bay, separated by 13 mutations, instead of Biak, as it was shown in the CR dataset. In both networks, one sequence, sampled in Biak, was included into the New Britain clade, as shown in the trees.

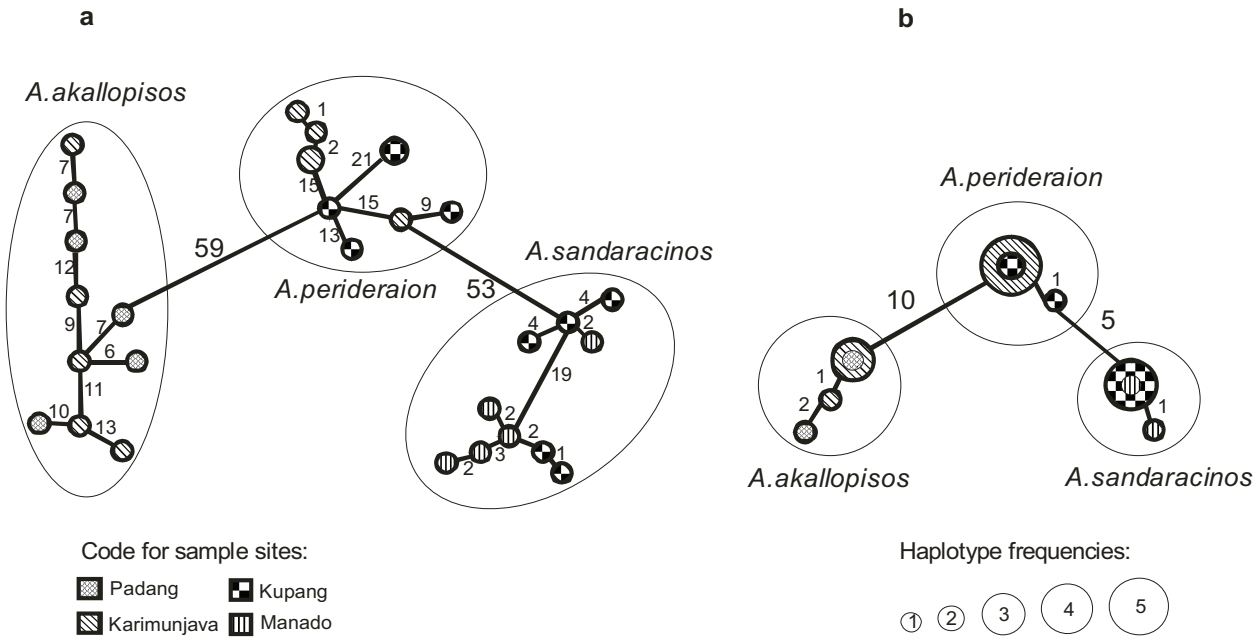


Fig. 4: Haplotype networks of (a) CR and (b) cyt b sequences of *Amphiprion akallopisos*, *A. perideraion* and *A. sandaracinos* from different locations in the Indo-Malay Archipelago. Pattern fillings of circles correspond to the different sample locations and numbers indicate mutational steps. The size of the circles indicate the haplotype frequencies.

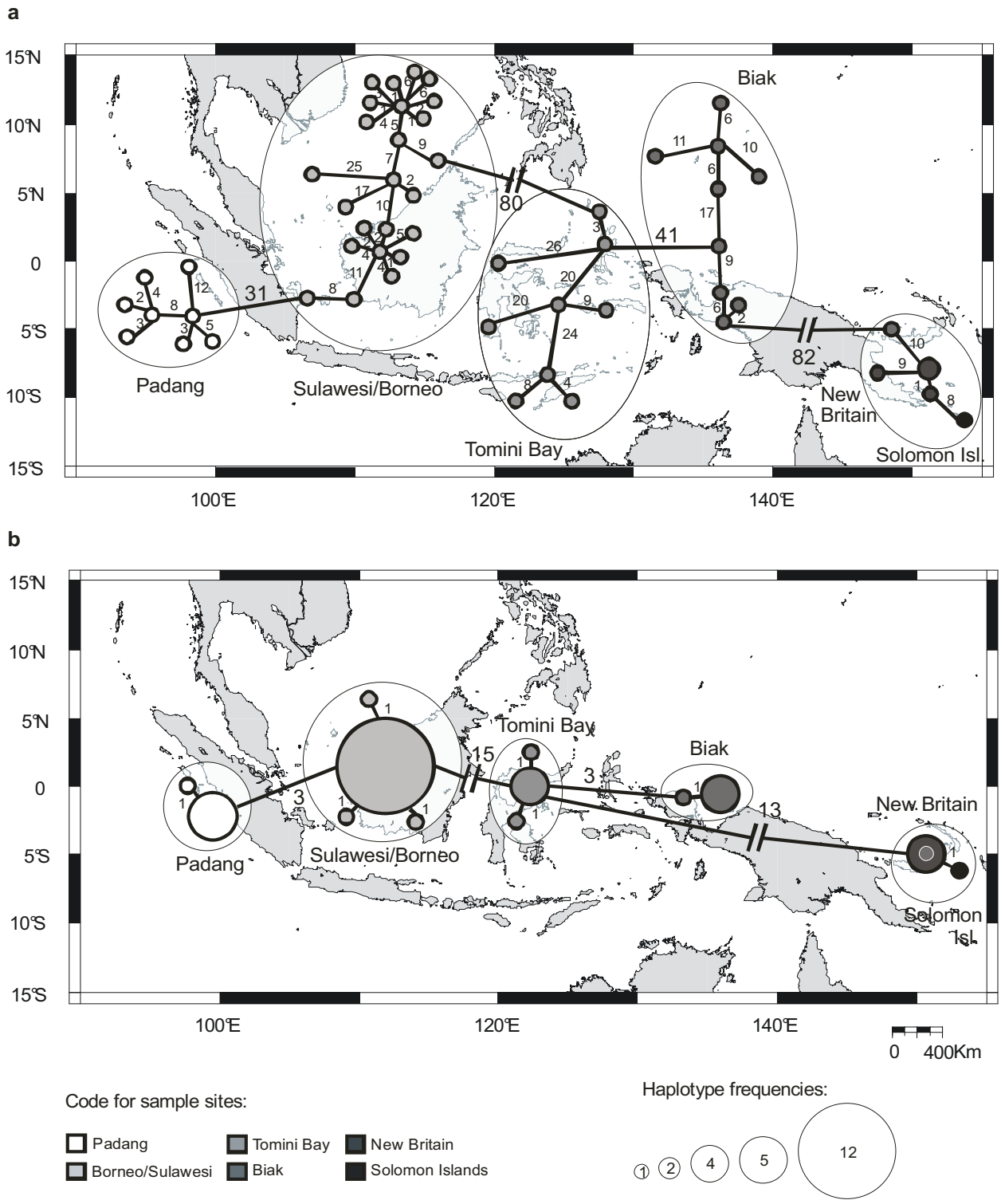


Fig. 5: Haplotype networks of (a) CR and (b) cyt b sequences of *A. ocellaris* and *A. percula* from different locations in the Indo-Malay Archipelago. Numbers indicate mutational steps. The size of the circles indicate the haplotype frequencies.

2.4.3. Sequence divergence

The sequence divergences for both markers within and among clades, as well as the genetic distances between clades after accounting for the diversity within them are shown in Table 2. The genetic distances of 6.6 % in CR and 1 % in cyt *b* sequences between *A. percula* from Tomini Bay and Biak was within the same range as the distances between *A. ocellaris* from Padang and other populations of this species (5.8 % and 0.9 %, respectively). Most of these values are between the maximum value within clades of species (*A. percula*; Togian Islands = 6 % in CR and 0.4 % in cyt *b*) and the minimum value among species (*A. perideraion-A. sandaracinos*, CR = 10.1 % and cyt *b* = 1.5 %) observed in the datasets. The genetic distances between *A. percula* from Tomini Bay/Biak and New Britain/Solomon Islands were 16.7–18 % in CR and 3.8–5 % in cyt *b* sequences. In comparison, the genetic differences in the *A. akallopisos/A. sandaracinos/A. perideraion* complex did not exceed 12.3 % in CR and 4.8 % in cyt *b* (Table 2).

2.4.4. Molecular clock

The Shimodaira–Hasegawa and Kishino–Hasegawa tests, conducted to compare the ML trees reconstructed with and without the molecular clock enforced, showed no significant difference for both markers (CR: $p = 0.288$ and $p = 0.715$, respectively; cyt *b*: $p = 0.238$ and $p = 0.847$, respectively). Therefore, the molecular clock was not rejected and the separation among the species' ancestors could be estimated. With the used mutation rate of 6.41 % per million years for the CR, the genetic distance of 11.1 % could be translated to approx. 1.7 million years divergence time between *A. akallopisos* and *A. perideraion*. This time frame fits into the divergence range assumed for the cyt *b* fragment (1.1–3.1 MYA), comprising the geological border between Pliocene and Pleistocene (Table 3). The genetic distance of 12.3 % between *A. akallopisos* and *A. sandaracinos* of the CR was translated to 1.9 million years and therefore revealed a slightly longer separation time between these species. The divergence range calculated for the cyt *b* fragment dated the split between *A. akallopisos* and *A. sandaracinos* as well into the Pleistocene-Pliocene border (1.7-4.8 MYA). *A. sandaracinos* and *A. perideraion* revealed, with 1.6 million years for the CR and a range of 0.5-1.5 million years for cyt *b*, a more recent split in the Pleistocene (Table 3).

Table 2: Sequence divergences (calculated with Paup*) of (a) CR (considering the GTR model) and (b) cyt *b* sequences (considering the TVM model) between (below diagonal), within taxa (diagonal), and genetic distances between taxa after accounting for diversities within them (bold, above diagonal), of the genus *Amphiprion* (Ao = pooled sequences of *A. ocellaris* from Borneo and Sulawesi, Aa = all sequences of *A. akallopisos*, Ap = all sequences of *A. perideraion*, As = all sequences of *A. sandaracinos*) from different locations in the Indo-Malay Archipelago (abbr. see Table 1)

	Ao	AoPa	AperTI	Aper Bk	Aper NB	Aper Solo	Aa	Ap	As
(a)									
Ao	0.043	0.058	0.174	0.185	0.169	0.184	0.197	0.209	0.203
AoPa	0.094	0.030	0.173	0.183	0.166	0.184	0.210	0.225	0.215
ArTI	0.225	0.218	0.060	0.066	0.167	0.179	0.238	0.248	0.224
ArBk	0.228	0.219	0.118	0.043	0.169	0.180	0.240	0.248	0.224
ArNB	0.198	0.189	0.205	0.198	0.016	0.020	0.251	0.261	0.248
ArSolo	0.205	0.199	0.209	0.201	0.028	0.000	0.258	0.252	0.264
Aa	0.233	0.240	0.283	0.276	0.274	0.273	0.030	0.111	0.123
Ap	0.251	0.261	0.299	0.290	0.290	0.273	0.147	0.042	0.101
As	0.236	0.241	0.265	0.257	0.267	0.275	0.149	0.133	0.023
(b)									
Ao	0.001	0.009	0.050	0.062	0.048	0.052	0.272	0.588	0.325
AoPa	0.010	0.001	0.060	0.072	0.058	0.062	0.242	0.343	0.285
ArTI	0.052	0.062	0.004	0.010	0.038	0.042	0.212	0.232	0.214
ArBk	0.063	0.073	0.012	0.001	0.046	0.050	0.237	0.252	0.231
ArNB	0.048	0.058	0.040	0.046	0.000	0.003	0.217	0.225	0.210
ArSolo	0.052	0.062	0.044	0.050	0.003	0.000	0.225	0.231	0.216
Aa	0.274	0.244	0.216	0.239	0.219	0.227	0.004	0.031	0.048
Ap	0.588	0.343	0.234	0.252	0.225	0.231	0.033	0.000	0.015
As	0.325	0.285	0.216	0.231	0.210	0.216	0.050	0.015	0.000

Table 3: Divergence times between clades of the genus *Amphiprion*
(Ao, pooled sequences of *A. ocellaris* from Borneo and Sulawesi; Aa, all sequences of *A. akallopisos*; Ap, all sequences of *A. perideraion*; As, all sequences of *A. sandaracinos*; further abbr. see Table 1)

Pairs of taxa	Control region	Cytochrome *b*	Geological era
Ao-AoPa	0.9	0.3 – 0.9	Pleistocene
Ao/AoPa-AperTI	2.7	1.8 – 6.0	Pliocene/end of Miocene
Ao/AoPa-AperBk	2.9	2.2 – 7.2	Pliocene/ end of Miocene
Ao/AoPa-AperNB/AperSolo	2.6-2.9	1.7 – 6.2	Pliocene/ end of Miocene
AperTI-AperBk	1.0	0.4 – 1.0	Pleistocene
AperTI-AperNB/AperSolo	2.6-2.8	1.4 – 4.2	Pliocene
AperBk-AperNB/AperSolo	2.6-2.8	1.6 – 5.0	Pliocene
Aa-Ap	1.7	1.1 – 3.1	Pleistocene/Pliocene
Aa-As	1.9	1.7 – 4.8	Pliocene
Ap-As	1.6	0.5 – 1.5	Pleistocene

[Miocene: 23-5.33 MYA, Pliocene: 5.33-1.8 MYA, Pleistocene: 1.8 MYA-11500 YA (Grabstein & Ogg 2004)]

The genetic distances between the sibling species *A. percula* and *A. ocellaris* were between 16.6–18.5 % in CR and 4.8–7.2 % in cyt *b*, which indicated that the split between them is approx. 1.7–7.2 (2.6–2.9 for CR) million years old. The divergence time between the Indian Ocean clade of *A. ocellaris* and the other populations within the Indo-Malay Archipelago reaches back 300,000 to 900,000 years (900,000 years for CR). This is similar to the separation of the populations of *A. percula* from Tomini Bay and Biak, which is 400,000 years to 1 million years old (1 million years in CR). The divergence between Biak/Tomini Bay and New Britain/Solomon Islands is 1.4–5 million years old (2.6–2.8 million years in CR), similar to the split between *A. ocellaris* and *A. percula* (Table 3).

2.5. Discussion

Molecular phylogenetic analyses of closely related species provide insights into their relationships, allowing us to verify their morphological taxonomic classification. Sometimes, such studies indicate that the previously assumed classification is wrong or not sufficient. This was the case in a study on the *Dascyllus trimaculatus* species complex (Bernardi et al. 2001), in which the authors revealed inconsistence between morphological and colouration traits, as well as their molecular phylogenetic relationships. In contrast to that, research on coral reef fishes of the genus *Thalassoma* (Costagliola et al. 2004) confirmed the morphological species definition, even though the colouration pattern observed in that group

of fishes questioned this. Additionally, such studies can provide insides into divergence and speciation processes, as well as geographic locations of these events.

Different mutation rates for the mitochondrial control region in fish have been estimated, ranging from 2-6.41 % per million years (Faber and Stepien, 1998; Campton et al., 2000; Donaldson and Wilson, 1999; Waters et al., 2001; Messmer et al., 2005). In this study, we used a mutation rate of 6.41 %, because it was obtained for the same CR fragment. The other studies used different fragments or the complete CR, resulting in lower mutation rates. The mutation rate for the cyt *b* region varies widely in literature, but for teleost fish species rates between 1.0 % and 2.8 % per million years were found (Ortí et al., 1994; Martin and Bermingham, 1998; Perdices et al., 2002; Chenoweth et al., 2002; Banford et al., 2004; Casey et al., 2004). CR divergence times were within the ranges calculated for cyt *b*. It shows a consistency for the divergence times calculations between the results of the two markers.

2.5.1. *Amphiprion akallopisos/perideraion/sandaracinos* species complex

The definition by morphological characters and colouration pattern of *A. akallopisos*, *A. perideraion*, and *A. sandaracinos* was supported by the molecular phylogenetic analysis in this study. Additionally, the comparably low sequence divergences in this group confirmed the close relatedness of these species. Based on the colouration pattern, it was expected that *A. akallopisos* and *A. sandaracinos* are sister species, but *A. sandaracinos* and *A. perideraion* were observed to be more closely related in the phylogenetic trees as well as in the haplotype networks. This confirms the findings of another study on the molecular phylogeny of anemonefishes (Santini and Polacco, 2006). The authors of this study assume that the main characters of the *A. akallopisos/ perideraion/sandaracinos* species complex (orange-pinkish colouration with a white band on the back, a slender body with a rounded caudal fin) evolved before the radiation into different species.

Molecular clock estimates on the divergence time among the *Phalerebus* species indicated that the separation of *A. akallopisos* and the other two species took place by the end of the Pliocene and beginning of the Pleistocene 1.1-4.8 million years ago (1.7-1.9 in CR). The species *A. perideraion* and *A. sandaracinos* diverged later, in the Pleistocene, 500,000 years to 1.5 million years ago (1.6 million years in CR).

By the end of the Pliocene and during the Pleistocene the sea level dropped, following the increasing glaciations, which created barriers for migration between the ocean basins. The ancestral population inhabiting the Indian Ocean gave rise to *A. akallopisos*, while the

ancestral population on the Pacific side went through a sympatric speciation or allopatric speciation in separate ocean basins, such as the South China Sea, the Sulu Sea, and the Sulawesi Sea during sea level low stands (Voris, 2000). This gave rise to *A. perideraion* and *A. sandaracinos*. The present distribution patterns of the species support these findings. *A. akallopisos* is distributed mainly in the Indian Ocean, but also present in the Java Sea and probably re-colonised the Sunda Shelf through the Sunda Strait after the glacial times. *A. sandaracinos* and *A. perideraion* both inhabit almost the whole Indo-Malay Archipelago. However, *A. sandaracinos* has a smaller distribution area (Fautin and Allen, 1994). Although, there is an overlap in host anemone acceptance (both fish species can occur in *Heteractis crispa*), it seems to be that they avoid competition by specialisation on different host anemone species (Elliott and Mariscal, 2001). *A. sandaracinos* represents a specialised and *A. perideraion* a rather generalised behaviour accepting two and four anemone species as hosts, respectively (Fautin and Allen, 1994). The speciation of the latter two species took place around 100,000 to 300,000 years later than the split between *A. akallopisos* and the ancestor of *A. sandaracinos* and *A. perideraion*.

The separation of the Pacific and Indian Ocean by sea level low stands triggered divergence and speciation also in other coral reef dwelling animals (McMillan and Palumbi, 1995; Williams, 2000; Kochzius et al. 2003).

2.5.2. *Amphiprion ocellaris/percula* species complex

All specimens from the Togian Islands (Tomini Bay) were clearly included in the *A. percula* clade. This is contrary to the distribution pattern proposed by Allen (1991) and Fautin and Allen, (1994), assuming the presence of *A. ocellaris* in Tomini Bay. Regarding Kuiter and Tonozuka (2005), both species occur in Tomini Bay, inhabiting different depth. *A. percula* is supposed to live in shallow water close to the coastline, whereas *A. ocellaris* inhabits the deeper areas. We sampled at different locations and different depths varying between one and 25 m, but could not confirm these findings, because the phylogenetic analysis clearly showed that all specimens belong to *A. percula*.

The specimens of *A. percula* collected at three different locations formed distinct geographic subclades, supported, by high bootstrap values. The haplotype networks both indicated a strong separation of the samples from New Britain (New Guinea) including the sample from the Solomon Islands, reaching back around 1.9-10 million years. In contrast to the CR analysis, the clade from New Britain and Solomon Islands is not connected to Biak in the

cyt *b* network, but to Togian Island. Obviously, the New Britain lineage is genetically very distinct and its connection to the other clades is not well resolved. This was as well shown in the inconsistencies and not resolved nodes in the phylogenetic trees. Possibly, there is a tendency of mutation saturation present for the strong diverged clades for the third codon positions of the cyt *b* gene (Farias et al. 2001) and/or in the highly variable CR sequences, as well indicated by the comparably small Ts/Tv ratios in both markers (3.9 in labroid fishes, Bernardi and Bucciarelli 1999, 3 in lionfishes, Kochzius et al. 2002, 3.93 in cichlid fishes, Farias et al. 2001). The divergence times between the Biak/Tomini Bay and the New Britain clade are similar to the separation between *A. ocellaris* and *A. percula*. Nelson et al. (2000) found the similar divergence time of 1.9-7.5 million years between *A. ocellaris* and *A. percula* using the same cyt *b* fragment as in the present study. Specimens of *A. percula* from Biak and from Tomini Bay are separated by a number of mutational steps similar to that one separating the clade of *A. ocellaris* from Padang (Indian Ocean) to their conspecifics. In the cyt *b* fragment the mutational steps of the abovementioned clades were even equal.

On the one hand, the genetic distances between some of the geographic groups of the *A. percula/ocellaris* complex were larger than between species in the subgenus *Phalerebus*. The clade of *A. percula* from New Britain and the Solomon Islands shows a divergence of 82 steps from its conspecifics, raising the question if they can be still regarded as one species.

On the other hand, the separation by 31 mutations between the *A. ocellaris* specimens from Padang (Indian Ocean) and the other *A. ocellaris* clade, was lower than between the *Phalerebus* species. The same could be observed between *A. percula* samples from Tomini Bay and from Biak.

Both, the genetic distance of CR (6.6 %) and cyt *b* (1 %) between *A. percula* from Biak and *A. percula* from the Togian Islands could correspond to strongly diverged populations, whereas sequence divergences of 16.7-18 % (CR) and 3.8-5 % (cyt *b*) present between *A. percula* from New Britain/Solomon Islands and the other *A. percula* clades are rather at species level. This view is supported by comparison with the lower genetic distances among the clearly distinct species in the subgenus *Phalerebus* (Table 2). The analysis revealed a sharp genetic break, although the strong South Equatorial Current (SEC) along the northern coast of New Guinea could indicate high gene flow. Although, part of the SEC joins the North Equatorial Countercurrent (NECC) northwest of New Guinea, a noticeable part of it branches off before it reaches Biak (Fig. 1b), which might prevent a continuous mixing (Godfrey 1996). Different current regimes might have also restricted connectivity along the northeastern coast of New Guinea during times of low sea level stands in the glacials. The

New Britain group might be rather connected to populations in the Great Barrier Reef to the South than to the other clades in the Northwest. A strong connectivity of New Britain and the Great Barrier Reef could not be found in another coral reef fish species (*Pseudochromis fuscus*, Messmer et al. 2005), however, the close relatedness to the neighbouring Solomon Islands, although only represented by one sequence, could indicate connectivity in southeast direction.

The fact that two mitochondrial lineages were found in Biak (one specimen corresponding to the New Britain Clade) indicates that occasional migration takes place resulting either in hybridisation and introgression, or a coexistence of these diverged lineages without genetic exchange. To resolve the question of hybridisation and the presence of a cryptic species, it would be necessary to use a nuclear genetic marker for further analyses.

The basic differentiation pattern of the *A. ocellaris/A. percula* complex, with the incertitude of the genetic isolation of the New Britain Clade, can be traced back to the separation of ocean basins during late Pliocene sea level low stands (Van Andel 1994). Due to climate oscillations in the late Pliocene, the sea level dropped by around 40-70 m during glacial periods (Van Andel 1994). This caused intermediate frequent disturbances that are likely to increase the probability of divergence and speciation (Roxburgh et al., 2003; Shea et al., 2004). Even the divergence of the New Britain clade might have been influenced by possible different current patterns during the Pliocene.

In the following Pleistocene, glaciations increased and the sea level dropped up to 120 m (Voris, 2000). During sea level low stands the Sunda Shelf was exposed and the population of *A. ocellaris* from Padang (Indian Ocean) was separated from the ones on the other side of the Sunda shelf. This separation, most probably initiated also the speciation in the *Phalerebus*-species complex (see above). Such a divergence of marine animals in the Indo-Malay Archipelago was also shown for other species, e.g. the mantis shrimp *Haptosquilla pulchella* (Barber et al., 2000, 2002), starfish species (Benzie, 1999; Williams, 2000) and the giant clam *Tridacna crocea* (Nuryanto and Kochzius, 2006). In butterflyfishes, different species assemblages were found in the Indian and Pacific Ocean with genetic variations of around 2 % between the species of the two different oceans. Comparably to the anemonefishes, butterflyfishes are also coral reef associated organisms, which seem to have undergone diversifications during sea level low stands in that area (McMillan and Palumbi, 1995). Additionally, the central part of the Indo-Malay Archipelago was separated from the Pacific coast of New Guinea, leading to a genetic break between populations. Similar genetic differentiation was revealed in other marine organisms, such as *Haptosquilla pulchella*

(Barber et al. 2002) and *Tridacna crocea* (Nuryanto & Kochzius 2006). The population of *A. percula* from Tomini Bay is very isolated and obviously only connected to other populations of this species in the East by currents going through the Moluccan Islands. This is especially notably, because at the neighbouring coastal areas of Sulawesi (e.g. Manado in the North, Kendari in the South) populations of *A. ocellaris* were found (unpublished data). The population of mantis shrimps in Tomini Bay was shown to be also very divergent (Barber et al. 2002), supporting the distinctiveness of that area (Wallace et al. 2002). A study on scad mackerel found a connection between populations in Tomini Bay and the Moluccan Islands (Arnaud et al. 1999). It would be necessary to analyse populations of that area to proof this zone as a connection between Tomini Bay and the Pacific coast of New Guinea.

The results of the present study suggest that there are possibly three species present within the *A. ocellaris/percula* species complex, which is not concordant to the current taxonomy based on morphological characters and colour pattern. Within the *A. akallopisos/A. perideraion/A. sandaracinos* species complex, genetic clades follow species boundaries defined by morphology and colour pattern. The latter results gave a helpful scaling within the genus *Amphiprion* for the interpretation of the *A. ocellaris/A. percula* species complex analyses. The species of the two subgenera (*Actinicola* and *Phalerebus*) are biologically very similar to each other, they are quite specialized in host selection (2-4 host anemones), share the same food sources, and behave and reproduce in a similar way. The species boundaries patterns revealed in this study were contrasting, though. Within the *A. ocellaris/A. percula* complex, there was a strong subdivision corresponding to geographical locations, but not following the species definitions and distributions assumed before. Whereas within the *Phalerebus* subgenus, species boundaries were concordant to their prior species classification, but did not reveal geographical clades.

This study shows, that important factors, for divergences of lineages until speciation of the genus *Amphiprion* in the „Coral Triangle", were sea level fluctuations since the Miocene (Van Andel 1994). This supports on the one hand the „centre of origin" theory for explaining the high biodiversity present in that region (Briggs 2000, 2005). On the other hand it shows, that through the separation between the ocean basins, divergences took place at each side of the barriers, which obviously also contributed to species richness in this marine biodiversity hotspot.

2.6. Acknowledgments

We thank the institutions and individuals that have made our study possible: German Federal Ministry of Education and Research (BMBF, Grant No. 03F0390B), which funded the project „Molecular Genetics as a Tool for the Management of Marine Ornamentals in Sulawesi (Indonesia)" in the framework of the SPICE project (Science for the Protection of Indonesian Coastal Marine Ecosystems); colleagues from the University of Bremen, especially A. Nuryanto for help during field work, and D. Blohm; S. Planes from the University of Perpignan (France) for providing samples from New Britain; U. John from the Alfred-Wegener-Institute Bremerhaven (Germany) for sequencing support; colleagues from Universitas Hasanuddin (Makassar, Indonesia) for logistical support during field work in Spermonde Archipelago, especially J. Jompa.

2.7. References

Allen, G.R., 1991. Riffbarsche der Welt. Mergus Verlag, Melle, Germany. pp. 1-272.

Allen, G.R., Werner, T.B., 2002. Coral reef fish assessment in the „coral triangle" of southeastern Asia. Environ. Biol. Fishes 65, 209-214.

Alvarado, B.J.R., Baker, A.J., Mejuto, J., 1995. Mitochondrial DNA control region sequences indicate extensive mixing of swordfish (*Xiphias gladius*) populations in the Atlantic Ocean. Can. J. Fish Aquat. Sci. 52, 1720-1732.

Arnaud, S., Bonhomme, F., Borsa, P., 1999. Mitochondrial DNA analysis of the genetic relationships among populations of scad mackerel (*Decapterus macarellus, D. macrosoma, D. russelli*) in South-East Asia. Mar. Biol. 135, 699-707.

Banford, H.M., Bermingham, E., Collette, B.B., 2004. Molecular phylogenetics and biogeography of transisthmian and amphi-Atlantic needlefishes (Blenoidae: *Strongylura* and *Tylosurus*): perspectives on New World marine speciation. Mol. Phylogenet. Evol. 31, 833-851.

Barber, P.H., Palumbi, S.R., Erdmann, M.V., Moosa, M.K., 2000. A marine Wallace's line? Nature 406, 692-693.

Barber, P.H., Palumbi, S.R., Erdmann, M.V., Moosa, M.K., 2002. Sharp genetic breaks among populations of *Haptosqulla pulchella* (Stomatopoda) indicate limits to larval transport patterns, causes and consequences. Mol. Ecol. 11, 659-674.

Benzie, J.A.H., 1999. Genetic structure of coral reef organisms: ghost of dispersal past. Am. Zool. 39, 131-145.

Bernardi, G., Bucciarelli, G., 1999. Molecular phylogeny and speciation of the surfperches (Embiotocidae, Perciformes). Mol. Phylogenet. Evol. 13, 77-81.

Bernardi, G., Holbrook, S.J., Schmitt, R.J., 2001. Gene flow at three spatial scales in a coral reef fish, the three-spot dascyllus, *Dascyllus trimaculatus*. Mar. Biol. 138, 457-465.

Bernardi, G., Holbrook, S.J., Schmitt, R.J., Crane, N.L., DeMartini, E., 2002. Species boundaries, populations and colour morphs in the coral reef three-spot damselfish (*Dascyllus trimaculatus*) species complex. Proc. R Soc. Lond. B 269, 599-605.

Briggs, J.C., 2000. Centrifugal speciation and centres of origin. J. Biogeogr. 27, 1183-1188.

Briggs, J.C., 2005. The marine East Indies: diversity and speciation. J. Biogeogr. 32, 1517-1522.

Campton, D.E., Bass, A.L., Chapman, F.A., Bowen, B.W., 2000. Genetic distinction of pallid, shovelnose, and Alabama sturgeon: emerging species and the US Endangered Species Act. Conserv. Genet. 1, 17-32.

Casey, SP., Hall, H.J., Stanley, H.F., Vincent, A.C.J., 2004. The origin and evolution of seahorses (genus *Hippocampus*): a phylogenetic study using the cytochrome *b* gene of mitochondrial DNA. Mol. Phylogenet. Evol. 30, 261-272.

Chenoweth, S.F., Hughes, J.M., Connolly, R.C., 2002. Phylogeography of the pipefish, *Urocampus carinirostris*, suggests secondary intergradation of ancient lineages. Mar. Biol. 141, 541-547.

Costagliola, D., Robertson, D.R., Guidetti, P., Stefanni, S., Wirtz, P., Heiser, J.B., Bernardi, G., 2004. Evolution of the coral reef fish *Thalassoma* spp. (Labridae). 2. Evolution of the eastern Atlantic species. Mar. Biol. 144, 377-383.

Donaldson, K.A., Wilson, R.R., 1999. Amphi-panamic geminates of snook (Percoidei: Centropomidae) provide a calibration of the divergence rate in the mitochondrial DNA control region of fishes. Mol. Phylogenet. Evol. 13, 208-213.

Elliott, J.K., Mariscal, R.N., 2001. Coexistence of nine anemonefish species: differential host and habitat utilization, size and recruitment. Mar. Biol. 138, 23-36.

Excoffier L, Laval LG, Schneider S (2005) Arlequin vers. 3.1: an integrated software package for population genetic data analysis. *Evolutionary Bioinformatics online*, 1, 47-50.

Faber, J.E., Stepien, C.A., 1998. Tandemly repeated sequences in the mitochondrial DNA control region and phylogeography of the pike-perches *Stizostedion*. Mol. Phylogenet. Evol. 10, 310-322.

Farias, I.P., Orti, G., Sampaio, I., Schneider, H., Meyer, A., 2001. The cytochrome *b* gene as a phylogenetic marker: the limits of resolution for analysing relationships among cichlid fishes. J. Mol. Evol. 53, 89-103.

Fautin, D.G., Allen, G.R., 1994. Anemonenfische und ihre Wirte. Tetra-Verlag, Melle. pp. 1-168.

Frankham, R., Ballou, J.D., Briscoe, D.A., 2002. Introduction to Conservation Genetics. Cambridge Univ. Press, UK. pp. 1-617.

Godfrey, J.S., 1996. The effect of the Indonesian throughflow on ocean circulation and heat exchange with the atmosphere: a review. J. Geophys. Res. 101, 12217-12237.

Goldman, N., Anderson, J.P., Rodrigo, A.G., 2000. Likelihood-based tests of topologies in phylogenetics. Syst. Biol. 49, 652-670.

Hall, T.A., 1999. BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Series 41, 95-98.

Jokiel, P., Martinelli, F.J., 1992. The vortex model of coral reef biogeography. J. Biogeogr. 19, 449-458.

Kishino, H., Hasegawa, M., 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J. Mol. Evol. 29, 170-179.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S.F., Villablanca, F.X., Wilson, A.C., 1989. Dynamics of mtDNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86, 6196-6200.

Kochzius, M., Soller, R., Khalaf, M.A., Blohm, D., 2003. Molecular phylogeny of the lionfish genera *Dendrochirus* and *Pterois* (Scorpaenidae, Pteroidae) based on mitochondrial DNA sequences. Mol. Phylogenet. Evol. 28, 396-403.

Kuiter, H.K., Tonozuka, T., 2004. Indonesian Reef Fishes. Part 2. PT Dive and Dives, Bali, Indonesia. pp. 376-377.

Lee, W.J., Howell, W.H., Kocher, T.D., 1995. Structure and evolution of teleost mitochondrial control regions. J. Mol. Evol. 41, 54-66.

Martin, A.P., Bermingham, E., 1998. Systematics and evolution of lower Central American Cichlids inferred from analysis of cytochrome *b* gene sequences. Mol. Phylogenet. Evol. 9, 192-203.

McMillan, W.O., Palumbi, S.R., 1995. Concordant evolutionary patterns among Indo-West Pacific butterflyfishes. Proc. R Soc. Lond. 260, 229-236.

Messmer, V., van Herwerden, L., Munday, P.L., Jones, G.P., 2005. Phylogeography of colour polymorphism in the coral reef fish *Pseudochromis fuscus*, from Papua New Guinea and the Great Barrier Reef. Coral Reefs 24, 392-402.

Nelson, J.S., Hoddell, R.J., Chou, L.M., Chan, W.K., Phang, V.P.E., 2000. Phylogeographic structure of false clownfish, *Amphiprion ocellaris*, explained by sea level changes on the Sunda shelf. Mar. Biol. 137, 727-736.

Nuryanto, A., Kochzius, M., 2006. Limited gene flow in the giant clam *Tridacna crocea* across the Indo-Malay Archipelago. In: Abstracts of the 6th European meeting of the International Society for Reef Studies (ISRS), Bremen, Germany, 19–22 September 2006

Ortí, G., Bell, M.A., Reimchen, T.E., Meyer, A., 1994. Global Survey of mitochondrial DNA sequences in the Threespine Stickleback: evidence for recent migrations. Evolution 48, 608-622.

Paraskeris, D., Magiorkinis, E., Magiorkinis, G., Kiosses, V.G., Lenny, P., Vandamme, A.-M., Rambaut, A., Hatzakis, A., 2004. Phylogenetic reconstructions of a known HIV-1 CRF04_cpx transmission network using maximum likelihood and Baysian methods. J. Mol. Evol. 59, 709-717.

Perdices, A., Bermingham, E., Montilla, A., Doadrio, I., 2002. Evolutionary history of the genus *Rhambia* (Teleostei: Pimelododae) in Central America. Mol. Phylogenet. Evol. 25, 172-189.

Pond, S.L.K., 2007. Evolutionary model selection with a genetic algorithm: a case study using stem RNA. Mol. Biol. Evol. 24, 159-170.

Posada, D., Crandall, K., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817-818.

Roxburgh, S.H., Shea, K., Wilson, J.B., 2004. The intermediate disturbance hypothesis: patch dynamics and mechanisms of species coexistence. Ecology 85, 359-371.

Santini, S., Polacco, G., 2006. Finding Nemo: molecular phylogeny and evolution of the unusual life style of anemonefish. Gene 385, 19-27.

Shea, K., Roxburgh, S.H., Rauschert, E.S.J., 2004. Moving from pattern to process: coexistence mechanisms under intermediate disturbance regimes. Ecol. Lett. 7, 491-508.

Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114-1116.

Swofford, D.L., 1998. PAUP*. Phylogenetic Analysis using Parsimony (*and other Methods). Version 4. Sinauer, Sunderland.

Tavaré, S., 1986. Some probabilistic and statistical problems on the analysis of DNA sequences. Lect. Math. Live Sci. 17, 57-86.

Thompson, J.G., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

Van Andel, T.H., 1994. New views on an Old Planet: A History of Global Change. Cambridge Univ. Press, UK. pp. 1-439..

Voris, H.K., 2000. Maps of Pleistocene sea levels in Southeast Asia: shorelines, river systems and durations. J. Biogeogr. 27, 1153-1167.

Wallace, C.C., Paulay, G., Hoeksema, B.W., 2002. Nature and origins of unique high diversity reef faunas in the Bay of Tomini, Central Sulawesi: The ultimate „centre of diversity"? Proc. International Coral Reef Symp., Bali, Indonesia, pp. 185-192.

Waters, J.M., Craw, D., Youngson, J.H., Wallis, G.P., 2001. Genes meet geology: fish phylogeographic pattern reflects ancient, rather than modern, drainage connections. Evolution 55, 1844-1851.

Williams, S.T., 2000. Species boundaries in the starfish genus *Linckia*. Mar. Biol. 136, 137-148.

Woodland, D.J., 1983. Zoogeography of the Siganidae (Pisces): an interpretation of distribution and richness patterns. Bull. Mar. Sci. 33, 713-717.

Geological history and oceanography of the Indo-Malay Archipelago shape the genetic population structure of the false clown anemonefish (*Amphiprion ocellaris*)

3. Geological history and oceanography of the Indo-Malay Archipelago shape the genetic population structure of the false clown anemonefish (*Amphiprion ocellaris*)

Molecular Ecology (2008) 17: 3999-4014

Janne Timm* and Marc Kochzius

Biotechnology and Molecular Genetics, FB2-UFT, University of Bremen, 28359 Bremen, Germany

* Corresponding author.
E-mail addresses: janne.timm@uni-bremen.de, janneket@gmx.de (J. Timm).

3.1. Abstract

Like many fishes on coral reefs, the false clown anemonefish, *Amphiprion ocellaris*, has a life history with two different phases: adults are strongly site attached, whereas larvae are planktonic. Therefore, the larvae have the potential to disperse, but the degree of dispersal potential depends primarily on the period of the larval stage, which is only 8-12 days in *A. ocellaris*. In this study, we investigated the genetic population structure and gene flow in *A. ocellaris* across the Indo-Malay Archipelago by analysing a fragment of the mitochondrial control region. Population genetic analysis, using AMOVA, revealed a significant and high overall Φ_{ST}-value of 0.241 ($p< 0.001$), clearly showing limited gene flow. Haplotype network analysis detected eight distinct clades corresponding mainly to different geographical areas, which were most probably separated during sea level low stands in the Pleistocene. The distribution of the clades among the different populations indicated slow partial re-mixing mainly in the central region of the archipelago. Major surface currents seem to facilitate larval dispersal, indicated by higher connectivity along major surface currents in the region (e.g. Indonesian Throughflow). Four main groups were found by the hierarchical AMOVA within the archipelago. These different genetic lineages should be managed and protected as separate ornamental fishery stocks and resource contributing to the genetic diversity of the area. Regarding the high diversity and the differentiation among areas within the Indo-Malay

Archipelago of *A. ocellaris* populations, the centre-of-origin theory is supported to be the main mechanism by which the high biodiversity evolved in this area.

Keywords: coral reef fish, Coral Triangle, mitochondrial control region, Pleistocene refuges, Southeast Asia

3.2. Introduction

Oceans seem to exhibit little barriers and therefore good opportunities for dispersal. However, studies on population genetics and live histories of marine organisms have shown that long-distance dispersal may be ecologically rare and the exchange of genetic material between distant populations is often restricted (Palumbi 2003). Land bridges or islands, changing current regimes (Benzie 1998), and salinity or temperature gradients (e.g. cold upwellings, Benzie 1999) may constitute such barriers, leading to genetic subdivision on regional scales or between ocean basins. Both historical oceanographic and geological changes are therefore strong contributing factors towards genetic population structuring present today (Harpending et al. 1998; Lessa et al. 2003). In particular, marine communities, depending on shallow coastal habitats such as coral reefs, might undergo high fluctuation in community composition. Temporary losses of the coastal habitat or a shift in the hydrological regime can alter population sizes and distribution ranges, causing extinction and recolonisation in certain regions (Fauvelot et al. 2003).

The Indo-Malay Archipelago, also called “coral triangle”, is located between the Indian and Pacific Ocean and harbours the world’s richest marine shallow water biodiversity (Briggs 2000; Allen & Werner 2002; Hoeksema 2007). This archipelago consists of over 25,000 islands of different sizes, shapes, and geological origins, spread over a distance of around 5,000 km in west-east dimension (Hall 1996; Tomascik et al. 1997). Coral reefs fringing the islands are separated by open water, frequently subjected to strong and complex currents (Wyrtki 1961), so that the region does not provide a continuous, but instead a rather patchy coral reef habitat. Although these properties could enhance genetic diversification in organisms with low dispersal capabilities, the origin of the high biodiversity in this area is generally still under debate. Alternative hypotheses suggest that species richness might either be the result of diversifications within the region (“centre of origin”; Briggs 2000, 2005), caused by the immigration of lineages evolved at the peripheries (“centre of acculumation”,

Jokiel & Martinelli 1992), or the result of an overlap of the faunas from the Indian and Pacific Ocean ("centre of overlap", Woodland 1983).

Numerous molecular phylogenetic and population genetic studies on different marine organisms have revealed a genetic discontinuity between the Indian and Pacific Ocean, explained by sea-level changes during glaciations in the Pliocene and Pleistocene, which resulted in a temporary land barrier (McMillan & Palumbi 1995; Williams & Benzie 1998; Duda & Palumbi 1999; Nelson et al. 2000; Kochzius et al. 2003; Froukh & Kochzius 2008; Timm et al. 2008). Indeed, such structuring of populations and even speciation processes have also been recorded in the seas within the archipelago, which underwent historical separation (Barber et al. 2002, 2006; Sugama et al. 2002; Lourie et al. 2005; Knittweis et al. 2008; Kochzius & Nuryanto 2008). Moreover, even pelagic fish species with migrating adults, like the Indian scad mackerel (*Decapterus russelli*), showed different genetic lineages (Perrin & Borsa 2001). However, the related round scad mackerel (*Decapterus macrosoma*) did not show such a differentiation (Borsa 2003). It thus seems that ecology and behaviour have a strong influence on the dispersal potential of organisms and therefore their capacity for exchange between populations.

Anemonefish depend on the occurrence of certain anemone species found in shallow water habitats of the Indo-Pacific (Allen 1991; Fautin & Allen 1994), because they rely on their host for protection. Like many coral reef fish, anemonefish have a two-phase life history where adults are strongly site attached and larvae are planktonic (Fautin & Allen 1994). The eggs of this group of fish are demersal, and dispersal ensues only once the larvae are hatched. The geographical dispersal potential of each species thus depends primarily on the length of the larval stage. A study on the saddleback anemonefish (*Amphirion polymnus*) has shown a high proportion of self-recruitment (Jones et al. 2005), suggesting low dispersal potential. The false clown anemonefish (*Amphiprion ocellaris*) is distributed throughout the Indo-Malay Archipelago and it is one of the most popular aquarium fish worldwide. It is still caught from the wild in large numbers (Wabnitz et al. 2003), but little is known about its dispersal capacity and the resulting connectivity between populations. *A. ocellaris* is a specialist, living in symbiosis with only three anemone species (Fautin & Allen 1994). They are inept swimmers, prone to predation if out of their host. The larval stage of these fish lasts only 8-12 days. Because of their dependence on the presence of the host anemones in coral reefs, the site-attached behaviour of the adults, demersal eggs and the short larval stage, this species has a low dispersal capacity and hence a strong genetic population structure can be expected. A previous study on this species, using a fragment of the mitochondrial cytochrome *b* gene (cyt

b) as a marker, revealed a significant genetic population structure in the Indo-Malay Archipelago, with a genetic break between the Indian and Pacific Ocean. Populations on the west coast of Sumatra and Thailand, as well as the area around Singapore, and populations in the central and eastern part of the archipelago, were differentiated (Nelson et al. 2000).

In this study, we aim to investigate the genetic population structure in *A. ocellaris* on three spatial scales to uncover connectivity in: (i) the Spermonde Archipelago, located at the southwestern tip of Sulawesi, where distances among sites are between 10 km and 80 km; (ii) Sulawesi, central Indo-Malay Archipelago, with distances up to 1,100 km; and (iii) the Indo-Malay Archipelago, with sampling locations as far apart as 4,600 km. For this purpose, we used a fragment of the mitochondrial control region (CR) as genetic marker, which is highly variable in fish (Lee et al. 1995). Therefore, it will provide a better resolution than the cyt *b* marker used in the previous study (Nelson et al. 2000). Additionally, more populations on a larger geographical scale have been investigated. Our results will also shed light on the evolutionary forces generating the high biodiversity in the "Coral Triangle".

3.3. Materials and methods

In the present study, a total of 421 tissue samples of the false clown anemonefish (*Amphiprion ocellaris*) were collected at three different scales: (i) Spermonde Archipelago, (ii) Sulawesi, and (iii) the Indo-Malay Archipelago (Fig. 6a Table 4).

For the small-scale analysis, 107 samples were taken at islands in the midshelf, outershelf and outer-rim area of the Spermonde Archipelago. The midshelf islands (Barranglompo, Barrangcaddi and Samalona) were 10-20 km away from the shore and within the 30 m isobath (20-30 m depth, Moll 1983). The outershelf (Lumulumu, Sarapokeke and Karrangkassi) and northern outershelf islands (Reangreang and Jangangangang) were 30-40 km away from the coastline and within the 50 m isobath (30-50 m depth, Moll 1983). The offshore islands (Suranti and Kapoposang) were at the outer rim of the archipelago, 60-80 km away from the shore. This region lies between shallower waters (30 m maximum) and the drop-off (100 m isobath), and exhibits higher hydrodynamic energy than the other areas (Renema & Troelstra 2001). The Spermonde Archipelago is located at the southwestern tip of Sulawesi, comprises around 160 small islands and covers an area of around 16,000 km^2 (Whitten et al. 2002). The sites included in this part of the study are between 10 km and around 80 km apart.

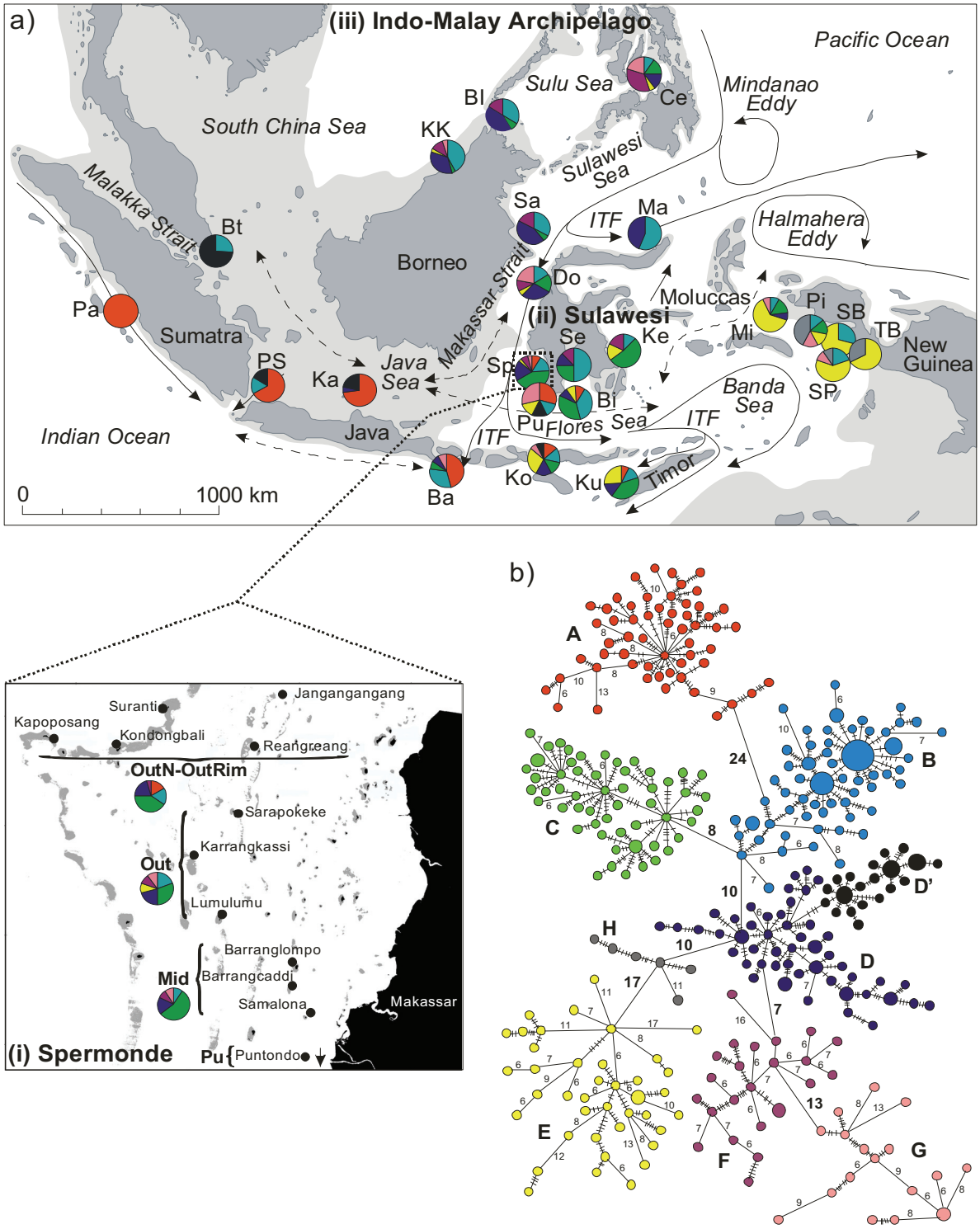


Fig. 6: (a) Sample sites of *A. ocellaris* on three different scales: (i) Spermonde Archipelago, (ii) Sulawesi, and (iii) Indo-Malay Archipelago. Abbreviations of sample sites are given in Table 1. Pleistocene maximum sea level low stand of 120 m is indicated by the light grey area (Voris 2000). Major surface currents are shown as arrows (solid, permanent; dashed, changing with monsoon seasons; Wyrtki 1961; Gordon & Fine 1996; ITF, Indonesian throughflow). Pie charts represent the proportion of clades defined in the network at the different sample sites. (b) Haplotype network of 359 partial control region sequences of *A. ocellaris*. Circles represent haplotypes, whereas the size corresponds to the frequency. Distinct clades (A-H) are colour coded. Numbers and hyphens indicate number of mutational steps between haplotypes.

Table 4: Samples of *A. ocellaris* from different locations in the Indo-Malay Archipelago, and the corresponding abbreviations (Abbr). Number of individuals (N_{ind}), number of haplotypes (N_{haplo}), N_{haplo}/N_{ind}-ratio, and the haplotype (h) and nucleotide (π) diversities are given per site. For Spermonde, only the values of the pooled shelf region are shown.

Sample site(s)	Region	Abbr	N_{ind}	N_{haplo}	N_{haplo}/N_{ind}	$h \pm$ S.D.	$\pi \pm$ S.D.
Spermonde	South Sulawesi	Sp	107	101	0.94	1.00 ± 0.002	0.063 ± 0.03
Kapoposang, Kondongbali, Suranti	Outer-rim	Out Rim	40	38	0.95	1.00 ± 0.01	0.066 ± 0.03
Samalona, Barrang-lompo, Barrangcaddi	Midshelf	Mid	11	10	0.91	0.98 ± 0.05	0.045 ± 0.03
Saropokeke, Karang-kassi, Lumulumu	Outershelf	Out	24	24	1.00	1.00 ± 0.01	0.066 ± 0.03
Reangreang, Jangangangang	Outershelf (North)	OutN	32	31	0.97	1.00 ± 0.01	0.062 ± 0.03
Sulawesi	(Spermonde incl.)		187	174	0.93	1.00 ± 0.0008	0.061 ± 0.03
Bira	South Sulawesi	Bi	11	11	1.00	1.00 ± 0.04	0.066 ± 0.04
Donggala	Central Sulawesi	Do	18	17	0.94	0.99 ± 0.02	0.063 ± 0.03
Kendari	Southeast Sulawesi	Ke	18	17	0.94	0.99 ± 0.02	0.053 ± 0.03
Manado	North Sulawesi	Ma	18	17	0.94	0.99 ± 0.02	0.043 ± 0.02
Pulau Sembilan	Bone Bay, Sulawesi	Se	8	8	1.00	1.00 ± 0.06	0.054 ± 0.03
Puntondo	South Sulawesi	Pu	7	7	1.00	1.00 ± 0.076	0.085 ± 0.05
Indo-Malay Archipelago	(all sites incl.)		421	385	0.92	1.00 ± 0.0003	0.074 ± 0.036
Bali	East Bali	Ba	13	13	1.00	1.00 ± 0.03	0.087 ± 0.05
Banggi Islands	North Borneo	BI	12	12	1.00	1.00 ± 0.03	0.041 ± 0.02
Batam	Malakka Strait	Bt	23	18	0.78	0.97 ± 0.02	0.030 ± 0.02
Cebu	Visayas	Ce	20	18	0.90	0.99 ± 0.02	0.051 ± 0.03
Karimunjava	Java Sea	Ka	18	18	1.00	1.00 ± 0.02	0.064 ± 0.03
Kota Kinabalu	Northwest Borneo	KK	24	21	0.88	0.98 ± 0.02	0.046 ± 0.02
Komodo	Komodo/Rinca	Ko	14	14	1.00	1.00 ± 0.03	0.071 ± 0.04
Kupang	Timor	Ku	15	15	1.00	1.00 ± 0.02	0.061 ± 0.03
Misool	Moluccas	Mi	13	13	1.00	1.00 ± 0.03	0.061 ± 0.03
Padang	West Sumatra	Pa	16	16	1.00	1.00 ± 0.02	0.031 ± 0.02
Pisang	West New Guinea	Pi	7	7	1.00	1.00 ± 0.076	0.063 ± 0.04
Pulau Seribu	Java Sea	PS	18	16	0.89	0.98 ± 0.03	0.064 ± 0.03
Sangalaki	East Borneo	Sa	18	18	1.00	1.00 ± 0.02	0.041 ± 0.02
Sebakor Bay	West New Guinea	SB	7	7	1.00	1.00 ± 0.076	0.053 ± 0.03
Sanggala/Papisol	West New Guinea	SP	10	9	0.90	0.98 ± 0.054	0.061 ± 0.03
Triton Bay (Kaimana)	West New Guinea	TB	6	6	1.00	1.00 ± 0.096	0.053 ± 0.03

On the second scale, 80 tissue samples, additional to the 107 Spermonde samples, were taken from six other sites around Sulawesi, which is one of the largest islands in the Indo-Malay Archipelago (around 180,000 km^2 land area) and has the longest coastline of all Indonesian islands (> 5,200 km^2 coastline), caused by the peculiar shape with three long and narrow peninsulas (Whitten et al. 2002). The distances between sites ranged from 40 km (Puntondo to Spermonde) to over 1100 km (Pulau Sembilan to Manado).

The third and largest scale includes a total of 421 samples from 26 locations (including the four shelf areas of Spermonde and all Sulawesi populations) throughout the Indo-Malay Archipelago, stretching more than 4,500 km from Padang (Sumatra, Indian Ocean) to Triton Bay (New Guinea, Banda Sea).

Fish specimens were collected under water with aquarium hand nets, fin clips were taken on-site, and the fish were subsequently released to their host anemone. The procedure ensures the survival of the fish. The tissue samples were preserved in 96 % ethanol and finally stored at 4°C.

Genomic DNA was isolated from the tissue using extraction kits from QIAGEN and Macherey-Nagel, following the manufacturers' protocols. A fragment of around 370 bp from the mitochondrial control region (CR) was amplified by polymerase chain reaction (PCR) using the primers CR-A (5'-TTCCACCTCTAACTCCCAAAGCTAG-3') and CR-E (5'-CCTGAAGTAGGAACCAGATG-3') (Lee et al. 1995). The PCRs with a volume of 25 µl contained 2.5 µl 10 × PCR buffer, 0.075 µmol Mg^{2+}, 0.25 µmol dNTP mix, 10 pmol of each primer, 0.5 U Taq polymerase and 10-30 ng genomic DNA of each sample. The temperature profile of the PCR was 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s. The terminal elongation at 72 °C took 2 min. PCR products were purified with the QIA-quick PCR Purification Kit (QIAGEN). Sequencing of both strands was conducted on an ABI PRISM 310 and 3100 Automated Sequencer (Applied Biosystems) with the PCR primers using the BigDye Terminator Cycle Sequencing Kit (version 1.1 and 3.1) according to the manufacturer's recommendations. Both PCR and the following cycle sequencing reactions were conducted in a PerkinElmer Thermocycler, Eppendorf Ep, and Eppendorf Ep S Mastercycler. The cycle sequencing reactions were purified with the NucleoSeq Kit (Macherey-Nagel).

The CR sequences were edited with help of the software Seqman (version 4.05, DNAStar). Multiple sequence alignments were carried out using Clustal W (Thompson et al. 1994) as implemented in the software Bioedit (version 7.0.0.1, Hall 1999).

The whole data set, as well as each subset, was tested for selective neutrality of the marker with Tajima's D (Tajima 1989, 1993) and Fu's Fs (Fu 1997) tests by the software Arlequin (version 3.1, Schneider et al. 2000) and Fu and Li's D and F-tests (Fu & Li 1993) by DnaSP (version 4.10.9; Rozas & Rozas 2006). For the tests conducted with DnaSP, one sequence of the sibling species *Amphiprion percula* was added as outgroup for a more precise calculation, as for these tests tree reconstructions are necessary which are rooted by the outgroup. With Arlequin, it was also possible to conduct Chakraborty's test of amalgamation (Chakraborty 1990), which tests for selective neutrality and population homogeneity.

In order to evaluate the polymorphism of the used marker, haplotype (h) and nucleotide (π) diversity (Nei 1987; Nei & Li 1989) for each sample site and for all sites were calculated with Arlequin. The historical demography of *A. ocellaris* was analysed at the three scales by mismatch distribution (Schneider & Excoffier 1999), the sum of square deviation (Rogers & Harpending 1992), and Harpending's raggedeness index (Harpending 1994), thus testing the model of sudden population expansion (Rogers 1995). The mismatch distribution, which is the distribution of the observed differences between haplotypes, is multimodal in populations under a demographic equilibrium and unimodal if a recent and fast demographic expansion of the population has taken place.

To reveal if there is a genetic structure within the data set (Indo-Malay Archipelago) and each subset (Spermonde and Sulawesi), an analysis of molecular variance (AMOVA) was conducted with the program Arlequin (version 3.1, Excoffier et al. 2005). In order to reveal detailed patterns of genetic divergences, pairwise Φ-statistics were calculated with the same program. The significance level was adjusted for the multiple comparisons (error α) according to the false discovery rate control described in Benjamini & Hochberg (1995). A hierarchical AMOVA was conducted to test spatial groupings of populations. A minimum-spanning network of the haplotypes was calculated with Arlequin and drawn by hand. To prevent an over-representation of haplotypes from Spermonde in the network, this large proportion of the data set (107 sequences alone, Table 4) was reduced to 45 sequences. The samples were chosen randomly, but each shelf region was represented nearly equally (10-12 sequences out of each region). The haplotype network could be divided into clades and the frequencies of the clades were calculated for each population, reflected by pie diagrams and drawn on a map of the region. Finally, a Mantel test (Mantel 1967) was calculated with the Isolation by Distance Web Service (IBDWS, Jensen et al. 2005), in order to test for a correlation of geographical and genetic distances.

3.4. Results

3.4.1. Spermonde Archipelago

The 107 sequences from Spermonde Archipelago represented 101 haplotypes. The N_{Haplo}/N_{Ind} ratio (0.94) as well as haplotype (1.00 ± 0.002) and nucleotide (0.063 ± 0.03) diversity was high. The pooled midshelf sites showed the lowest haplotype (0.98 ± 0.05) and nucleotide (0.045 ± 0.03) diversity, as well as the lowest N_{haplo}/N_{ind} ratio (0.91) (Table 4).

Tajima's D-test for selective neutrality, and Chakraborty's test of amalgamation were not significant ($p = 0.18$ and $P = 0.53$, respectively; Table 5). Fu's Fs test ($p = 0.004$), as well as Fu and Li's F and D-tests (both $P < 0.05$) were all significant (Table 5). Fu's Fs test is, next to genetic hitchhiking, sensitive to sudden population expansions (Fu 1997), especially if it has a high negative value, which was the case for the samples from Spermonde (Fs = -23.9, Table 5). The Fu and Li's F and D-tests are rather sensitive to substructures in the data set, as well as to background selection, and decreasing population size (Fu 1996, 1997).

The mismatch distribution showed, both in the sum of squared deviation ($p = 0.50$) and in the Harpending's raggedness index ($p = 0.99$), no significant deviation from the simulated sudden demographic expansion model (Table 5). However, the graph of the observed mismatch distribution values showed a multimodal curve (Fig. 7a), possibly due to population substructures (Schneider & Excoffier 1999; Ray et al. 2003).

Table 5: Results of different neutrality tests, the sum of squared deviation (SSD), and the Harpending's raggedness index (HRI) for three datasets of mitochondrial control region sequences of *A. ocellaris*. *p*-values are given in parenthesise.

	(i) Spermonde	(ii) Sulawesi	(iii) Indo-Malay Archipelago
Neutrality test			
Tajima's D	-0.95 ($p = 0.18$)	-1.37 ($p = 0.06$)	-1.29 ($p = 0.09$)
Fu's Fs	-23.9 ($p = 0.004$)	-23.7 ($p < 0.001$)	-23.5 ($p < 0.02$)
Chakraborty's	0.001 ($p = 0.53$)	0.001 ($p = 0.15$)	0.0014 ($p = 0.0002$)
Fu+Li's D	-3.2 ($p < 0.05$)	-4.3 ($p < 0.02$)	-3.4 ($p < 0.02$)
Fu+Li's F	-2.8 ($p < 0.05$)	-3.6 ($p < 0.02$)	-3.0 ($p < 0.02$)
Mismatch distribution			
SSD	0.004 ($p = 0.50$)	0.002 ($p = 0.53$)	0.0016 ($p = 0.70$)
HRI	0.001 ($p = 0.99$)	0.001 ($p = 0.98$)	0.0004 ($p = 0.99$)

The overall AMOVA showed no significant structure within Spermonde Archipelago ($\Phi_{ST} = -0.005$, $p = 0.585$, Table 6). However, the pairwise comparison (Table 7) showed, that a

significant differentiation could be found between the pooled midshelf sites and the pooled outershelf sites (Φ_{ST} = 0.06, p = 0.027). The hierarchical AMOVA revealed a significant structure (Φ_{CT} = 0.020, p = 0.023, Table 6) when the sample sites were divided into the three groups: (i) all midshelf sites, (ii) all outershelf sites, and (iii) all sites of the northern outershelf and outer-rim. This would support a differentiation of the midshelf and outershelf populations and an unrestricted gene flow between the northern outershelf and outer-rim areas. A Mantel test did not detect isolation by distance.

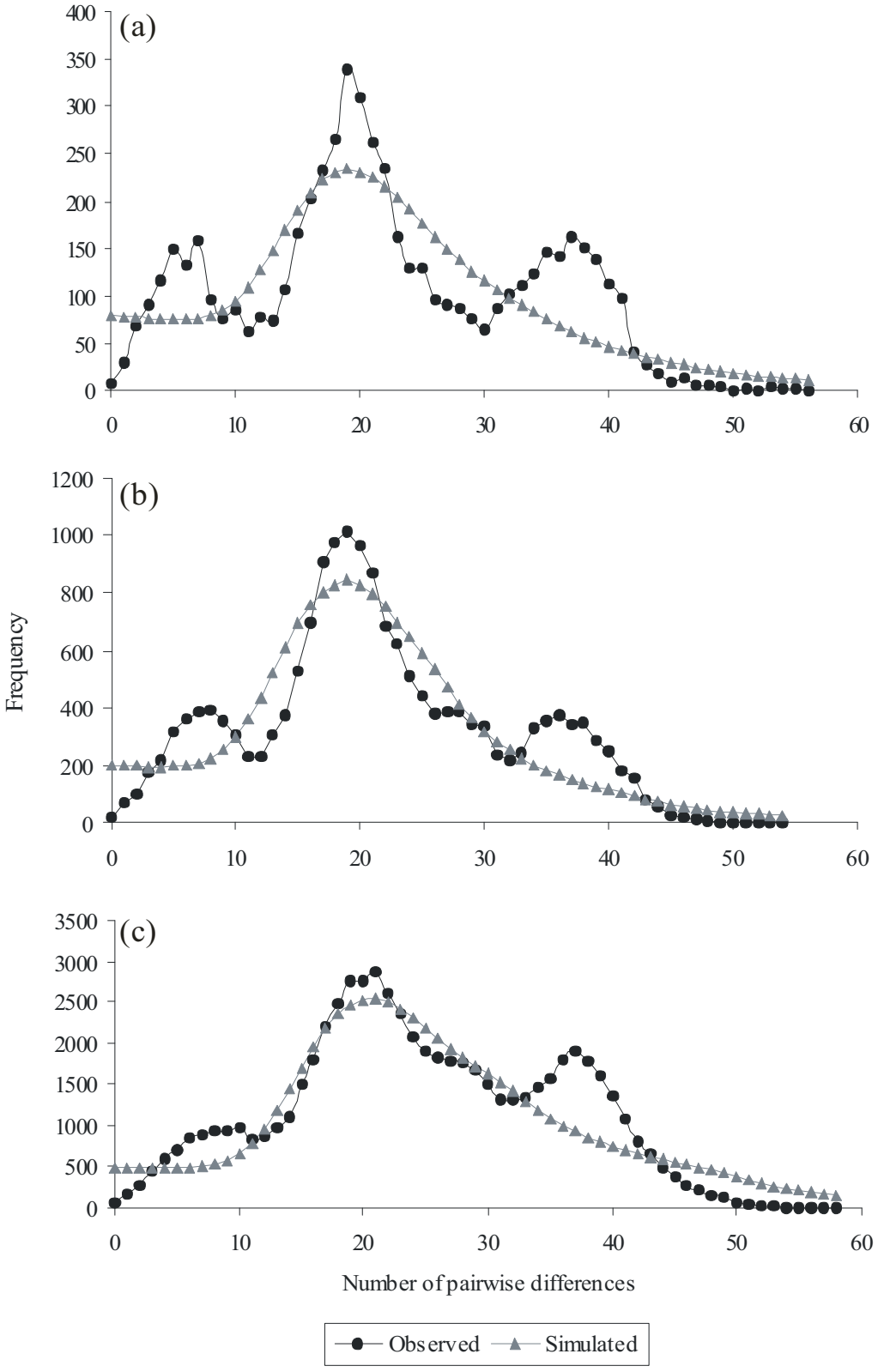


Fig. 7: Mismatch distribution of mitochondrial control region Sequences from *A. ocellaris* from three different data sets: (a) Spermonde Archipelago (b) Sulawesi, and (c) Indo-Malay Archipelago.

Table 6: Hierarchical AMOVA based on mitochondrial control region sequences from *A. ocellaris* with alternative groupings of sample sites in the Spermonde Archipelago, Indonesia (for abbreviations see Table 4).

Grouping	Φ_{CT}-value	*p*-value
4 groups		
(Out)(Mid)(OutN)(OutRim)	0.010	0.136
3 groups		
(Mid)(Out)(OutN, OutRim)	0.020	0.023
(Mid)(Out, OutN)(OutRim)	-0.001	0.378
(Mid)(Out, OutRim)(OutN)	0.004	0.230
(Mid, Out)(OutN)(OutRim)	-0.009	0.724
2 groups		
(Mid)(Out, OutN, OutRim)	0.013	0.081
(Mid,Out)(OutN,OutRim)	-0.001	0.403
(Mid, Out, OutN)(OutRim)	-0.007	0.628
without grouping	Φ_{ST} = -0.005	0.585

3.4.2. Sulawesi

One hundred and seventy-four haplotypes were detected in 187 sequences, indicating a high genetic diversity. The overall haplotype diversity (*h*) was 1.00 (± 0.0008). The nucleotide diversity (π) varied between 0.043 (± 0.023) in Manado and 0.085 (± 0.048) in Puntondo and was in total 0.61 (± 0.03) for this data set. The N_{haplo}/N_{ind} ratio was with 0.93 nearly as high as in the small-scale data set (Table 4).

Tajima's D-test of selective neutrality was not significant ($p = 0.08$). Fu's Fs test was significant ($p < 0.001$), suggesting a sudden population expansion, which is supported by the nonsignificant sum of squared deviation ($p = 0.53$) and Harpending's raggedness index ($p = 0.98$), even though the multimodal mismatch distribution deviates slightly from the simulated one (Fig. 7b). Fu and Li's F and D-tests were also significant (both $p < 0.02$), but not Chakraboty's test of amalgamation ($p = 0.15$, Table 5).

The overall AMOVA on this scale showed a significant population structure ($\Phi_{ST} = 0.039$, $p < 0.001$). The sample sites in Spermonde were divided into three groups for this and the following analyses, taking into account the results obtained with the small-scale data set. The pairwise comparison (Table 7) revealed that the most northern site in Sulawesi (Manado) shows a significant differentiation to all sites except to Pulau Sembilan, which is located in Bone Bay (over 1000 km distance). Donggala, at the western coast of central Sulawesi is significantly different to its neighbouring populations in Spermonde and Manado, both over 500 km away, as well as to Kendari, located at the southeastern tip of Sulawesi (over 1000 km distance). The latter population is differentiated to all other sites except its neighbours Pulau Sembilan (347 km distance) and Bira (361 km distance). The population in Pulau Sembilan is

not significantly different to Manado, Donggala and Bira, even though the former two are geographically far away from Bone Bay. Bira is only significantly different to Manado and Spermonde and generally features low Φ_{ST}-values. Spermonde is significantly different to all sites around Sulawesi, but the group of the northern outershelf sites and the outer-rim area shows the lowest values to all other sites, whereas the midshelf sites showes the highest (Table 7). The midshelf sites showed a surprisingly high value to Puntondo (0.222, p = 0.0002), located at the southern edge of the Spermonde Archipelago, only about 40 km away.

Table 7: Pairwise Φ_{ST}-values from different populations of *Amphiprion ocellaris* in the Indo-Malay Archipelago. For Spermonde, the pairwise Φ_{ST}-values of the separate calculation with all Spermonde samples (n=107) are included and marked by the darker grey shading. The light grey shading indicates the Sulawesi pairings. All significant values are indicated with * (significance level adjusted to a mean α of 0.025 for the whole dataset and 0.0271 for the Spermonde dataset) and the non-significant values are written in italics.

	SpOutN-OutRim	Sp Out	SpMid	Pu	Do	Ma	Bi	Se	Ke	Sa	Pa	Ka	Ba	Ko	Ku	BI	Bt	PS	KK	Ce	Mi	Pi	SB	SP
Sp Out	*0.01*																							
SpMid	*0.002*	0.06*																						
Pu	0.14*	0.15*	0.22*																					
Do	0.15*	0.16*	0.19*	*0.04*																				
Ma	0.18*	0.19*	0.27*	0.15*	0.08*																			
Bi	0.09*	0.11*	0.13*	*0.05*	*0.03*	0.09*																		
Se	0.10*	0.14*	0.14*	0.13*	0.07	*0.04*	*-0.01*																	
Ke	0.15*	0.18*	0.16*	0.18*	0.10*	0.18*	*-0.01*	*0.04*																
Sa	0.17*	0.18*	0.25*	0.19*	0.05	*0.05*	*0.05*	*0.05*	0.13*															
Pa	0.45*	0.50*	0.64*	0.37*	0.53*	0.62*	0.52*	0.63*	0.60*	0.64*														
Ka	0.32*	0.34*	0.43*	*0.13*	0.34*	0.42*	0.30*	0.40*	0.42*	0.43*	0.14*													
Ba	0.15*	0.17*	0.23*	*-0.01*	0.15*	0.21*	*0.08*	0.15	0.21*	0.22*	0.24*	*0.05*												
Ko	0.14*	0.13*	0.19*	*-0.01*	*0.02*	0.13*	*-0.01*	0.10*	0.07*	0.08*	0.47*	0.25*	0.08											
Ku	0.12*	0.15*	0.15*	*0.08*	0.06*	0.16*	*-0.02*	*0.06*	*-0.01*	0.10*	0.54*	0.34*	0.14*	*0.002*										
BI	0.19*	0.19*	0.29*	0.17*	*0.04*	0.10	*0.04*	0.10	0.13*	*-0.01*	0.65*	0.41*	0.21*	*0.05*	0.10*									
Bt	0.29*	0.31*	0.43*	0.32*	0.24*	0.23*	0.26*	0.28*	0.33*	0.17*	0.71*	0.49*	0.35*	0.24*	0.30*	0.21*								
PS	0.28*	0.29*	0.39*	*0.10*	0.30*	0.37*	0.24*	0.34*	0.36*	0.38*	0.17*	*-0.01*	*0.02*	0.21*	0.29*	0.37*	0.46*							
KK	0.15*	0.19*	0.20*	0.16*	0.09*	*0.03*	0.09*	*0.01*	0.15*	*0.04*	0.61*	0.42*	0.22*	0.13*	0.13*	0.11*	0.19*	0.38*						
Ce	0.15*	0.20*	0.18*	0.11*	0.04	0.14*	0.08*	0.06	0.12*	0.08*	0.59*	0.40*	0.21*	0.10*	0.10*	0.11*	0.26*	0.37*	0.05					
Mi	0.26*	0.26*	0.30*	0.14*	0.14*	0.28*	0.13*	0.23*	0.18*	0.25*	0.60*	0.42*	0.25*	0.07	0.08	0.24*	0.42*	0.38*	0.26*	0.20*				
Pi	0.17*	0.18*	0.21*	0.09	*0.03*	0.17*	*0.03*	0.09	*0.07*	0.13*	0.62*	0.40*	0.17*	*0.03*	*0.02*	0.11*	0.37*	0.35*	0.17*	0.11*	*0.07*			
SB	0.30*	0.30*	0.38*	0.19*	0.19*	0.35*	0.19*	0.29*	0.25*	0.32*	0.67*	0.46*	0.28*	0.12*	0.16*	0.31*	0.50*	0.42*	0.31*	0.24*	*-0.03*	0.15		
SP	0.27*	0.27*	0.32*	0.14*	0.13*	0.29*	0.15*	0.24*	0.21*	0.26*	0.62*	0.42*	0.25*	0.09*	0.12*	0.24*	0.44*	0.39*	0.27*	0.20*	*-0.03*	*0.07*	*-0.05*	
TB	0.31*	0.31*	0.41*	0.18*	0.19*	0.36*	0.20*	0.32*	0.27*	0.33*	0.67*	0.45*	0.27*	0.11	0.16*	0.32*	0.51*	0.42*	0.34*	0.27*	*-0.04*	*0.08*	*-0.01*	*-0.04*

Table 8: Hierarchical AMOVA based on mitochondrial control region sequences from *A. ocellaris* with alternative groupings of sample sites from Sulawesi, Indonesia. Spermonde is represented by three groups (Mid, Out, OutN-OutRim; for abbreviations see Table 4).

Grouping	Φ_{CT}-value	*p*-value
3 groups		
(Ma)(Do, Sp, Pu, Bi, Se)(Ke)	0.051	0.039
(Ma)(Do, Sp, Pu, Bi)(Se, Ke)	0.045	0.019
(Ma, Do)(Sp, Pu, Bi, Se)(Ke)	0.042	0.019
(Ma)(Do, Sp, Pu, Se)(Bi, Ke)	0.045	0.014
(Ma, Do)(Sp, Pu, Bi)(Se, Ke)	0.039	0.010
(Ma)(Do, Sp, Pu)(Bi, Se, Ke)	0.043	0.002
(Ma, Do)(Sp, Pu)(Bi, Se, Ke)	0.040	0.002
4 groups		
(Ma)(Do, Sp, Pu)(Bi, Se)(Ke)	0.041	0.014
(Ma, Do)(Sp, Pu)(Bi, Se)(Ke)	0.038	0.013
(Ma)(Do, Sp)(Pu, Bi)(Se, Ke)	0.032	0.016
(Ma)(Do, Sp)(Pu, Bi, Se)(Ke)	0.030	0.030
without grouping	$\Phi_{ST} = 0.039$	< 0.001

Puntondo is significantly different to most of the sites. In general, the differentiations of sites around Sulawesi follow an isolation-by-distance model (Mantel test: $r = 0.46$, $P = 0.02$). The hierarchical AMOVA showed the highest, significant Φ_{CT}-value for the following grouping: (i) Ma; (ii) Do, Sp, Pu, Bi, Se; (iii) Ke (Table 8).

3.4.3. Indo-Malay Archipelago

The 421 sequences analysed for this large scale showed 253 polymorphic sites and resulted in 385 haplotypes. Two-thirds (76.62%) of the 278 substitutions were transitions, leading to a ts/tv ratio of 3.28. The alignment of 362 bp contained 73 indels. Only nine haplotypes were shared among populations (2.3%) and the other (97.7%) were private haplotypes, present only in one population. Most of the private haplotypes were singletons, that is, only represented by a single individual, and mean haplotype (1.00 ± 0.0003) as well as mean nucleotide (0.074 ± 0.036) diversity was accordingly very high. The N_{haplo}/N_{ind} ratio was nearly as high as in the other two analyses (0.92). The range of haplotype and nucleotide diversities was from 0.97 to 1.00, and 0.03–0.087, respectively. The lowest genetic diversity could be detected in Batam, with a haplotype diversity of 0.97 ± 0.02 and a nucleotide diversity of 0.03 ± 0.02, whereas the highest values for nucleotide diversity were found in Puntondo and Bali (0.085 ± 0.048 and 0.087 ± 0.046, respectively). The lowest N_{haplo}/N_{ind} ratio was detected in Batam (0.78) (Table 4).

The neutrality of the marker was evaluated with several tests. Tajima's D-test was again not significant ($p = 0.09$), whereas Fu's Fs as well as Fu and Li's F and D-tests were significant (all $p < 0.02$). Most notably, Fu's Fs test showed a high negative value (-23.5, $p < 0.02$), indicating sudden population expansion. The mismatch distribution showed again a slightly multimodal curve as was the case for the previous two data sets (see Fig. 7c), but the sum of square deviation and Harpending's raggedness index were not significant ($p = 0.70$ and $p = 0.99$, respectively). Chakraborty's test of amalgamation was significant for this data set ($p < 0.0002$) (Table 5).

Table 9: Hierarchical AMOVA results based on mitochondrial control region sequences from *A. ocellaris* with best groupings (a total of 27 groupings were tested) for each category (numbers of groups tested) of populations in the Indo-Malay Archipelago. Spermonde is represented by three distinct groups (Mid, Out, OutN-OutRim; for abbreviations see Table 4), here all included in Sp. All *p*-values were < 0.001.

Grouping	Φ_{CT}- value
4 groups	
(KK, BI, Ce, Sa, Ma, Do, Sp, Pu, Bi, Ko, Ku, Se, Ke, Ba)(Bt) (Mi, Pi, SB, SP, TB)(Pa, PS, Ka)	0.252
(KK, BI, Ce, Sa, Ma, Do, Sp, Pu, Bi, Ko, Ku, Se, Ke)(Bt) (Mi, Pi, SB, SP, TB)(Pa, PS, Ka, Ba)	0.247
5 groups	
(KK, BI, Ce, Sa, Ma, Do)(Sp, Pu, Bi, Se, Ko, Ku)(Bt) (Mi, Pi, SB, SP, TB)(Pa, PS, Ka, Ba)	0.214
(KK, BI, Ce, Sa, Ma)(Do, Sp, Pu, Bi, Se, Ko, Ku)(Bt) (Mi, Pi, SB, SP, TB)(Pa, PS, Ka, Ba)	0.212
(KK, BI, Ce, Sa, Ma, Do)(Sp, Pu, Bi, Se, Ko, Ku, Ba)(Bt) (Mi, Pi, SB, SP, TB)(Pa, PS, Ka)	0.212
6 groups	
(KK, BI, Ce, Ma, Sa, Do)(Sp, Pu, Bi, Se, Ko, Ku, Ke)(Bt) (Mi, Pi, SB, SP, TB)(Pa, PS, Ka)(Ba)	0.215
(KK, BI, Ce, Ma, Sa, Do)(Sp, Pu, Bi, Ko, Ku)(Ke, Se)(Bt) (Mi, Pi, SB, SP, TB)(Pa, PS, Ka, Ba)	0.212
7 groups	
(KK, Ce, BI)(Ma, Sa, Do)(Sp, Pu, Bi, Se, Ke, Ko, Ku)(Bt) (Mi, Pi, SB, SP, TB)(Pa)(PS, Ka, Ba)	0.210
(KK, Bt)(Ce, BI)(Ma, Sa, Do)(Sp, Pu, Bi, Se, Ke, Ko, Ku, Ba) (Mi, Pi, SB, SP, TB)(Pa)(PS, Ka)	0.203
without grouping	Φ_{ST} = 0.241

An AMOVA revealed a strong genetic structure with a significant Φ_{ST}-value of 0.241 ($p < 0.001$, Table 9). The groups in Spermonde Archipelago, which showed significant differentiation among each other in the hierarchical AMOVA of the small-scale analysis, also showed different patterns of differentiation to other sites in the Indo-Malay Archipelago. The

group of midshelf sites had higher Φ_{ST}-values than the other Spermonde groups. The northern outershelf/outer-rim group showed the lowest Φ_{ST}-values to populations outside the Spermonde Archipelago (Table 7). All groups in Spermonde showed their lowest values to Bira and Pulau Sembilan, indicating connectivity, but restricted gene flow to these close sites (186 km and 268 km, respectively).

The populations along the southwestern coast of New Guinea (Pi, SB, SP, TB) and of the island Misool in the Molluccas (Mi) showed no significant Φ_{ST}-values among each other. However, the southeastern sites (SB, SP and TB) in the Banda Sea were strongly differentiated from all other populations in the Indo-Malay Archipelago (Φ_{ST}-values: 0.2–0.3, Table 7. The lowest Φ_{ST}-values could be found between these sites and Komodo (Ko) as well as Kupang (Ku), whereas the highest differentiation could be observed to Padang (Pa, Indian Ocean) and Batam (Bt, Malakka Strait). The population of Pisang (Pi) seems to have had more genetic exchange with nearby populations of the Indo-Malay Archipelago, indicated by nonsignificant and low Φ_{ST}-values (Table 7). The population from Padang (Indian Ocean) showed the highest Φ_{ST}-values of all populations of up to 0.7. These values were below 0.2 only to Pulau Seribu (PS) and Karimunjava (Ka), and the differentiation to Bali ($\Phi_{ST} = 0.24$) was only slightly higher. Pulau Seribu and Karimunjava (Java Sea) had very high Φ_{ST}-values to nearly all other populations, except among each other and to Padang and Bali (Indian Ocean). The population of Batam at the Malakka Strait also had very high Φ_{ST}-values in the pairwise comparisons, and the strongest differentiation could be observed between Batam and Padang (Table 7). The sample sites located in the centre of the study area and along the Indonesian Throughflow (ITF) showed generally many nonsignificant and low Φ_{ST}-values in the pairwise comparisons (Table 7).

The hierarchical AMOVA resulted in several groupings with significant Φ_{CT}-values between 0.165 and 0.252. The best groupings found for each category (number of groups tested) are shown in Table 9 The highest value (0.252) was found with four groups: (i) South China Sea (Bt), (ii) Moluccas (Mi) and New Guinea (Pi, SB, SP, TB), (iii) Java Sea (Ka, PS) and Indian Ocean (Pa), and (iv) all remaining populations located in the central part of the Indo-Malay Archipelago (Bi, Do, Ke, Ko, Ku, Ma, Se, Sp, Ba), northern Borneo (BI, KK, Sa), and the Visayas (Ce). When Bali (Ba) was included in the Java-Sea/Indian Ocean-group, the Φ_{CT}-value was nearly as high (0.247). The highest value ($\Phi_{CT} = 0.214$) for five groups was found when the central group was divided into a northern (BI, Ce, KK, Ma, Sa, Do) and southern (Bi, Ke, Ko, Ku, Se, Sp) group. An isolation-by-distance analysis revealed a strong and significant correlation of the genetic and geographical distances ($r = 0.74$, $P < 0.001$).

The haplotype network with a reduced sample size for Spermonde (n = 45), showed eight distinct clades, separated by 7-24 mutational steps (Fig. 6b). Clade D included a subclade (D′), separated by only three mutational steps. Clades B and D contained the highest number of shared haplotypes and clade B included the two most common haplotypes. Clade D exhibits the most central position in the network. Clade A was separated from the other clades by the highest number of mutations. Clade H was the smallest one, containing only six sequences, each representing one haplotype.

The geographical distribution of clades and their frequency at each sample site is given in Fig. 6(a). Clade A mainly occurred in the Indian Ocean (Ba and Pa) and Java Sea (Ka and PS), but was also found in lower frequencies in South Sulawesi (Pu, Bi and Sp), Komodo (Ko), and Timor (Ku). Clade C showed high frequencies in southern and central Sulawesi (Bi, Do, Ke, Se, Sp), Komodo (Ko), Timor (Ku), and the Visayas (Ce). It was also present in lower frequencies in Bali (Bi), northern Borneo (BI, KK, Sa), as well as New Guinea (Mi, Pi), but could not be found to the west of Bali. Clade E was found in very high frequencies along the coast of New Guinea (Pi, SB, SP, TB) and Misool (Mi). In southern and central Sulawesi (Bi, Do, Ke, and Sp) as well as Komodo (Ko) and Timor (Ku), it was less frequent. It was absent in the Indian Ocean (Ba and Pa), Java Sea (Ka and PS), as well as Batam (Bt; Malakka Strait). The widely spread clades B and D were present in 86 % and 73 % of the sample sites, respectively. Clade B was most common in northern Borneo (BI, KK, and Sa), around Sulawesi (Bi, Do, Ke, Ma, Se, and Sp), as well as Pulau Seribu (PS, Java Sea), Batam (Bt, Malakka Strait) and Bali (Ba, Indian Ocean). Clade D showed the highest frequency in Batam (75 %), the Java Sea (Ka and PS), northern Borneo (BI, KK) as well as around the Sulawesi Sea (Ma and Sa). Clade F haplotypes had a rather small geographical distribution and could be only found in the northern populations (BI, Ce, KK, and Sa) as well as at some sites around Sulawesi (Do, Ke, Se, Sp). Clade G was most frequent in sites downstream of the ITF (Ba, Do, Ko, Sp), as well as in the Visayas (Ce), Misool (Mi) and two of the sample sites in New Guinea (Pi and SP). Haplotypes of clade H were only present at three sites in New Guinea (Pi, SP, TB).

3.5. Discussion

Studies on population genetics and molecular systematics in the “Coral Triangle” have drawn different pictures of isolation and divergence processes as well as connectivity patterns in this diverse and complex area. Organisms with high dispersal potential, like pelagic fish species

(e.g. *Decapterus macrosoma*, Borsa 2003) and sea stars (*Linckia laevigata*, Williams & Benzie 1998; *Acanthaster planci*, Benzie 2000) showed genetic population differentiation with two major lineages one dominant in the Pacific and the other in the Indian Ocean. In a similar manner, the most prominent differentiation in the present study was between the Indian Ocean (Pa) and the populations of the Java Sea (PS and Ka) on the one hand, and the regions east and north of them on the other hand, showing such a genetic break also in *A. ocellaris*. Many marine species have shown complex structures, revealing more than two distinct groups in the Indo-West Pacific (Chenoweth et al. 1998; Williams & Benzie 1998; Gopuerko et al. 1999; Benzie et al. 2002), and even within the Indo-Malay Archipelago (Barber et al. 2002; Lourie et al. 2005; Rohfritsch & Borsa 2005; Knittweis et al. 2008; Kochzius & Nuryanto 2008; Timm et al. 2008). These complex regional substructures are the result of the species' biology that influences the dispersal potential, such as behaviour (e.g. swimming behaviour of larvae, Bradbury & Snelgrove 2001), and lifehistory traits (e.g. larval stage duration, Doherty et al. 1995; Hellberg 1996; Bradbury & Snelgrove 2001).

In anemonefish species (e.g. *Amphiprion polymnus*), populations with high proportions of self-recruitment have been observed (Jones et al. 2005), and the major break between the Pacific and Indian Oceans was also shown in *A. ocellaris* using a fragment of the mitochondrial cyt *b* gene (Nelson et al. 2000). Anemone fishes are strongly site-attached, which leads to a strong genetic population structure on even much smaller scales, which is also facilitated by complex geography (Hall 1996) and current patterns (Wyrtki 1961) in the Indo-Malay Archipelago.

The results of the present study on *A. ocellaris* in the Indo-Malay Archipelago at three different scales clearly showed genetic population substructuring, exceeding a simple model with diverged populations coming from the Indian and Pacific Ocean. At least four distinct groups could be revealed (Table 9): (i) Indian Ocean and Java Sea, (ii)

Central Indo-Malay Archipelago, (iii) southwestern coast of New Guinea, and (iv) Batam.

The measured nucleotide and haplotype diversities where at the upper end of the range of diversity found in other coral reef fish for the control region (Fauvelot et al. 2003; Messmer et al. 2005; Bay et al. 2006). This finding supports on the one hand the highly polymorphic character of the sequence marker in the studied species, on the other hand it underlines that the organism itself shows high levels of diversity. Additionally, the high proportion of private haplotypes indicates restriction in migration among populations (Barber et al. 2002). The nonsignificant Tajima's D-tests for all data sets support the neutrality of the marker, as assumed by various previous studies using the mitochondrial control region in different fish

species (Chenoweth et al. 1998; Arnaud et al. 1999; Bernardi et al. 2001; Fauvelot et al. 2003; Kochzius & Blohm 2005; Froukh & Kochzius 2007). In all three data sets, Fu's Fs test was significant, suggesting that either selection in form of genetic hitchhiking, or sudden demographic expansion must have occurred in recent history (Fu 1997). The statistics, carried out on the mismatch distribution of the sequences to test for a sudden demographic expansion (Rogers 1995), revealed that the population of *A. ocellaris* indeed experienced sudden demographic and spatial expansion in recent history. A previous decrease in population size and spatial distribution possibly happened because of a loss of shallow marine coastal habitat during low sea level stands in the Pleistocene (Voris 2000). The fact that the curves of the mismatch distributions did not strictly follow the simulated curves under the sudden expansion model (Roger 1995), but were slightly multimodal, underlines the finding of substructures in the data sets with low migration among demes (Schneider & Excoffier 1999; Ray et al. 2003). This is supported by significant Fu and Li's neutrality tests, with alternative theories of population reduction, background selection and population substructuring (Fu & Li 1993). A mere population reduction is unlikely, because of (i) the oceanographic history of the study area, whereas a scenario of a population reduction followed by an expansion is far more likely; and (ii) because of the significant Fu's Fs test and the nonsignificant sum of square deviation (SSD) and Harpending's raggedness index (HRI) analyses.

3.5.1. Spermonde

It was not possible to detect an overall population structure by the AMOVA in Spermonde Archipelago. This result was probably due to only very low differences between sites, which in some cases had no divergences at all. However, especially the midshelf sites had low sample sizes, which hindered the detection of significant values. However, despite the low sample numbers, it was possible to identify significant differentiations between the pooled midshelf and outershelf sites. Interestingly, it was not possible to find a significant value between the pooled midshelf and the outer-rim and northern outershelf sites, although the geographical distances are higher. The driving factor of divergences was thus not isolation by distance. This was supported by the nonsignificant Mantel test (Mantel 1967), indicating no significant correlation of geographical and genetic distances. Despite the results of the overall AMOVA, the hierarchical AMOVA showed significant groupings, leading to three groups in the Spermonde Archipelago. The midshelf sites formed a distinct group, whereas the outer-rim and outershelf sites were genetically better connected to each other, but could still be

divided into separate groups. The outer-rim and northern outershelf sites showed an unrestricted gene flow among them. This pattern can be explained by the location of the Spermonde Archipelago at the ITF and the current regimes within the archipelago. The outer-rim and northern outershelf sites are located at or close to the shelf drop-off and are therefore influenced directly by strong currents coming from the ITF, facilitating the exchange among these sites. The ITF might also provide a high input of larvae from upstream populations, and increasing the possibility, that larvae from the outer-rim and northern outershelf drift away, to neighbouring as well as more distant downstream populations in the ITF. At the outershelf sites, the current regimes might be different, leading to a restricted gene flow with outer-rim and northern outershelf sites, while still maintaining a connection to the ITF, resulting in some exchange to outside populations. Since different clades are present in the outershelf area of the archipelago, coastal currents could play a role (Fig. 6a, detail). The midshelf area is probably less connected to other areas and receives obviously a negligible larval input from the ITF. Midshelf larvae are thus more constrained to settle in this area and a higher proportion of self-recruitment might be provoked. High self-recruitment was found in *A. polymnus* in a small-scale study at Schumann Island, New Guinea (Jones et al. 2005). Low genetic exchange to neighbouring and distant populations is also reflected by the lower diversity indices shown for the pooled midshelf sites, which indicate less variability in the population and closer relationships among haplotypes. In addition to that, a higher fishing pressure as well as habitat destruction due to the proximity to Makassar might reduce population size, which could also play a role in causing lower diversity indices in the midshelf group. A similar population structure was found in the mushroom coral *Heliofungia actiniformis* in the same area (Knittweis et al. 2008). In contrast, studies on the stomatopod *Haptosquilla pulchella* (Barber et al. 2002) and boring giant clam, *Tridacna crocea* (Kochzius & Nuryanto 2008), did not show a significant genetic structure in Spermonde.

3.5.2. Sulawesi

A significant but still not very strong population structure was found around Sulawesi. The northern population in Manado was strongly differentiated, as well as Kendari located at the southeastern tip of Sulawesi (Fig. 6a). Most of the populations located at the centre of the study area were well connected to each other. The main driving factor for the restricted connectivity among sites at this scale was geographical distance, as supported by the significant Mantel test. Another factor leading to the present genetic population patterns is the presence or absence of strong currents, which facilitate larval dispersal (Cowen et al. 2006).

The population in Donggala is better connected to Manado, for example, than Kendari, although the geographical distances are similar. Donggala is located at the west coast of Sulawesi, in the Makassar Strait, directly at the ITF, presumably facilitating the input of larvae from upstream populations, such as Manado. The population in Kendari at the eastern coast of Sulawesi is much more remote and not directly connected to the ITF, with minor currents only allowing for exchange with neighbouring populations like Pulau Sembilan and Bira. Because of the lack of strong currents along the eastern coast, gene flow between Kendari and Manado is restricted. Pulau Sembilan shows no significant Φ_{ST}-values to the neighbouring populations of Kendari and Bira, which might indicate unrestricted gene flow. However, Pulau Sembilan also shows no significant Φ_{ST}-value to Manado, which is very distant. Therefore, it should be noted that the sample size in Pulau Sembilan is rather low, which might have prevented the detection of restricted gene flow. A study on the boring giant clam, *Tridacna crocea*, showed restricted gene flow between Bira and Pulau Sembilan (Kochzius & Nuryanto 2008).

3.5.3. Indo-Malay Archipelago

In contrast to Spermonde and Sulawesi, the data set including all sample sites across the Indo-Malay Archipelago showed a significant Chakraborty's test of amalgamation. As neutrality of the marker is assumed due to the other tests on neutrality and previous studies, the alternative hypothesis is favoured, stating that the genetic pattern developed by a mixture of previously separated evolving gene pools of the species (Chakraborty 1990). This pattern can as well be explained by the isolation of genetic lineages during glacial periods, especially in the Pleistocene (Voris 2000), when ocean basins were separated through land barriers. The different groups from various regions came into secondary contact, when re-colonising new habitats with rising sea level, causing a pattern of amalgamation of gene pools. This demographic and spatial population expansion was also indicated by the significant Fu's Fs neutrality test and the mismatch distribution statistics. Obviously, the dispersal capacity in this strongly localised species is low, due to its biology (site-attached adults; demersal eggs; short larval stage of 8–12 days; Fautin & Allen 1994). Therefore, the imprint of isolation during sea level low stands is still present in the genetic population structure of *A. ocellaris*.
Strong population structuring was also indicated by the high Φ_{ST}-value of the overall AMOVA for the Indo-Malay Archipelago. The pairwise comparisons showed especially strong genetic differentiations of the Indian Ocean (Pa) and Malakka Strait (Bt), but also of

the Java Sea populations (Ka, PS) and New Guinea (Pi, SB, SP, TB). The most northern sites (Visayas and northern Borneo) seemed to be differentiated as well. However, although not directly located next to the ITF, they are connected to the southern sites along the ITF. The grouping with the highest Φ_{CT}-value in the hierarchical AMOVA underlines this pattern of high gene flow along the ITF. Although the population of Bali was included into the central group with the highest Φ_{CT}-value, other combinations of sites in the hierarchical AMOVA showed different outcomes for the affiliation of Bali. On one hand, this site is, influenced by the ITF, which flows through Lombok Strait between Bali and Lombok (Wyrtki 1961). On the other hand, Bali seems to have an affiliation to the Java Sea and Indian Ocean, indicated by relatively low pairwise Φ_{ST}-values and a large proportion of clade A haplotypes.

A substructure could be detected within the central group, indicated by the distribution and frequency of clades, as well as the results of the pairwise comparison. It consists of samples from northern Borneo (KK, BI), Visayas (Ce), Sulawesi Sea (Sa, Ma), and Makassar Strait (Do). The Visayas in particular have a high proportion of clade F haplotypes, indicating that this clade might have evolved in the Sulu Sea during sea level low stands. A similar division between northern and southern locations was revealed in populations of the seahorse, *Hippocampus kuda*, in the same area (Lourie et al. 2005). Clades B and D (Fig. 6) seem to be ancestral lineages, because they were highly abundant, show the highest number of shared haplotypes and were found at nearly all sites. Their major refugial areas during glacial periods were probably in the Pacific (clade B) and South China Sea (clade D, including D′).

The population of Puntondo is part of the central group in the hierarchical AMOVA, but has a high proportion of haplotypes of clade A, which is mostly present at sites in the Indian Ocean and Java Sea. Due to its location at the southwestern-most tip of Sulawesi it seems to be on the one hand connected to populations along the ITF and on the other hand to the Java Sea populations by seasonally changing currents going through the Flores and Java Sea into the South China Sea (Wyrtki 1961). The high genetic diversity in this population also underlines the genetic connectivity to different areas of the Indo-Malay Archipelago.

The population of Batam (Malakka Strait) seems to be quite distinct from all others, shown by the high Φ_{ST}-values, the hierarchical AMOVA, and the subclade D′, to which the majority of the haplotypes found in Batam belong. The location of Batam is relatively distant from the other sites included in the study. Therefore, isolation by distance might be a reason for the high genetic differentiation. During the transgression of the sea level after the last glacial maximum, recolonisation was first possible from the South China Sea, and later also from the Andaman Sea through the Malakka Strait (Voris 2000). Since Batam seems to have low

affinities to Kota Kinabalu at the northwestern coast of Borneo, it might be recolonised from populations originating from the Asian mainland coast of the South China Sea. However, in order to reveal the origin of the population in Batam, additional sample sites from the Andaman Sea and Asian mainland coast of the South China Sea are necessary. The population of Kota Kinabalu is obviously influenced by the Sulu Sea populations, as indicated by the hierarchical AMOVA and the haplotype network. Batam also showed the lowest genetic diversity. Both results could be explained by a bottleneck or founder effect. Batam is very close to Singapore and therefore easily accessible for the international marine ornamental trade. Several years ago, the already high demand on the popular ornamental fish *A. ocellaris* increased and "Nemo" was caught intensively in Batam. A local dive operator reported that it was virtually impossible to find this species during that time. However, at the time of sampling, *A. ocellaris* populations had apparently recovered and this species was quite abundant. Despite the absence of catch statistics for marine ornamentals in Batam, our data on genetic structure and diversity indeed indicate that the population in Batam experienced a severe bottleneck, most probably due to over-exploitation.

The population in Padang is also very distinct, but in contrast to Batam it has a high haplotype diversity. Therefore, a population bottleneck is unlikely. However, the low nucleotide diversity of the population indicates a close relatedness between haplotypes, underlining the isolation from other populations of the archipelago. This differentiation was also found in the haplotype network and the distribution and frequency of clades. Clade A was mainly found in Padang and the Java Sea, reflecting the major genetic break between Indian and Pacific Ocean previously found in *A. ocellaris* with cty *b* sequence data (Nelson et al. 2000) and in other fish (Borsa 2003; Kochzius et al. 2003), as well as other marine organisms in that region (William & Benzie 1998; Barber et al. 2002; Benzie 2000; Knittweis et al. 2008; Kochzius & Nuryanto 2008). The Indian Ocean side was isolated from the Pacific Ocean side of the archipelago by land, which enforced an isolated evolution for thousands of years and a secondary contact between populations from the different areas of the archipelago. The decreasing frequencies of clade A haplotypes present in the populations of the Java and Flores Sea (Fig. 6a) are indicating a recolonisation of the Sunda shelf (Java Sea) mainly through the Sunda Strait. A similar genetic differentiation between the Java Sea and populations to the East was found in mantis shrimp (Barber et al. 2002), the sea horse species *Hippocampus trimaculatus* (Lourie et al. 2005), as well as the boring giant clam, *Tridacna crocea* (Kochzius & Nuryanto 2008). The authors suggest that the separation of different seas during the Pleistocene lead to a genetic differentiation and diversification of lineages, as proposed by McManus (1985). This

is accordant with the centre-of-origin theory and supported by the genetic population structure found in *A. ocellaris*. However, in the Java Sea (Ka, PS), partial mixing of lineages can be observed, indicated by the presence of haplotypes from different origins (Fig. 6a).

The populations from the southwestern coast of New Guinea (Pi, SB, SP, TB) and Misool (Mi) also form a distinct group and are represented mainly by clade E (Fig. 6a), indicating a low connectivity to the western part of the Indo-Malay Archipelago. This distinct group was probably even more isolated during times of low sea level stands, caused by a combination of changes and/or reduction of ocean currents (Williams et al. 2002), and additional land barriers in the Moluccas (Voris 2000). The distribution of clade E haplotypes shows that a connection to populations in the Flores Sea (Ko, Ke) and Timor (Ku) is present today. Despite this, mixing of lineages in New Guinea and neighbouring populations seems to be low, as indicated by the high and significant pairwise Φ_{ST}-values and the distribution of clade H (Fig. 6a), which only occurs in populations at the coast of New Guinea (Fig. 6a).

Present-day population structure is the result of many factors acting upon the genetic composition throughout time. By looking at dominant historical processes and prevailing oceanographic conditions in the study area, it is possible to interpret recent population structures. The genetic population structure of *A. ocellaris* found in this study seems to be caused by historical geographical isolation of populations, geographical distances between sites, as indicated by a significant Mantel test, and the prevailing currents leading to a better connectivity along the ITF, for example. The division into two subgroups of the group in the central Indo-Malay Archipelago is thus either due to a gradual increase of geographical distance between sites, a result of former reduction of exchange by the constriction of the Makassar Strait during the Pleistocene (Voris 2000), or a combination of these factors.

Overall, the high genetic diversity and occurrence of different genetic lineages within the Indo-Malay Archipelago shows that diversifications within the area contribute to the high marine biodiversity in the “Coral Triangle”, thus supporting the centre-of-origin theory (Briggs 2000, 2005). Although, obviously evolutionary processes are taking place within the archipelago, it was shown in other studies that also diversifications and speciation processes at the periphery and the overlap of ocean-specific marine faunas influence the biodiversity in the area (Benzie 1999). Thus, the theories might not be mutually exclusive, but a combination of the different processes leading to the high biodiversity in the region is the most probable scenario (Hoeksema 2007).

3.5.4. Conservation

Coral reef organisms are heavily exploited for the aquarium trade, especially in the main exporting countries Indonesia and Philippines (Wood 2001). The false clown anemonefish (*A. ocellaris*) is one of the most popular ornamental fish species (Wabnitz et al. 2003). Considering the general fishing pressure on coral reefs and on some species in particular, it is important that fisheries are monitored and managed to ensure sustainability of these resources and therefore prevent over-exploitation. Among various conservation efforts, the application of marine protected areas (MPA) was shown to be one of the most promising for coral reefs, as these ecosystems are highly complex and the organisms depend on a balanced system. MPAs may not only help to protect marine biodiversity but as well enhance fishing yields in adjacent areas (Palumbi 2004). Population genetic studies on coral reef organisms contribute to conservation efforts by giving information about the connectivity among reefs and the genetic diversity of populations. The molecular methods used in the present study give a general picture of the dispersal patterns and the genetic diversity in *A. ocellaris*. The present study has shown that gene flow, and therefore larval dispersal, was restricted on a small spatial scale in Spermonde archipelago. Thus, the dispersal distance in the studied species is probably only within an order of a few tens of kilometres. Larval dispersal of marine organisms can vary greatly, and was estimated to be in the order of 25-150 km, but it was suggested that a single-generation dispersal of only a few tens of kilometres may be common in many marine organisms. For such organisms, MPAs should be sized to ensure effectively self-seeding, because exchange with populations several hundreds of kilometres away is ecologically rare (Palumbi 2003). Thus, for *A. ocellaris* it would be on the one hand necessary to protect areas large enough for self-seeding, for example certain shelf areas in Spermonde which are not well connected to areas outside the archipelago (e.g. Midshelf Spermonde). On the other hand, it was shown by the data of this study that some populations with access to major currents (e.g. ITF) within the Indo-Malay Archipelago, show higher dispersal distances along such passageways. Species with longer dispersal distances need an alternative reserve design, protecting populations in distance that ensure exchange between MPAs, for example in MPA networks (Palumbi 2003). For this strategy, it is important to know connectivity patterns of the species. This would as well count for populations of *A. ocellaris* and imply to ensure exchange possibilities among populations by MPA networks, spaced according to the observed patterns. Along the ITF for example, the protected areas may be spaced further apart, whereas in areas with less connectivity, like the eastern coast of Sulawesi, it would be necessary to keep protected areas closer to ensure larval exchange. As well, the four main

lineages revealed by the hierarchical AMOVA, should be considered as four stocks for the ornamental fishery and sources for genetic diversity, which should be managed separately.

3.6. Acknowledgements

We would like to thank the German Federal Ministry of Education and Research (BMBF, grant no. 03F0390B), which funded the project "Molecular Genetics as a Tool for the Management of Marine Ornamentals in Sulawesi (Indonesia)" in the framework of SPICE (Science for the Protection of Indonesian Coastal Marine Ecosystems); Centre for Tropical Marine Ecology (Bremen, Germany) for project co-ordinated, especially C. Richter; GEO Magazine (Hamburg, Germany) for financing the research expedition to Misool and New Guinea; A. Nuryanto from the Jenderal Soedirman University (Purwokerto, Indonesia) for help during field work; colleagues from Universitas Hasanuddin (Makassar, Indonesia) for logistical support during field work in Spermonde Archipelago, especially J. Jompa; the colleagues from the University of Bremen, especially D. Blohm. The SPICE project is conducted and permitted under the governmental agreement between the German Federal Ministry of Education and Research (BMBF) and the Indonesian Ministry for Research and Technology (RISTEK), Indonesian Institute of Sciences (LIPI), Indonesian Ministry of Maritime Affairs and Fisheries (DKP), and Agency for the Assessment and Application of Technology (BPPT). This work was carried out in co-operation with Hassanuddin University (UNHAS, Makassar, Indonesia), Agricultural University Bogor (IPB, Bogor, Indonesia), and Jenderal Soedirman University (Purwokerto, Indonesia).

3.7. References

Allen GR (1991) Riffbarsche der Welt. Mergus Verlag, Melle, Germany, 1–272.

Allen GR, Werner TB (2002) Coral reef fish assessment in the "coral triangle" of southeastern Asia. *Environmental Biology of Fishes*, 65, 209-214.

Arnaud S, Bonhomme F, Borsa P (1999) Mitochondrial DNA analysis of the genetic relationships among populations of scad mackerel (*Decapterus macarellus*, *D. macrosoma*, *D. russelli*) in South-East Asia. *Marine Biology*, 135, 699-707.

Barber PH, Palumbi SR, Erdmann MV, Moosa MK (2002) Sharp genetic breaks among populations of *Haptosqulla pulchella* (Stomatopoda) indicate limits to larval transport patterns, causes and consequences. *Molecular Ecology*, 11, 659-674.

Barber PH, Erdmann MV, Palumbi SR (2006) Comparative phylogeography of three codistributed stomatopods: origins and timing of regional lineage diversification in the coral triangle. *Evolution*, 60, 1825–1839.

Bay LK, Crozier RH, Caley MJ (2006) The relationship between population genetic structure and pelagic larval duration in coral reef fishes on the Great Barrier Reef. *Marine Biology*, 149, 1247-1256.

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal *of the Royal Statistical Society Series B (Methodological)*, 57, 289–300.

Benzie JAH (1998) Genetic structure of marine organisms and SE Asian biogeography. In: *Biogeography and Geological Evolution of SE Asia* (eds Hall R, Holloway JD), pp. 197-209. Backhuys Publishers, Leiden, The Netherlands.

Benzie JAH (1999) Genetic structure of coral reef organisms: ghost of dispersal past. *American Zoologist*, 39, 131-145.

Benzie JAH (2000) The detection of spatial variation in widespread marine species: methods and bias in the analysis of population structure in the crown of thorns starfish (Echinodermata: Asteroidea). *Hydrobiologia*, 420, 1-14.

Benzie JAH, Ballment E, Forbes AT et al. (2002) Mitochondrial DNA variation in Indo-Pacific populations of the giant tiger prawn, *Penaeus monodon*. *Molecular Ecology*, 11, 2553-2569.

Bernardi G, Holbrook SJ, Schmitt RJ (2001) Gene flow at three spatial scales in a coral reef fish, the three-spot dascyllus, *Dascyllus trimaculatus*. *Marine Biology*, 138, 457-465.

Borsa P (2003) Genetic structure of the round scad mackerel *Decapterus macrosoma* (Carangidae) in the Indo-Malay archipelago. *Marine Biology*, 142, 575-581.

Bradbury IR, Snelgrove PVR (2001) Contrasting larval transport in demersal fish and benthic invertebrates: the role of behaviour and advective processes in determining spatial pattern. *Canadian Journal of Fisheries and Aquatic Sciences*, 58, 811–823.

Briggs JC (2000) Centrifugal speciation and centres of origin. *Journal of Biogeography*, 27, 1183-1188.

Briggs JC (2005) The marine East Indies: diversity and speciation. *Journal of Biogeography*, 32, 1517-1522.

Chakraborty R (1990) Mitochondrial DNA polymorphism reveals hidden heterogeneity within some Asian populations. *American Journal of Human Genetics*, 86, 497-506.

Chenoweth SF, Hughes JM, Keenan CP, Lavery S (1998) When oceans meet: a teleost shows secondary intergradation at an Indian–Pacific interface. *Proceedings of the Royal Society B: Biological Sciences*, 265, 415-420.

Cowen RK, Paris CB, Srinivasan A (2006) Scaling of connectivity in marine populations. *Science*, 311, 522-527.

Doherty PJ, Planes S, Mather P (1995) Gene flow and larval duration in seven species of fish from the Great Barrier Reef. *Ecology*, 76, 2373-2391.

Duda TF, Palumbi SR (1999) Population structure of the black tiger prawn, *Penaeus monodon*, among western Indian Ocean and western Pacific populations. *Marine Biology*, 134, 705-710.

Excoffier L, Laval LG, Schneider S (2005) Arlequin vers. 3.1: an integrated software package for population genetic data analysis. *Evolutionary Bioinformatics online*, 1, 47-50.

Fautin DG, Allen GR (1994) *Anemonenfische und Ihre Wirte*. Tetra-Verlag, Melle, Germany.

Fauvelot C, Bernardi G, Planes S (2003) Reductions in the mitochondrial DNA diversity of coral reef fish provide evidence of population bottlenecks resulting from Holocene sea-level change. *Evolution*, 57, 1571-1583.

Froukh T, Kochzius M (2007) Genetic population structure of the endemic fourline wrasse (*Larabicus quadrilineatus*) suggests limited larval dispersal distances in the Red Sea. *Molecular Ecology*, 16, 1359-1367.

Froukh T, Kochzius M (2008) Species boundaries and evolutionary lineages in the blue green damselfishes *Chromis viridis* and *Chromis atripectoralis* (Pomacentridae). *Journal of Fish Biology*, 72, 451-457.

Fu Y (1996) New statistical tests of neutrality for DNA samples of a population. *Genetics*, 143, 557-570.

Fu Y (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, 147, 915-925.

Fu Y-X, Li W-H (1993) Statistical tests of neutrality of mutations. *Genetics*, 133, 693-709.

Gopuerko D, Hughes JM, Keenan CP (1999) Mitochandrial DNA evidence for rapid colonisation of the Indo-West Pacific by the mudcrab *Scylla serrata*. *Marine Biology*, 134, 227-233.

Gordon AL, Fine RA (1996) Pathways of water between the Pacific and Indian oceans in the Indonesian seas. *Nature*, 379, 146-149.

Hall TA (1999) bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95-98.

Hall R (1996) Reconstructing Cenozoic SE Asia. In: Hall R, Blundell D (eds) Tectonic Evolution of Southeast Asia. *Geological Society of London*, Special Publication, No. 106, pp. 153-184.

Harpending HC (1994) Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human Biology*, 66, 591-600.

Harpending HC, Batzer MA, Gurven M *et al*. (1998) Genetic traces of ancient demography. *Proceedings of the National Academy of Sciences USA*, 95, 1961-1967.

Hellberg ME (1996) Dependence of gene flow on geographic distance in two solitary corals with different larval dispersal capabilities. *Evolution*, 50, 1167-1175.

Hoeksema BW (2007) Delineation of the Indo-Malayan centre of maximum biodiversity: the coral triangle. In: *Biogeography, Time, and Place: Distributions, Barriers, and Islands* (ed. W Renema), pp. 117-178. Springer, The Netherlands.

Jensen JL, Bohonak AJ, Kelley ST (2005) Isolation by distance, web service. *BMC Genetics*, 6, 1-6.

Jokiel P, Martinelli FJ (1992) The vortex model of coral reef biogeography. *Journal of Biogeography*, 19, 449-458.

Jones GP, Planes S, Thorrold SR (2005) Coral reef fish larvae settle close to home. *Current Biology*, 15, 1314-1318.

Knittweis L, Krämer WE, Timm J, Kochzius M (2008) Genetic structure of *Heliofungia actiniformis* (Scleractinia: Fungiidae) populations in the Indo-Malay Archipelago: implications for live coral trade management efforts. *Conservation Genetics*, doi: 10.1007/s10592-008-9566-5.

Kochzius M, Blohm D (2005) Genetic population structure of the lionfish *Pterois miles* (Scorpaenidae, Pteroinae) in the Gulf of Aqaba and northern Red Sea. *Gene*, 347, 295-301.

Kochzius M, Nuryanto A (2008) Strong genetic population structure in the boring giant clam *Tridacna crocea* across the Indo-Malay Archipelago: implications related to evolutionary processes and connectivity. *Molecular Ecology*, doi: 10.1111/j.1365-294X.2008.03803.x.

Kochzius M, Söller R, Khalaf MA, Blohm D (2003) Molecular phylogeny of the lionfish genera *Dendrochirus* and *Pterois* (Scorpaenidae, Pteroidae) based on mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, 28, 396-403.

Lee WJ, Howell WH, Kocher TD (1995) Structure and evolution of teleost mitochondrial control regions. *Journal of Molecular Evolution*, 41, 54-66.

Lessa EP, Cook JA, Patton JL (2003) Genetic footprints of demographic expansion in North America, but not Amazonia, during the late Quaternary. *Proceedings of the National Academy of Sciences, USA*, 100, 10331-10334.

Lourie SA, Green DM, Vincent ACJ (2005) Dispersal, habitat differences, and comparative phylogeography of Southeast Asia seahorses (Syngnathidae: *Hippocampus*). *Molecular Ecology*, 14, 1073-1094.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*, 27, 209-220.

McManus JW (1985) Marine speciation, tectonics, and sea level changes in Southeast Asia. *Proceedings of the 5th International Coral Reef Congress, Tahiti*, 4, 133-138.

McMillan WO, Palumbi SR (1995) Concordant evolutionary patterns among Indo-West Pacific butterflyfishes. *Proceedings of the Royal Society B: Biological Sciences*, 260, 229-236.

Messmer V, Van Herwerden L, Munday PL, Jones GP (2005) Phylogeography of colour polymorphism in the coral reef fish *Pseudochromis fuscus*, from Papua New Guinea and the Great Barrier Reef. *Coral Reefs*, 24, 392-402.

Moll H (1983) *Zonation and diversity of Scleractinia on reefs off S.W. Sulawesi, Indonesia*. PhD Thesis, University of Leiden, The Netherlands.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Nei M, Li J (1989) Variances of the average numbers of nucleotide substitutions within and between populations. *Molecular Biology and Evolution*, 6, 290-300.

Nelson JS, Hoddell RJ, Chou LM, Chan WK, Phang VPE (2000) Phylogeographic structure of false clownfish, *Amphiprion ocellaris*, explained by sea level changes on the Sunda shelf. *Marine Biology*, 137, 727-736.

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

Palumbi SR (2004) Marine reserves and ocean neighborhoods: the spatial scale of marine populations and their management. *Annual Reviews of Environment and Resources*, 29, 31-68.

Perrin C, Borsa P (2001) Mitochondrial DNA analysis of the geographic structure of Indian scad mackerel in the Indo-Malay archipelago. *Journal of Fish Biology*, 59, 1421-1426.

Ray N, Currat M, Excoffier L (2003) Intra-deme molecular diversity in spatially expanding populations. *Molecular Biology and Evolution*, 20, 76-86.

Renema W, Troelstra SR (2001) Larger foraminifera distribution on a mesotrophic carbonate shelf in SW Sulawesi (Indonesia). *Palaeogeography Palaeoclimatology Palaeoecology*, 175, 125-146.

Rogers AR (1995) Genetic evidence for a pleistocene population expansion. *Evolution*, 49, 608-615.

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

Rohfritsch A, Borsa P (2005) Genetic structure of Indian scad mackerel *Decapterus russelli*: Pleistocene vicariance and secondary contact in the central Indo-West Pacific seas. *Heredity*, 95, 315-326.

Rozas J, Rozas R (2006) DnaSP, DNA sequence polymorphism: an interactive program for estimating population genetic parameters from DNA sequence data. *Bioinformatics*, 6, 621-625.

Schneider S, Excoffier L (1999) Estimation of past demographic parameters from distribution of pairwise differences when the mutation rates vary among sites: application to human mitochondrial DNA. *Genetics*, 152, 1079-1089.

Sugama K, Haryanti Benzie JAH, Ballment E (2002) Genetic variation and population structure of the giant tiger prawn, *Penaeus monodon*, in Indonesia. *Aquaculture*, 205, 37-48.

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123, 585-595.

Tajima F (1993) Unbiased estimation of evolutionary distance between nucleotide sequences. *Molecular Biology and Evolution*, 10, 677-688.

Thompson JG, Higgins DG, Gibson TJ (1994) clustal w: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673-4680.

Timm J, Figiel M, Kochzius M (2008) Contrasting patterns in species boundaries and evolution of anemonefishes (Amphiprioninae, Pomacentridae) in the centre of marine biodiversity. *Molecular Phylogenetics and Evolution*, doi: 10.1016/j.ympev.2008.04.024.

Tomascik T, Mah JA, Nontji A, Moosa MK (1997) *The Ecology of the Indonesian Seas - Part One*. Periplus Editions Ltd, Hong Kong.

Voris HK (2000) Maps of Pleistocene sea levels in Southeast Asia: shorelines, river systems and durations. *Journal of Biogeography*, 27, 1153-1167.

Wabnitz C, Taylor M, Green E, Razak T (2003) *From Ocean to Aquarium*. UNEP-WCMC, Cambridge, UK.

Whitten T, Mustafa M, Henderson GS (2002) *The Ecology of Sulawesi*. *Ecology of Indonesia Series*, Vol. IV. Periplus Editions Ltd, Hong Kong.

Williams ST, Benzie JAH (1998) Evidence of a biogeographic break between populations of a high dispersal starfish: congruent regions within the Indo-West Pacific defined by color morphs, mtDNA, and allozyme data. *Evolution*, 52, 87-99.

Williams ST, Jara J, Gomez E, Knowlton N (2002) The marine Indo-West Pacific break: contrasting the resolving power of mitochondrial and nuclear genes. *Integrative & Comparative Biology*, 42, 941-952.

Wood E (2001) *Collection of Coral Reef Fish for aquaria: Global Trade, Conservation Issues and Management Strategies*. Marine Conservation Society, Ross-on-Wye,UK.

Woodland DJ (1983) Zoogeography of the Siganidae (Pisces): an interpretation of distribution and richness patterns. *Bulletin of Marine Sciences*, 33, 713-717.

Wyrtki K (1961) *Physical Oceanography of the Southeast Asian Waters*. NAGA Report Vol. 2, The University of California, Scripps Institution of Oceanography, La Jolla, California.

Microsatellite analysis revealed population patterns similar to findings of mitochondrial control region analysis in the false clown anemonefish (*Amphiprion ocellaris*)

4. Microsatellite analysis revealed population patterns similar to findings of mitochondrial control region analysis in the false clown anemonefish (*Amphiprion ocellaris*)

Janne Timm*, Serge Planes and Marc Kochzius

Biotechnology and Molecular Genetics, FB2-UFT, University of Bremen, 28359 Bremen, Germany

* Corresponding author.
E-mail addresses: janne.timm@uni-bremen.de, janneket@gmx.de (J. Timm).

4.1. Abstract

Many studies, using various marker systems, have been conducted on the genetic population structure of marine organisms to reveal connectivity among locations and dispersal capabilities. Although mitochondrial sequence markers are widely used, their accuracy is controversially discussed. In the present study, microsatellites are applied to analyse the genetic population structure of the false clown anemonefish (*Amphiprion ocellaris*) in the Indo-Malay Archipelago. The results are compared to those obtained with a fragment of the mitochondrial control region (Timm & Kochzius 2008). The major findings of the microsatellite dataset were congruent to the mitochondrial control region results, with three strongly differentiated lineages and an indication to further separations in the Indo-Malay Archipelago. Similar to the mitochondrial DNA (mtDNA) analysis, microsatellite data showed a correlation of genetic structure to historical ocean basin separation during sea level low stands in the Pleistocene, geographic distance, and dominant current patterns. However, these patterns revealed by microsatellites are not as pronounced as in the mtDNA analysis, which might indicate the reflection of the recent structure with microsatellites and traces of historical patterns still detectable in the mitochondrial control region. The microsatellite analysis did not give a better resolution on the small scale as expected. This study supports the applicability of the used mitochondrial marker for population genetic analyses, and

recommends the comparison of different marker systems for precise information on different time scales.

4.2. Introduction

Many studies on the genetic population structure of organisms have been conducted in recent years with various marker systems, such as allozymes, mitochondrial (mt) and nuclear (nc) DNA sequences, and size based DNA markers, e.g. RFLP (restriction length polymorphism), AFLP (amplified fragment length polymorphism), RAPD (random amplified polymorphic DNA), and microsatellites (Parker et al. 1998).

Mitochondrial markers are widely used for population genetic analyses, as certain regions show high variability and the procedure for amplification and sequencing is comparably easy. For invertebrates, traditionally the COI region is often chosen, because although protein coding, this region revealed many informative substitutions within species (Barber et al. 2002, Benzie et al. 2002, 2003, Kochzius & Nuryanto 2008,). For fish species, the cytochome *b* gene is frequently used for phylogenetic (Farias et al. 2001, Kochzius et al. 2003, Casey et al. 2004, Santini & Polacco 2006, Concheiro Pérez et al. 2007), as well as phylogeographic and population genetic studies (Nelson et al. 2000, Lourie et al. 2005, Chenoweth et al. 2002, Perrin & Borsa 2001, Árnason 2004). The non-coding mitochondrial control region (CR) is even more variable in vertebrates and has been proven to give good resolutions for populations of fish species (Arnaud et al. 1999, Bernardi et al. 2001, Kochzius & Blohm 2005, Froukh & Kochzius 2007, Timm & Kochzius 2008).

The use of mitochondrial DNA for population genetic studies was criticised, because it was shown for several major groups of animals that predictions on genetic diversity based on their biology were met using nuclear marker systems like allozymes, but not by mitochondrial markers (Bazin et al. 2006). This finding was controversially discussed and it was concluded that caution should be taken when interpreting results from genetic markers, especially mtDNA (Berry 2006, Wares et al. 2006, Eyre-Walker 2006, Mulligan et al. 2006). Comparing different marker systems using the same populations is a possibility to see how the different markers behave and if they are e.g. influenced by selection. Several studies were already conducted, using not only different sequence markers, but different marker systems, often representing mitochondrial and nuclear lineages (Williams 2000, Williams & Benzie 1998, Borsa 2003, Rohfritsch & Borsa 2005, Carlsson et al. 2004). The main results obtained by the different markers were often very similar (Reilly et al. 1999, Williams 2000, Borsa 2003,

Rohfritsch & Borsa 2005), but in some studies disparate results were found (DeInnocentiis et al. 2001), sometimes caused by differences in resolution (Shaw et al. 1999). Actually, in many cases, the microsatellite analyses gave the best resolution for recent differentiations, compared to allozymes or sequence markers (Bentzen et al. 1996, Reilly et al. 1999, Shaw 1999). Especially mitochondrial sequence markers may provide rather a historic than a recent picture of gene flow.

Microsatellites are simple repetitive sequences found throughout the eukaryote nuclear genome (Tautz 1989). They are among the most variable markers, because their mutation rate of 10^{-2} to 10^{-6} /locus/generation (Weber & Wong 1993, Vázquez et al 2000) is higher than most other marker systems. They usually show high resolutions on the population level (Bentzen et al. 1996 Reilly et al. 1999, Shaw et al. 1999) and are therefore used for recent patterns of population differentiation and even for parentage analyses (Kellogg et al. 1995, Norris et al. 2000, Jones & Ardren 2003).

The present study was conducted in the Indo-Malay Archipelago, located between the Indian and Pacific Ocean, and the Asian and Australian continent. It consists of thousands of different sized islands and peninsulas, forming complex geographic structures and that cause very complex current patterns (Wyrtki 1961). It is an interesting region for studies of evolution and population dynamics, because of its geological history with fusions of land masses of different origin (Hall 1996), and the drying up of large parts of the shallow Sunda shelf during the glacials (Voris 2000). It is an area where the highest marine shallow water biodiversity is found worldwide (Briggs 2000, Allen & Werner 2002, Hoeksema 2007).

An extensive study on the population structure of the anemonefish species *A. ocellaris* in the Indo-Malay Archipelago has been conducted using a fragment of the mitochondrial control region (Timm & Kochzius 2008). In this study a structure of four major groups of populations was found in the archipelago, probably representing lineages that were separated in different seas and ocean basins during sea level low stands in the Pleistocene (Voris 2000). Sub-structures due to isolation by distance and major currents were shown on smaller scales, like the island of Sulawesi. On the smallest scale analysed in that study, with distances of only 10 to 80 km between sites, only a very shallow differentiation could be revealed with the mitochondrial control region.

Due to their biology, anemonefishes have a low dispersal capability. They live in symbiosis with anemones and largely depend on them for protection. Therefore, adults do not disperse. Their live history involves demersal eggs which are protected and brooded by the adults, and a short pelagic larval duration (PLD) of only 8 to 12 days (Fautin & Allen 1994).

A. ocellaris is a host specialist, living in symbiosis with only three anemone species (*Heteractis magnifica, H. crispa, Stichodactyla mertensii*; Fautin & Allen 1994), and therefore, it is suggested that it has an even lower dispersal potential than species that can utilize many anemone species (e.g. *A. clarkii*, was found to live with 10 anemone species, Fautin & Allen 1994).

In the present study, conducted with a microsatellite set of 6 loci, the genetic population structure of *A. ocellaris* in the Indo-Malay Archipelago was reconstructed. This was done to verify the population structure previously found with (CR), in order to test the reliability of this mitochondrial marker. Since microsatellites are ought to give good resolution for populations on a small scale, the second aim of this study was to generate a better picture of the population structure in the Spermonde Archipelago.

4.3. Materials and Methods

4.3.1. Sample locations and collection

For studying diversity and population structure, as well as for a better insight into the dispersal capacity of the False Clown Anemonefish (*Amphiprion ocellaris*), a total of 432 tissue samples were collected at three different scales.

For the small scale analysis, 90 samples were taken at islands of the midshelf (Barranglompo, Barrangcaddi and Samalona), outershelf (Lumulumu), northern outershelf (Reangreang and Jangangangang), and outer-rim area (Suranti, Kondongbali, and Kapoposang) of the Spermonde Archipelago (Table 10; Fig.8, detail). The midshelf islands were within the 30 m isobath (20 to 30 m depth, Moll 1983), 10 to 20 km away from the shore. The outershelf and northern outershelf islands were 30 to 40 km away from the coastline and within the 50 m isobath (30 to 50 m depth, Moll 1983). The outer-rim area was 60 to 80 km away from the shore and located between shallower waters (30 m max.) and the shelf edge (100 m isobath). The shelf edge is exposed to the Indonesian Throughflow (ITF) and exhibits higher hydrodynamic energy than the other areas (Renema & Troelstra 2001). The sample sites of this part of the study were 10 to around 100 km apart. The Spermonde Archipelago is located in the South East of Sulawesi, comprises around 160 small islands and covers an area of around 16,000 km^2 (Whitten et al. 2002).

For the second scale analysis, the samples from the Spermonde Archipelago were supplemented with 175 samples from six additional sample sites around the island of

Sulawesi. Sulawesi is located in the centre of the Indo-Malay Archipelago and has a remarkably long coastline (>5,200 km²; Whitten et al. 2002). Its long and narrow peninsulas originated from different land masses that collided (Hall 1996), and formed the peculiar shape with two large bays (Bone Bay and Tomini Bay). The sample sites on this scale were 40 km (Puntondo to Spermonde) to over 1,100 km (Pulau Sembilan to Manado) apart.

For the third scale analysis, all 432 samples from different regions of the Indo-Malay Archipelago were included (Table 10, Fig. 8). The maximum geographic distance between sites was more than 4,500 km from Padang (Sumatra, Indian Ocean) to Triton Bay (New Guinea, Banda Sea).

Fin clips were collected under water with aquarium nets and the fish were subsequently released to their host anemone. The tissue samples were preserved in 96% Ethanol and finally stored at 4 °C.

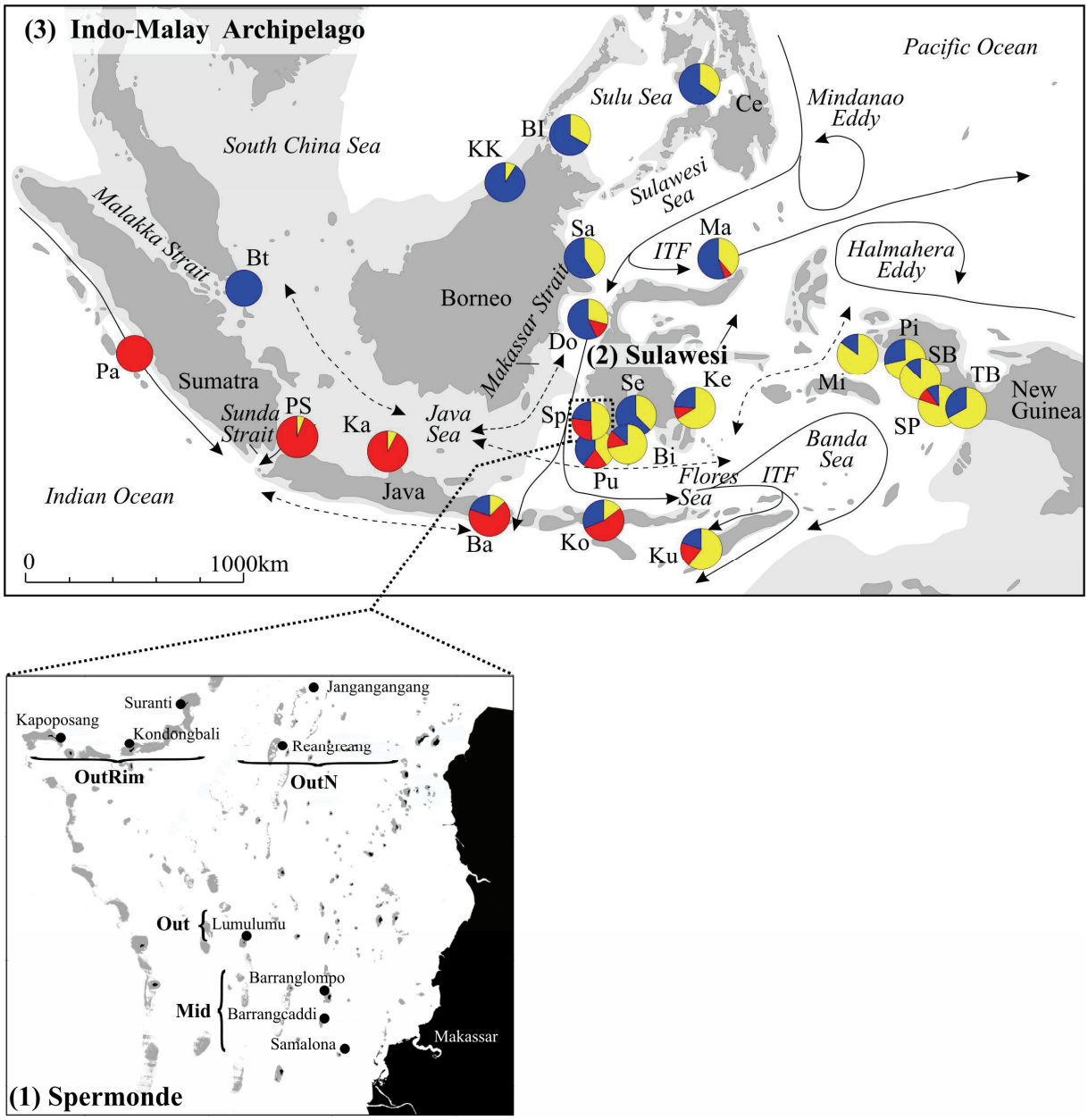


Fig. 8: Map of the study area with names of the major islands, seas and straits, as well as the abbreviations of the sample sites. For each sample site the frequencies of the different groups in the false Clown Anemonefish *A. ocellaris* from the analysis with the programme STRUCTURE (ver. 2.2., Pritchard et al. 2000a) are indicated by different colours in the pie diagrammes. Major surface currents are given by arrows (solid= uni-directional, dashed= reversing with the monsoon seasons). The light grey shading indicates the land area during the Pleistocene maximum sea level low stand of 120 m (after Voris 2000).

4.3.2. DNA extraction, amplification, and fragment analysis

Genomic DNA was isolated from the tissue using extraction kits from Qiagen (Hilden, Germany) and Macherey-Nagel (Düren, Germany), following the manufacturers' protocols.

11 microsatellite loci isolated for the Sattleback Anemonefish (*Amphiprion polymnus*, Quenouille et al. 2004) and 13 loci isolated for the Eastern Clown Anemonefish (*Amphiprion percula*) were tested in *A. ocellaris*. Finally, six polymorphic microsatellite loci were chosen, three from the *A. polymnus* (45, 65 and 120) and three from the *A. percula* (CF9, CF27 and CF42) set, and amplified by PCR. The following PCR conditions were used: the reaction volume of 10 µl contained 1 µl 10 x PCR buffer, 0.0125 µmol Mg^{2+}, 0.002 µmol dNTP mix, 5 pmol of each primer (the forward primer was fluorescence labelled with HEX or FAM; see Table 11), 0.1 U Taq Polymerase and 10-20 ng genomic DNA of each sample for each locus. The temperature profile of the PCR was 94 °C for 2 min, followed by 35 cycles of 94 °C for 45 s, the primer specific annealing temperature (Table 11) for 45 s and 72 °C for 60 s. The terminal elongation at 72 °C took 2 minutes. The fragment analyses of the small scale dataset were performed in a 6.5 % polyacrylamid gel (PAGE), and bands were detected and analysed by a Li-Cor genetic analyser and SAGA 2 software (Li-Cor, Biosciences).
The fragment analyses for the second and third scale datasets were conducted with an ABI 3100 Automated Sequencer (Applied Biosystems, Darmstadt, Germany) using an internal 500 Rox Size Standard (Applied Biosystems). The fragment lengths were analysed and corrected with the software Genemapper (Applied Biosystems). To ensure a consistent allele scoring, part of the samples analysed with PAGE were again analysed with the ABI Automated Sequencer and raster- shifting was adjusted.

4.3.3. Data analysis

To obtain the input file formats for the various programmes used for analysis, the software CONVERT (ver. 1.31, Glaubitz 2004) was used, which works with Exel files as input.
The gene diversity for each population was detected by FSTAT (ver. 2.9.3.2., Goudet 1995), as well as the allelic richness in proportion to the smallest sample size of six individuals in this dataset. The expected and observed heterozygosities for each population, and the *p*-values to test for a significant difference between them, were calculated with the programme Arlequin (ver.3.1, Excoffier et al. 2005), in order to test for the Hardy-Weinberg Equilibrium (HWE). The mean heterozygosities over all loci per population and its standard deviations were as well calculated with Arlequin. The software Genepop (Web-Service, Raymond & Rousset 1995) was used to test for linkage disequilibrium among loci in each population and for genic (focussing on alleles) and genotypic (focussing on genotypes) differentiation among populations by a χ^2-test procedure (Goudet et al.1996).

To scan each dataset for the presence of null alleles, the programme Microchecker (ver. 1.0., Van Oosterhout et al. 2004) was used. The programme takes into account the heterozygosity rates, and the frequency and distribution of alleles.

To reveal if there is a genetic structure within the dataset (Indo-Malay Archipelago) and each subset (Spermonde and Sulawesi), a Bayesian analysis implemented in the software STRUCTURE (ver. 2.2., Pritchard et al. 2000a) was performed, testing for different numbers of clusters in the dataset and giving the corresponding probabilities. The settings used were 100,000 replicates and 80,000 burn-ins, but for unstable runs (when the deviation between repeated runs was high), it was increased as high as 1,000,000 replicates and 100,000 burn-ins. Values of *k* tested (number of clusters) varied between 1 and 6 for Spermonde and Sulawesi, and 1 and 10 for the Indo-Malay Archipelago, and each number of *k* was tested at least three times (to find out the deviation between repeated runs). When different clusters were found, all individuals were assigned to the most probable corresponding cluster and their frequencies were calculated for each population. These frequencies were drawn on a map of the study area as pie-diagrams to visualise the distributions of the clusters. As well, an Analysis of Molecular Variance (AMOVA) was done to test for population structures with the programme Arlequin (ver.3.1, Excoffier et al. 2005). In order to reveal detailed patterns of genetic divergences, pairwise F-statistics were calculated with the same programme and a hierarchical AMOVA was conducted to test spatial groupings of populations. For all procedures in which multiple tests were conducted (e.g. pairwise comparison of populations for F-statistics and population differentiation, as well as linkage disequilibrium), a correction of the significance level was done with the false discovery rate control (Benjamini & Hochberg 1995). It is a statistical method to correct for type I errors in significance calculations of multiple tests.

Finally, a Mantel Test (Mantel 1967) was calculated with the Isolation by Distance Web Service (IBDWS, Jensen et al. 2005) in order to test for a correlation of geographic and genetic distances.

Table 10: Sample sites of *A. ocellaris* from different regions in the Indo-Malay Archipelago, and the corresponding abbreviations (Abbr.). Number of individuals (N_{ind}), number of alleles ($N_{alleles}$), the mean gene diversities over all loci (Gene Div.) with the standard deviations (SD) are given per site. For Spermonde, only the values of the pooled shelf region are shown.

Sample site(s)	Region	Abbr.	N_{ind}	$N_{alleles}$	Gene Div. ± SD	Allelic rich. + SD
Spermonde	South Sulawesi	Sp	90	78	0.78 ± 0.42	5.05 ± 2.10
Samalona, Barranglompo, Barrangcaddi	Midshelf	Mid	16	48	0.79 ± 0.45	4.95 ± 1.71
Lumulumu	Outershelf	Out	6	27	0.64 ± 0.40	4.26 ± 1.67
Reangreang, Janganganggang	Outershelf (North)	OutN	32	61	0.80 ± 0.44	5.40 ± 1.73
Kapoposang, Suranti	Outer-rim	Out Rim	36	66	0.79 ± 0.43	5.28 ± 1.65
Sulawesi	(Spermonde incl.)		175	109		
Puntondo	South Sulawesi	Pu	10	46	0.75 ± 0.43	5.78 ± 2.22
Donggala	Central Sulawesi	Do	21	65	0.69 ± 0.39	4.88 ± 3.11
Manado	North Sulawesi	Ma	18	51	0.80 ± 0.47	4.35 ± 2.48
Bira	South Sulawesi	Bi	7	37	0.80 ± 0.46	5.76 ± 2.18
Kendari	Southeast Sulawesi	Ke	21	53	0.75 ± 0.42	5.11 ± 2.31
Pulau Sembilan	Bone Bay,Sulawesi	Se	8	33	0.73 ± 0.43	5.30 ± 2.21
Indo-Malay Archipelago	(all sites incl.)		432	124		
Sangalaki	West Borneo	Sa	29	63	0.70 ± 0.39	4.88 ± 2.79
Bali	South Bali	Ba	15	50	0.80 ± 0.45	5.17 ± 1.88
Komodo	Komodo/Flores	Ko	13	45	0.79 ± 0.45	5.22 ± 1.96
Kupang	Timor	Ku	20	50	0.72 ± 0.41	4.99 ± 2.01
Karimunjava	Java Sea	Ka	24	60	0.81 ± 0.44	5.48 ± 1.48
Pulau Seribu	Java Sea	PS	18	47	0.80 ± 0.45	5.19 ± 1.68
Padang	West Sumatra	Pa	17	54	0.78 ± 0.45	4.99 ± 1.95
Batam	Riau	Bt	23	27	0.49 ± 0.29	3.29 ±1.50
Kota Kinabalu	Northeast Borneo	KK	23	52	0.64 ± 0.37	3.76 ± 2.94
Banggi Islands	North Borneo	BI	12	46	0.77 ± 0.43	4.51 ± 2.80
Cebu	Cebu, Visayas	Ce	20	47	0.68 ± 0.41	3.47 ± 2.83
Misool	Maluccas	Mi	13	49	0.83 ± 0.47	4.95 ± 2.43
Pisang	West New Guinea	Pi	7	35	0.77 ± 0.45	4.71 ± 2.37
Sebakor Bay	West New Guinea	SB	7	39	0.75 ± 0.43	4.47 ± 2.54
Sanggala/Papisol	West New Guinea	SP	10	45	0.87 ± 0.52	4.80 ± 2.70
Kaimana/Triton Bay	West New Guinea	TB	6	35	0.80 ± 0.47	5.50 ± 2.93

4.4. Results

4.4.1. Microsatellite characterisation

The number of alleles in the six microsatellite loci varied between eight and 34, whereat the highest numbers of alleles (30 and 34, Table 11) were found in the microsatellite loci CF27 and CF42 that also showed the highest heterozygosities. The numbers of alleles were lowest in the loci 120 and 65, and the former showed together with locus 45 the lowest heterozyosities. The observed heterozygosites were in the same range as the expected heterozygosities in all loci, mostly varying only slightly towards a lower observed heterozygosity. The variability of the loci detected in the present dataset of *A. ocellaris* samples is appropriate for population genetic analyses and genetic diversity measures.

Table 11: Microsatellites for *A. ocellaris*: Motif, fluorescence label (Label), annealing temperature (Ann. Temp.), and the range of the fragment lengths (length), as well as the number of alleles ($N_{alleles}$), and the observed (H_o) and expected (H_e) heterozygosites of the whole dataset.

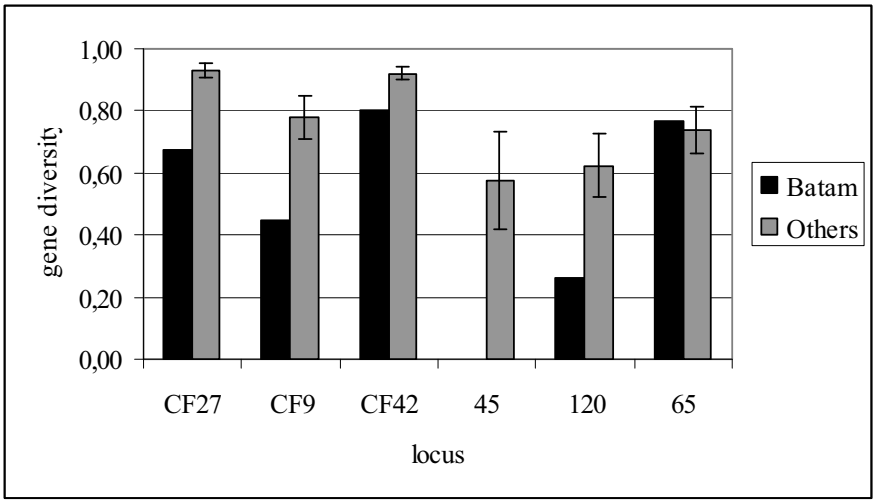
Locus	motif	Label	Ann.Temp.	length	$N_{alleles}$	H_o	H_e
CF9	$(TCAA)_8TGAA(TCAA)_{15}$	HEX	60°C	229-301bp	18	0.739	0.764
CF27	$(TCTA)_{16}$	HEX	62°C	171-301bp	34	0.864	0.916
CF42	$(TCTG)_{18}$	FAM	60°C	162-234bp	30	0.878	0.914
45	$(GT)_{35}$	FAM	54°C	210-250bp	19	0.521	0.548
120	$(GT)_{17}N_{20}(GT)_{12}$	HEX	62°C	450-464bp	8	0.587	0.607
65	$(GT)_{12}$	FAM	60°C	241-261bp	10	0.752	0.742

4.4.2. Genetic variability

The mean gene diversity over all loci ranged from 0.49 (in Batam) to 0.87 (in Sanggala/Papisol) per population. The mean allelic richness ranged from 3.29 (in Batam) to 5.78 (in Puntondo). For both diversity measurements of the different populations, Batam showed the lowest values (Table 10). As the six loci have different levels of variability, the standard deviations over all loci are very high. When the single locus diversities of the populations are considered, the reduced gene diversity and allelic richness of Batam (Riau) is obvious for all loci except locus 65, which showed the lowest values in Sebakor Bay (West

New Guinea) and Cebu (Visayas) (Appendix 1). Comparisons of the values in Batam for both measurements to the mean value of all other populations with the Mann-Whitney test (Mann & Whitney 1947, Sokal & Rohlf 1969) showed that the values in Batam were significantly lower and outside the standard deviation range (Fig. 9). Locus 45 revealed an extreme genetic reduction in Batam with only one allele present and therefore a gene diversity of Zero. This locus showed the highest diversity in the populations of Padang, the Java Sea (Pulau Seribu and Karimunjava), Bali, Komodo and Kupang. Locus 120 showed also the highest diversity in Padang and the Java Sea, but not in Komodo and Kupang. Additionally, this locus showed a high diversity in Puntondo, most sites in New Guinea, and Misool. The allele with 464 bp length of locus 120 was present only in the Indian Ocean (Padang and Kupang) (Appendix 1).

a)



b)

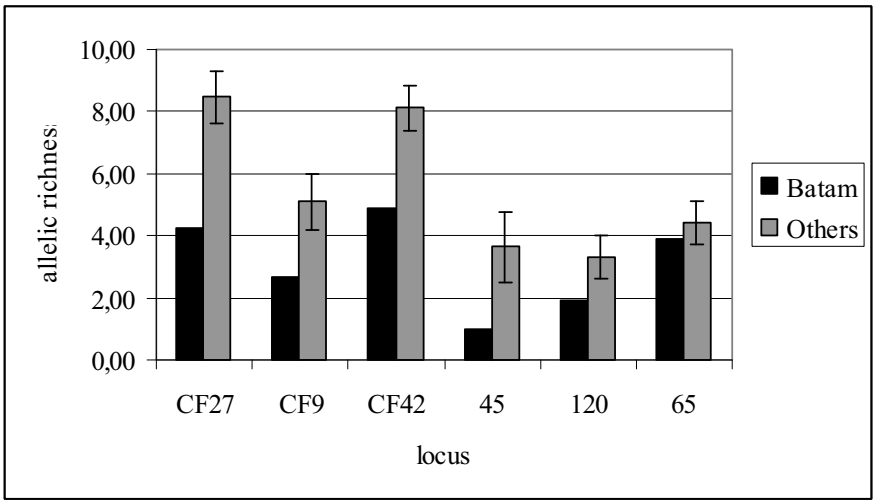


Fig. 9: a) Gene diversity and b) allelic richness of the *A. ocellaris* population in Batam and the mean of all other populations (incl. SD) for each locus.

Table 12: Expected (H_e) and observed (H_o) heterozygosities and *p*-values of deviation from Hardy Weinberg Equilibrium (HWE) of different *A. ocellaris* populations, as well as the mean expected and observed heterozygosities over all loci with the corresponding standard deviations (SD), and the *p*-values of the global test for deviations from HWE with expectations of heterozygote deficit (Het.def.). For abbreviations see Table 10.

	Locus CF27			Locus CF9			Locus CF42			Locus 45			Locus 120			Locus 65			Over all loci				Global HWE (Het.def.)	
	He	Ho	*p*	He	Ho	*p*	He	Ho	*p*	He	Ho	*p*	He	Ho	*p*	He	Ho	*p*	He	Ho	SD He	SD Ho	*p*	SD
SpMid	0.91	0.81	*0.002*	0.77	0.69	*0.856*	0.92	0.94	*0.290*	0.68	0.69	*0.966*	0.65	0.81	*0.229*	0.72	0.87	*0.570*	0.78	0.80	0.105	0.090	*0.765*	0.017
SpOut	0.89	1.00	*0.617*	0.74	0.67	*0.124*	0.91	1.00	*0.588*	0.56	0.33	*0.192*	0.30	0.33	*1.000*	0.68	0.83	*0.793*	0.68	0.69	0.207	0.279	*0.335*	0.011
SpOutN	0.94	0.88	*0.559*	0.84	0.81	*0.240*	0.92	0.94	*0.542*	0.70	0.56	*0.006*	0.60	0.63	*0.514*	0.78	0.78	*0.631*	0.80	0.77	0.119	0.132	*0.041*	0.012
SpOutRim	0.93	0.97	*0.559*	0.82	0.78	*0.195*	0.92	0.89	*0.074*	0.67	0.67	*0.018*	0.62	0.53	*0.142*	0.76	0.75	*0.563*	0.79	0.76	0.116	0.144	*0.088*	0.013
Sp	0.93	0.91	*0.066*	0.81	0.77	*0.239*	0.92	0.92	*0.008*	0.67	0.61	*0.001*	0.60	0.60	*0.122*	0.76	0.79	*0.853*	0.78	0.77	0.121	0.127	*0.081*	0.018
Pu	0.90	1.00	*0.063*	0.80	0.70	*0.396*	0.95	1.00	*1.000*	0.36	0.30	*0.106*	0.63	0.40	*0.053*	0.87	1.00	*0.837*	0.75	0.73	0.203	0.293	*0.551*	0.020
Bi	0.92	1.00	*1.000*	0.89	0.86	*0.796*	0.88	0.86	*0.760*	0.63	0.43	*0.105*	0.66	0.57	*0.681*	0.79	1.00	*0.870*	0.80	0.79	0.115	0.214	*0.558*	0.015
Do	0.96	0.95	*0.517*	0.75	0.71	*0.512*	0.95	0.95	*0.663*	0.39	0.45	*1.000*	0.42	0.40	*0.302*	0.80	0.75	*0.287*	0.71	0.70	0.228	0.217	*0.335*	0.032
Ma	0.95	0.94	*0.090*	0.77	0.72	*0.783*	0.89	0.83	*0.090*	0.59	0.56	*0.148*	0.48	0.53	*0.009*	0.70	0.65	*0.405*	0.73	0.71	0.162	0.148	*0.005*	0.002
Ke	0.94	0.95	*0.712*	0.80	0.86	*0.418*	0.90	0.86	*0.117*	0.45	0.45	*0.306*	0.65	0.57	*0.000*	0.79	0.86	*0.865*	0.76	0.76	0.165	0.181	*0.423*	0.025
Se	0.91	0.50	*0.018*	0.83	1.00	*0.764*	0.89	0.88	*0.448*	0.46	0.63	*0.488*	0.59	0.88	*0.138*	0.71	0.75	*0.921*	0.73	0.77	0.164	0.168	*0.168*	0.012
Sa	0.94	0.97	*0.969*	0.76	0.79	*0.247*	0.93	0.93	*0.835*	0.32	0.38	*1.000*	0.54	0.52	*0.051*	0.73	0.76	*0.561*	0.70	0.72	0.218	0.212	*0.553*	0.029
BI	0.95	0.92	*0.608*	0.72	0.67	*0.655*	0.92	0.92	*0.541*	0.62	0.58	*1.000*	0.58	0.58	*0.600*	0.82	0.75	*0.180*	0.77	0.74	0.140	0.140	*0.340*	0.022
KK	0.93	1.00	*0.911*	0.61	0.48	*0.107*	0.92	1.00	*0.924*	0.28	0.26	*0.417*	0.41	0.39	*1.000*	0.70	0.78	*0.072*	0.64	0.65	0.242	0.292	*0.977*	0.005
Bt	0.67	0.74	*0.080*	0.45	0.48	*0.824*	0.80	0.70	*0.653*	monomorph			0.26	0.30	*1.000*	0.76	0.61	*0.053*	0.49	0.47	0.287	0.255	*0.224*	0.014
Pa	0.94	0.94	*0.636*	0.70	0.71	*0.769*	0.91	0.81	*0.380*	0.78	0.94	*0.719*	0.73	0.77	*0.511*	0.77	0.82	*0.540*	0.80	0.83	0.089	0.086	*0.659*	0.021
PS	0.88	0.61	*0.006*	0.71	0.72	*0.446*	0.92	0.94	*0.872*	0.80	0.78	*0.903*	0.72	0.67	*0.807*	0.77	0.61	*0.252*	0.80	0.72	0.079	0.116	*0.060*	0.009
Ka	0.90	0.88	*0.682*	0.75	0.67	*0.022*	0.88	0.75	*0.029*	0.79	0.88	*0.877*	0.71	0.75	*0.825*	0.82	0.79	*0.120*	0.81	0.79	0.068	0.074	*0.032*	0.008
Ba	0.91	0.67	*0.008*	0.81	0.73	*0.705*	0.92	0.80	*0.123*	0.80	0.67	*0.142*	0.68	0.53	*0.386*	0.66	0.73	*0.673*	0.80	0.69	0.101	0.083	*0.012*	0.003
Ko	0.89	0.77	*0.108*	0.77	0.77	*0.049*	0.92	0.85	*0.574*	0.75	0.33	*0.004*	0.55	0.69	*0.376*	0.79	0.77	*0.723*	0.78	0.70	0.120	0.168	*0.008*	0.003
Ku	0.89	0.75	*0.341*	0.84	0.90	*0.640*	0.92	0.94	*0.601*	0.61	0.55	*0.618*	0.62	0.47	*0.007*	0.70	0.60	*0.047*	0.76	0.70	0.126	0.176	*0.003*	0.001
Ce	0.95	0.87	*0.449*	0.72	0.75	*0.242*	0.93	0.90	*0.565*	0.55	0.35	*0.155*	0.54	0.40	*0.094*	0.60	0.69	*0.900*	0.71	0.66	0.170	0.213	*0.070*	0.014
Mi	0.94	0.77	*0.023*	0.76	0.69	*0.197*	0.94	0.85	*0.396*	0.57	0.50	*0.841*	0.75	0.69	*0.019*	0.75	0.92	*0.423*	0.79	0.74	0.127	0.134	*0.065*	0.010
Pi	0.95	1.00	*1.000*	0.82	0.71	*0.788*	0.90	0.86	*0.728*	0.54	0.71	*1.000*	0.74	0.86	*1.000*	0.65	0.57	*0.248*	0.77	0.79	0.142	0.137	*0.293*	0.014
SB	0.95	1.00	*1.000*	0.73	0.88	*0.925*	0.94	0.75	*0.120*	0.52	0.63	*1.000*	0.77	0.75	*0.052*	0.58	0.63	*0.086*	0.75	0.77	0.164	0.133	*0.382*	0.023
SP	0.92	0.90	*0.783*	0.85	0.60	*0.120*	0.96	0.90	*0.407*	0.58	0.67	*0.615*	0.70	0.67	*0.595*	0.75	0.80	*0.916*	0.79	0.76	0.133	0.118	*0.142*	0.013
TB	0.97	0.83	*0.178*	0.91	0.83	*0.596*	0.92	1.00	*1.000*	0.55	0.33	*0.194*	0.62	0.50	*0.209*	0.80	0.67	*0.750*	0.80	0.69	0.160	0.224	*0.015*	0.002

As the heterozygosities are correlated to the diversity measurements, they showed as well a considerable variation among loci, with the highest values again presented by the loci CF27 and CF42 (Table 12). Over all loci, the expected heterozygosities varied between 0.491 (in Batam) and 0.806 (in Karimunjava) and the observed between 0.471 (in Batam) and 0.832 (in Padang). In most cases of variation between the expected and observed heterozygosities, the populations showed a heterozygotes deficit, but only few populations showed a significant deviation from HWE, indicated by significant *p*-values after correction for multiple tests (Table 12). The highest number of significant deviations from HWE in single locus calculations was found in the loci CF27 (five populations) and 45 (four populations). Locus 65 showed no deviations from HWE. The global test for deviations from HWE over all loci revealed significant *p*-values in Manado, Bali, Komodo, Kupang, and Triton Bay, all correlated with a heterozygote deficit, although the diversity measurements were high in these populations. Deviations from HWE could as well result from selection, population mixing, or non-random mating (Rousset & Raymond 1995). As the deviations from HWE were not consistent among any of the loci, all populations seem to be in HWE.

As the heterozygote deficit might as well be due to the presence of null alleles, next to the possibility that the populations are not in HWE, the loci were tested for null alleles for each population. In locus CF27 the possible presence of null alleles was indicated in three populations (Se, PS, Ba), and in locus 45 in one population (Ko). Therefore, the heterozygote deficit in Bali and Komodo might be due to the presence of null alleles in the loci CF27 and 45, respectively.

The test for linkage disequilibrium among loci showed that some combinations of loci presented a significant *p*-value after correction for multiple tests. All combinations appeared only once though, and for most only in a single population. Only in Manado, six loci combinations were significant, indicating rather a pattern concerning this population than a general linkage of these loci (Table 13).

Table 13: Significant loci combinations for linkage disequilibrium and corresponding populations in *A. ocellaris*. The *p*-values are given after corrections of false discovery rates (Benjamini & Hochberg 1995).

Loci combination	*p*- value	Population
CF9 - 45	0.008	Kupang
CF9 - 65	0.017	Karimunjava
CF9 - CF27	<0.001	Manado
CF9 - CF42	0.011	Manado
CF27 - 45	0.004	Manado
CF27 - 65	<0.001	Manado
CF27 - 120	<0.001	Spermonde
CF27 - CF42	<0.001	Manado
CF42 - 65	0.011	Donggala
CF42 - 120	0.022	Manado
120 - 65	0.013	Pulau Seribu

Even though indications of significant linkage disequilibrium were found in some populations for certain loci combinations and the loci CF27 and 45 might have null alleles in a few populations (max. three out of 23), all loci were used for further calculations.

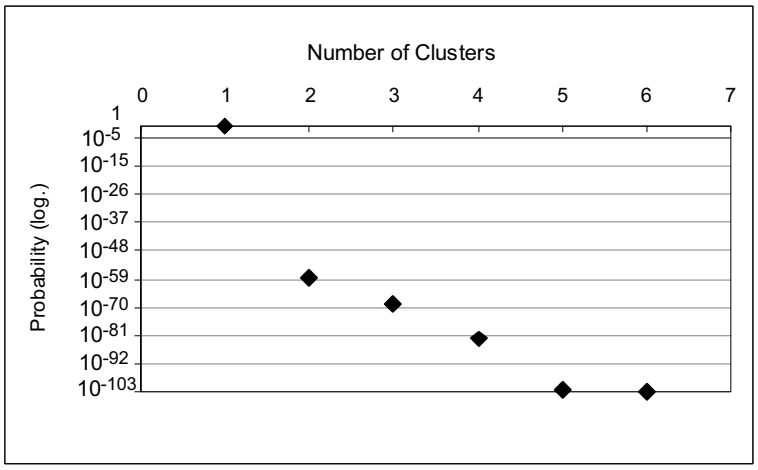
4.4.3. Population differentiation

4.4.3.1. Spermonde Archipelago

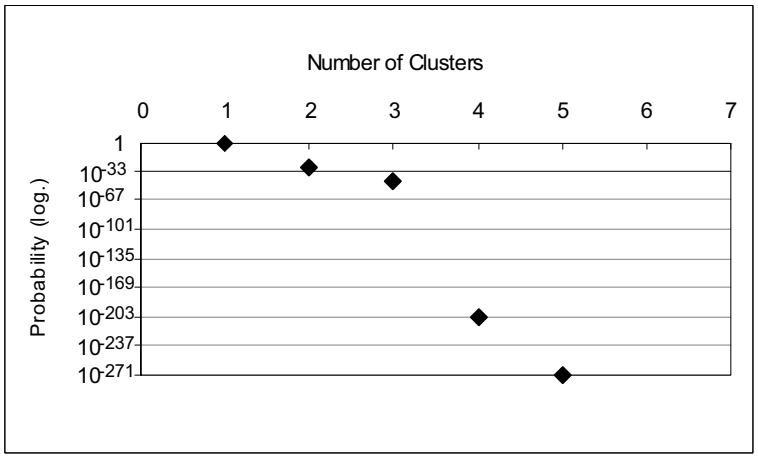
The Bayesian analysis to reveal population structuring gave the highest probability for one cluster (Fig. 10a), indicating no structure in Spermonde Archipelago. Also the over all AMOVA (F_{ST}= -0.0009, p= 0.603), a hierarchical AMOVA (grouping by shelf area), and the pairwise comparisons showed no significant F-statistics underlining an unrestricted gene flow in Spermonde Archipelago. Even the sensitive measurements of genic and genotypic differentiations showed no evidence for a population structure among shelf areas. For further analyses (second and third scale), all samples from Spermonde Archipelago were pooled.

The Isolation-By-Distance analysis showed no significant correlation of the geographic and genetic distances between sites (r = 0.48, p = 0.178).

a)



b)



c)

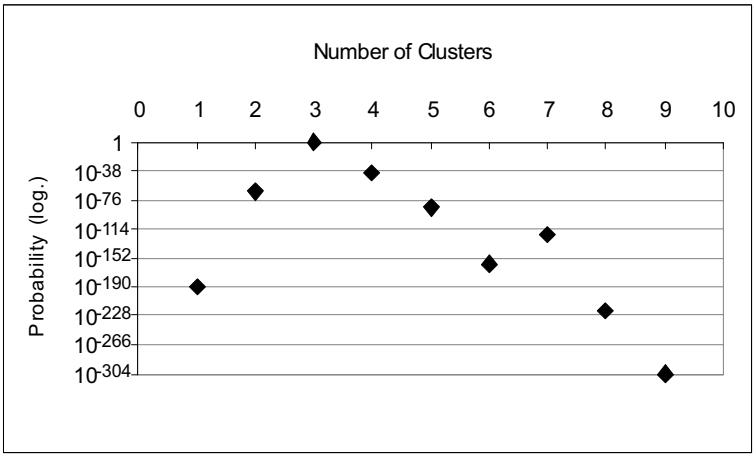


Fig. 10: Probability of the number of clusters in populations of *A. ocellaris* from a) Spermonde Archipelago, b) around Sulawesi Island, and c) in the Indo-Malay Archipelago calculated by STRUCTURE (ver. 2.2., Pritchard et al. 2000a).

4.4.3.2. Sulawesi

For the second scale analysis, including sites around the island of Sulawesi, the highest probability for the structure analysis was given for one cluster, so no population structures were detected by the Bayesian approach. The probabilities for two and three clusters were only slightly lower therefore shallow substructures might be present, not detectable by this analysis (Fig. 10b).

The AMOVA revealed a significant structure in the dataset, although the F_{ST}-value was rather low (F_{ST} = 0.0145, $p < 0.001$). The hierarchical AMOVA resulted in four significant groupings (Table 14), whereat the highest F_{CT}-value was observed for two groups (Sulawesi Sea/Makassar Strait and Southeast Sulawesi including Bira). All values were lower than the F_{ST}-value without grouping.

Table 14: Hierarchical AMOVA for *A. ocellaris* populations around the island of Sulawesi.

Number of groups	Groupings	F_{CT}-value	*p*-value
2 Groups	[Ma, Do, Sp, Pu][Bi, Se, Ke]	0.01317	0.03519
3 Groups	[Ma, Do, Sp][Bi, Se, Ke][Pu]	0.01188	0.01564
	[Ma, Do, Sp, Pu][Bi, Se][Ke]	0.01068	0.03030
	[Ma, Do][Sp, Pu][Bi, Se, Ke]	0.00847	0.04985

The pairwise comparison among populations around Sulawesi revealed significant differentiation among Kendari and most other sites except its closest neighbours Bira and Sembilan Islands, as well as among Donggala and Spermonde, Bira, and Kendari. Surprisingly, Manado as the most northern population, showed the only significant value towards Kendari (Table 15). The population in Sembilan Islands was not significant different to any other population around Sulawesi, although it is far away to some sites, for example Manado, and quite remotely located in the Bone Bay.

The genic and genotypic differentiation showed a more detailed structure around Sulawesi, with mainly significant differentiations among sites. Non-significant G-values were found between Manado and Donggala, which are neighbouring populations but still around 500 km apart, and between Bira and the populations in Puntondo, the Sembilan Islands as well as Kendari. Kendari shows in this analysis a strong differentiation as well to nearly all sites around Sulawesi, except to Sembilan Islands and Bira. In Sembilan Islands there was no significant differentiation detected to any of the other sites (Appendix 2).

In this dataset, there was no correlation of the geographic and genetic distances found by the Mantel-Test (r = 0.20, p = 0.195).

Table 15: Pairwise F_{ST}-values for 23 populations of *A .ocellaris* in the Indo-Malay Archipelago. Significant values (after correction for multiple tests following the false discovery rate procedure, Benjamini & Hochberg 1995) are marked with an asterisk. For abbreviations see Table 10.

	Sp	Pu	Bi	Do	Ma	Ke	Se	Sa	BI	KK	Bt	Pa	PS	Ka	Ba	Ko	Ku	Ce	Mi	Pi	SB	SP
Pu	0.012																					
Bi	0.013	0.028																				
Do	0.012*	0.010	0.036*																			
Ma	0.007	0.015	0.019	0.013																		
Ke	0.020*	0.035*	0.002	0.034*	0.025*																	
Se	0.006	0.022	-0.007	0.024	-0.006	0.000																
Sa	0.017*	0.013	0.027*	-0.001	0.006	0.032*	0.003															
BI	0.012	0.023	0.007	0.025*	0.005	0.021	-0.009	0.029*														
KK	0.029*	0.022	0.076*	0.012	0.011	0.060*	0.034*	0.006	0.045*													
Bt	0.095*	0.078*	0.178*	0.077*	0.103*	0.136*	0.124*	0.070*	0.135*	0.047*												
Pa	0.090*	0.124*	0.102*	0.137*	0.136*	0.139*	0.137*	0.155*	0.119*	0.186*	0.256*											
PS	0.045*	0.078*	0.057*	0.080*	0.077*	0.085*	0.066*	0.094*	0.067*	0.125*	0.193*	0.009										
Ka	0.055*	0.080*	0.063*	0.099*	0.086*	0.093*	0.081*	0.112*	0.069*	0.135*	0.196*	0.015*	-0.004									
Ba	0.011	0.039*	0.003	0.033*	0.029*	0.031*	0.015	0.041*	0.020	0.063*	0.131*	0.045*	0.010	0.018								
Ko	0.002	0.029	0.011	0.017	0.015	0.030*	0.010	0.017	0.017	0.035*	0.101*	0.076*	0.026	0.037*	-0.012							
Ku	0.016*	0.036*	0.005	0.043*	0.016	0.007	0.004	0.028*	0.024	0.055*	0.147*	0.131*	0.073*	0.075*	0.033*	0.018						
Ce	0.002	0.020	0.009	-0.002	-0.012	0.019*	-0.009	-0.007	-0.005	0.014	0.107*	0.130*	0.063*	0.084*	0.008	-0.003	0.020					
Mi	0.029*	0.045*	0.013	0.050*	0.029*	0.008	0.023	0.049*	0.032*	0.076*	0.193*	0.136*	0.086*	0.095*	0.052*	0.043*	0.000	0.026*				
Pi	0.030*	0.058*	0.039	0.052*	0.038*	0.028*	0.039*	0.048*	0.035	0.065*	0.208*	0.133*	0.084*	0.100*	0.053*	0.044*	0.021	0.031	0.001			
SB	0.033*	0.051*	0.038	0.038*	0.026	0.030*	0.036*	0.035*	0.052*	0.046*	0.178*	0.159*	0.100*	0.120*	0.059*	0.042*	0.015	0.023	-0.008	-0.013		
SP	0.019*	0.035	0.000	0.045*	0.024	0.005	0.000	0.035*	0.020	0.063*	0.176*	0.114*	0.063*	0.078*	0.031	0.023	0.011	0.022	-0.009	0.002	0.003	
TB	0.027	0.036	0.001	0.040*	0.041*	-0.003	0.012	0.038*	0.023	0.069*	0.187*	0.140*	0.092*	0.092*	0.029	0.024	0.013	0.035	0.005	0.006	0.023	0.000

4.4.3.3. Indo-Malay Archipelago

The Bayesian analysis revealed a structure of three clusters in the large scale dataset including all sample sites (Fig. 10c). The frequencies of the clusters in each population showed that one cluster (red, Fig. 8) was mainly presented in Padang, Indian Ocean, and the Java Sea (PS, Ka), but also in large proportions in Bali and Komodo. Lower frequencies were found in southwest Sulawesi and Donggala (Makassar Strait), and even lower ones in only three other sites. The second cluster (yellow, Fig. 8) was found with highest frequencies in the sites along the western coast of New Guinea, Molukkas (Misool), southeast Sulawesi (Kendari), south Sulawesi (Bira) and Timor (Kupang). Slightly lower frequencies could be observed at other sites in Sulawesi, and also at sites in northern Borneo and Cebu. Low frequencies were as well found further west in Komodo, Bali, Karimunjava and Pulau Seribu. The last cluster (blue, Fig. 8) was dominant in the South China Sea, northern Borneo and Cebu, but as well present with high frequencies in North Sulawesi and Bone Bay (Sembilan Islands). Lower frequencies were present along the way of the Indonesian Throughflow (ITF), including New Guinea and Misool.

AMOVA of the whole dataset supported the occurrence of a population structure by a significant F_{ST}-value of 0.0478 ($p < 0.001$). The structure on the large scale was therefore three- to fourfold higher than around Sulawesi. In the hierarchical AMOVA, the highest F_{CT}-value was given when the dataset was divided into three groups: (1) Batam (South China Sea), (2) Padang, Pulau Seribu and Karimunjava (Indian Ocean and Java Sea), and (3) all other populations (Table 16). When Bali was assigned to the Indian Ocean/Java Sea group, the value was only slightly lower. Testing of four groups revealed the highest value when New Guinea and Misool were separated from the large central group and when Padang and the Java Sea populations were divided into two separate groups (both F_{CT}= 0.06745, Table 16). Testing five groups resulted in a combination of the two latter groupings, with an only slightly lower F_{CT}-value (Table 16).

Table 16: The highest F_{CT}-values of the hierarchical AMOVA, testing different groupings of the large scale microsatellite dataset (Indo-Malay Archipelago) of *A. ocellaris*. All shown groupings were highly significant (*p*-values <0.001).

Number of groups	Groupings	F_{CT}-value
3 Groups	[Bt][Pa, PS, Ka][all others]	0.08229
	[Bt][Pa, PS, Ka, Ba][all others]	0.07296
4 Groups	[Bt][Pa, PS, Ka][Mi, Pi, SB, SP, TB][all others]	0.06745
	[Bt][Pa][PS, Ka][all others]	0.06745
5 Groups	[Bt][Pa][PS, Ka][Mi, Pi, SB, SP, TB][all others]	0.06714

The groupings of the hierarchical AMOVA were supported in the pairwise comparisons by high and significant F_{ST}-values between Batam and all other sites, as well as Padang that showed the only non-significant value to Pulau Seribu, and the lowest significant values to Karimunjava and Bali. Pulau Seribu and Karimunjava exhibited high significant values to nearly all other sites (Table 15). Kota Kinabalu in northern Borneo had high F_{ST}-values, except to the sites in northern Sulawesi, Cebu, and Sangalaki. Surprisingly, the F_{ST}-value to Banggi Islands, which is closest, was significant, but not very high. The sites in New Guinea and Misool had as well high F_{ST}-values to the other regions, except to southeastern Sulawesi (Bi, Ke, Se) and Timor (Ku). Generally, the northernmost sites of the central group (north of Spermonde, east of Batam) showed many low or non-significant values among each other. Cebu exhibited significant F_{ST}-values to Kendari and Misool, as well as to the other major groups (Batam and the Indian Ocean/Java Sea). The southern sites (south of Donggala, east of Karimunjava) also revealed many non-significant and low F_{ST}-values among each other. The sites in New Guinea and Misool showed only non-significant F_{ST}-values among each other (Table 15).

The test for population differentiation (χ^2-test for genic and genotypic differentiation), revealed a stronger structure, with even more significant G-values (Goudet et al. 1996; Appendix 2). Especially, the genic differentiation showed significant G-values among most sites. Non-significant G-values were found among Manado and the other northern sites, including Donggala in the Strait of Makassar. Bira had non-significant G-values to most southern and southeastern sites (Pu, Ke, Se, Ba, Ko, Ku, Mi, SP, TB), and Sembilan Islands showed very little significant G-values, similarly to the pairwise comparison of Arlequin. Again, the New Guinea sites and Misool showed no significant differentiation among each other (Appendix 2).

The Mantel-Test revealed a significant, but not very high correlation between the geographic and genetic distances ($r = 0.441$, $p < 0.001$). This correlation was already indicated by higher

differentiation values between long distant locations and low or non-significant F_{ST}- and G-values within regions.

4.5. Discussion

4.5.1. Microsatellite characterisation

Microsatellite loci that have been isolated for the *A. polymnus* and *A. percula* have been tested for *A. ocellaris* and six were selected for the conducted analyses.
The used microsatellite loci showed a high variability in their diversity, therefore the mean values over all loci had a large standard deviation. Generally, the three tetranucleotide microsatellites of *A. percula* showed higher diversity levels in *A. ocellaris* than the three dinucleotide loci of *A. polymnus*, although dinucleotide motives are suggested to generally display higher mutation rates (Chakraborty et al. 1997). However, in a study on human populations relatively higher mutation rates in tetra-nucleotide motif microsatellites were observed (Ellegren 2000). Apart from that, the utilised loci behaved similar in most analyses, but locus 65 showed different patterns in diversity between populations.
The number of alleles, as well as the diversity were similar for *A. ocellaris* and *A. polymnus* in the loci 120 and 65, whereas locus 45 revealed a larger number of alleles in *A. ocellaris*, which is most probably due to the larger dataset (432 compared to 100 individuals in *A. polymnus*, Quenouille et al. 2004) and a wider geographical range of sampling (in Quenouille et al. 2004 all samples were from New Guinea). Surprisingly, the heterozygosities of locus 45 were lower compared to *A. polymnus* (Quenouille et al. 2004), which might be due to the strong dominance of one allele found in *A. ocellaris*. The heterozygosities for locus 120 were similar in both studies and in locus 65 higher heterozygosities could be found in *A. ocellaris*. Since the microsatellite loci of *A. percula* are not published yet, there was no analyzed dataset at hand for comparison.

4.5.2. Genetic variability

The gene diversity, in most studies expressed as the expected heterozygosity, in *A. ocellaris* (0.491-0.806) was in a similar range as the expected heterozygosities of other marine fish species (0.86 in *Gadus morhua*, Bentzen et al. 1996; 0.69-0.93 in *Dicentrarchus labrax*, García De Leon et al. 1997; 0.75-0.85 in *Epinephelus marginatus*, De Innocentiis et al. 2001;

0.48-0.66 in five other species of *Epinephelus*, Nugroho et al. 1998). Only the population of Batam showed a reduced diversity compared to the other populations of *A. ocellaris* as well as to most of the other studies on different marine fish species. A reduction in diversity was previously found in Batam by the analysis of the mitochondrial control region of the same populations of *A. ocellaris* (Timm & Kochzius 2008). This reduction was even more pronounced in five of the six used loci in this study. In concordance to the previous study, Batam seems to have undergone a population reduction or local extinction, probably due to over exploitation for the aquarium trade. The demand for the species *A. ocellaris* in the aquarium trade and its exploitation is very high - it is one of the most traded marine aquarium fish species worldwide (Wabnitz et al. 2003). The island of Batam is located in direct proximity to Singapore, so caught fish could be easily shipped to the wholesalers in Singapore for the international market. Local divers reported that *A. ocellaris* could not be found at their dive sites after its popularity increase due to the movie "Finding Nemo" in 2003. However, during our sampling in 2005, we could find it on coastal coral reefs, although the reefs were highly impacted by sedimentation. This reduction or even extinction of the local *A. ocellaris* population, followed by either a re-colonization or growth of the remaining local population, could explain the low diversity of the population in Batam. The decrease of genetic diversity caused by over-exploitation was also found in other commercially important fish populations (e.g. Hauser et al. 2002).

In most populations, the expected and observed heterozygosities were similar, with a tendency to a heterozygote deficit, which was not significant in the test for deviation from HWE. This indicated that most of the populations were in HWE, with the exception of Manado, Bali, Komodo, Kupang and Triton Bay. The latter populations all showed a deviation from HWE due to a significant heterozygote deficit in the global test. Interestingly, these populations showed relatively high gene diversities and average observed heterozygosities. The deviation from HWE might be due to other factors than a real deficiency of heterozygote individuals, like the presence of null alleles in some loci, or the genetic exchange to other populations that causes a violation of the assumptions for the Hardy-Weinberg calculation. This violation might be expressed in a deviation from HWE (Rousset & Raymond 1995, Györffy et al. 2004). In the populations of Bali and Komodo, the possible presence of null-alleles was revealed in the loci CF27 and 45, respectively. These populations are in a central location for lineage mixing, based on the distribution of major clusters found with the Bayesian analysis. The Indian Ocean/Java Sea cluster is presented with quite high frequencies in the populations Bali and Komodo, but also the other two

clusters, one coming from the North-West and one from the East, were well mixed in these populations. Kupang had a higher proportion of the eastern cluster genotypes, but as well considerable frequencies of the other two clusters. Such lineage mixing was also present in a few other populations, but obviously did not lead to a deviation from HWE.

In the population of Manado, no null alleles were detected and it is located rather far away from the other populations, making lineage mixing/migration causing deviation from HWE more difficult. This population showed linkage disequilibrium in some combinations of loci. High degrees of linkage disequilibrium were found in small, relatively isolated and demographically stable populations in humans (Laan & Pääbo 1997, Zavattari et al. 2000). A relative stability in population size could be assumed for the population of Manado, as northern Sulawesi was only little effected by the sea level changes in the Pleistocene, but the assumption that Manado population is notably smaller than many of the other sites would need further studies on population sizes.

Triton Bay is at the easternmost site sampled in the present study and southernmost of the locations in New Guinea. In this population only two of the three major groups are presented, therefore lineage mixing should not be very pronounced. Notably, the sample size of this population is one of the lowest in the dataset, which could have an influence on the HWE-calculations (Györffy et al. 2004).

4.5.3. Population differentiation

4.5.3.1. Spermonde Archipelago

The significant but shallow structure in Spermonde Archipelago shown in the population genetic analysis using the mitochondrial control region (Timm & Kochzius 2008), was revealed only by an hierarchical AMOVA analysis grouping the islands of the different shelf areas together (midshelf, outershelf, northern outershelf and outer rim). Additionally, two comparisons between pairs of shelf areas (midshelf - outershelf and outershelf - northern outershelf/outer rim) gave significant F_{ST}-values. When these analyses were applied to the microsatellite dataset, they did not give any significant differentiation value, although their diversity measures were high.

One of the possible reasons that the previously found shallow population structure in the Spermonde Archipelago could not be found with the microsatellite dataset, might be a homoplasy of alleles, because of limitations to the allele size, which leads to an under-estimation of genetic differentiation (Nauta & Weissing 1996, Garza et al. 1995). It is not

probable for this study, because on the other scales differentiations could be found, and were varying between relatively light, and strong restrictions in gene flow, which would not be the case if a saturation of the allele size was present in the dataset. Another reason for generally low F_{ST}-values could be the high variability among the diversity measures of the different loci (Hedrick 2005). In this case, the values would be low, but it would not necessarily result in non-significant values. The calculation of a different genetic statistic, the G_{ST}-value was presented to be less sensible to variability among loci, but the overall G_{ST}-value was as well very low for the Spermonde dataset and nearly the same for the large scale dataset. A problematic factor for detecting population structures could alsoways be selection on certain loci (Garza et al. 1995, Barrière & Félix 2007). This possibility is unlikely, because (1) selection would not affect all loci, but most of them show similar results in the present study, and (2) on the larger scales the results were very similar to the CR data (Timm & Kochzius 2008). For some populations of the present study we could only analyse small sample sizes. Small sample sizes together with a large total number of alleles can lead to the reduction of statistical power, but not to the value itself (Ruzzante 1998). The sample sizes in Spermonde were low for some areas (pooled outershelf = 6, and pooled midshelf = 16). Actually, these were the most diverged populations in the previous study on the CR. The northern outershelf and outer-rim groups consisted of larger number of individuals (32 and 36, respectively), but these two sites were not significantly different from each other in the CR analysis. A significant population structure similar to the CR dataset might not be detectable, because of the low sample sizes in the outershelf and midshelf areas. The resolution of the microsatellites was not higher than that of the CR.

4.5.3.2. Sulawesi

A low but significant population structure was revealed for Sulawesi, although not in all analyses. The populations around Sulawesi were asigned to two groups: (1) North-West, and (2) South-East (Table 5). The pairwise comparison revealed that Bira in the South seems to be connected to both groups. This population structure follows the major current patterns around the island of Sulawesi, with the strong Indonesian Throughflow (ITF) from the North through the Makassar Strait along the western coast of the island, reversing major currents at the southern coastline, and only very little water movement up the eastern coast of Sulawesi (Wyrtki 1961). These patterns can explain the strong differentiation of Kendari, because little exchange of water and therefore larvae occurs. Exchange is common only between close sites, probably through minor coastal currents or larval movement (Bradbury & Snelgrove 2001).

On the contrary, the long distances along the western coastline are overcome easily by the strong ITF, leading to a better connectivity among sites.

The CR analysis showed a similar genetic pattern, but Manado was differentiated and the isolation by distance analysis was significant. Seemingly, a stronger gradient along the western coast of Sulawesi was detected in the mitochondrial DNA, leading to a stronger separation among long distanced sites, which was intangible in the microsatellite dataset. This slight deviation of the microsatellite results from those previously found in the CR dataset could be due to a reflection of the situation at different times. Since microsatellites can detect very recent genetic structures, and mitochondrial markers are said to reflect rather historical patterns Manado might have experienced a stronger separation during sea level low stands. Although the ITF was present during the Pleistocene (Kuhnt et al. 2004), it was probably largely reduced by a constriction of the Makassar Strait (Voris 2000), reducing the connection between northern and southern Sulawesi. With the sea level rise this reduction disappeared and the strong ITF enhanced lineage mixing between sites, reflected by no obvious differentiation among Manado and southern Sulawesi in the microsatellite dataset. Bira is probably influenced by the reversing currents from Flores Sea to the Java Sea and vice versa, so exchange in both directions is possible. The population of Sembilan Islands was not differentiated to any other site in the microsatellite analysis, which was rather due to low statistical power because of the low sample size (only 8 individuals) than to a high connectivity. In the CR analysis, the results for Sembilan Islands were similar and low statistical resolution was also expected (Timm & Kochzius 2008).

4.5.3.3. Indo-Malay Archipelago

The populations across the Indo-Malay Archipelago could be assigned to three groups based on the microsatellite data, instead to four revealed by the CR analysis (Timm & Kochzius 2008). The samples from New Guinea were included into the central group, the groups of Batam and the Indian Ocean/Java Sea were identical (highest F_{CT}-value in the hierarchical AMOVA). Again, this deviation between the two datasets could be due to different resolution in the marker systems. Whereas the CR analysis detected the separation of populations in the different geographically isolated sea areas, the microsatellite dataset revealed mixing of lineages in the centre of the archipelago. The Bayesian analysis showed that the population in Bali is influenced by the populations in Indian Ocean/Java Sea populations, similar to the CR analysis. Bali has also affinities to Komodo that was rather correlated to the central group in the CR data, as well as in the hierarchical AMOVA of the microsatellite dataset. Obviously,

there is a gradient of Indian Ocean/Java Sea clade frequencies to the East, indicating a lineage mixing in that direction along the currents going from the Java Sea towards the Flores Sea. The occurrence of the Indian Ocean/Java Sea clade in Donggala suggests that dispersal against the ITF is probably facilitated by a seasonal northward current in Makassar Strait along the coast of eastern Borneo (Wyrtki 1961).

Batam and Padang showed a strong differentiation in the present study, very similar to the separation revealed in the CR analysis (Timm & Kochzius 2008), although these two populations have very different genetic structures. Batam is strongly separated to all other populations studied and additionally it has a reduced genetic diversity revealed by the microsatellite and the CR analyses. In the microsatellite analysis the population in Batam has a quite low F_{st}-value to Kota Kinabalu, which is also located in the South China Sea (northern Borneo). Kota Kinabalu could be rather grouped with the other populations of northern Borneo. Possibly, both populations in the South China Sea share a common history with a separation of this sea basin from the Sulu Sea in the Northeast and the Flores Sea, as well as the Indian Ocean in the South and West, during sea level low stands in glacial epochs (Voris 2000). Since the last glacial in the Pleistocene, connectivity between Kota Kinabalu and the close by populations of the Sulu Sea was possible and this more recent influence might have resulted in a grouping of these populations. Unfortunately, no other populations from the South China Sea or places North-West of the Malakka Strait were analysed, so a connection between Batam and other locations could not be tested.

Padang showed, next to a strong differentiation, high diversities in the microsatellite analysis. The CR study revealed a high haplotype, but low nucleotide diversity, underlining the separation of a large Indian Ocean population of *A. ocellaris* during sea level low stands, leading to the evolution of many different but closely related haplotypes. The only connection to populations within the archipelago seems to be through the Sunda Strait to Pulau Seribu and Karimunjava, and further East to Bali and to some extent Komodo. The latter two might as well be somewhat connected to Padang along the Indian Ocean side of Java, as there are major currents in that region. As Padang and the Java Sea populations are strongly differentiated in both marker systems, exchange between this genetic lineage and the rest of the archipelago is still restricted or very slow.

The differentiation of the populations from the western coast of New Guinea and the island of Misool found in the CR study was reflected in the present study by many significant and quite high pairwise F_{ST}-values in most comparisons. The Bayesian analysis and hierarchical AMOVA could not detect this separation, but when 4 groups were tested in the hierarchical

AMOVA, the two highest F_{ct}-values were shown with the New Guinea/Misool populations as a separate group and with Padang as an extra group (same F_{CT}-value). When five groups were tested, consequently a combination of the best results for four groups came up with following groupings: (1) Indian Ocean (Padang), (2) Java Sea (Pulau Seribu, Karimunjava), (3) Batam, (4) New Guinea and Misool, and (5) Central Indo-Malay Archipelago (all other populations). The less pronounced differentiation of the New Guinea/Misool group in the microsatellite study could indicate a slow but ongoing lineage mixing with southern Sulawesi and Kupang. The stronger separation in the CR would reflect formerly stronger restrictions in exchange to the central part of the Archipelago, probably caused by reduced currents and more land barriers in the area during sea level low stands.

Generally, the results of the microsatellite analysis on the middle and large scale could support the findings of the CR study done with mostly the same individuals of *A. ocellaris*. Although estimations of genetic structure are based on two marker systems with different origin (mitochondrial and nuclear DNA) and to some extent different algorithms, both analyses revealed a very similar population structure in the Indo-Malay Archipelago. The differentiation of lineages from the Indian and the Pacific Ocean side of the archipelago were already detected in many coral reef organisms (McMillan & Palumbi 1995, Williams & Benzie 1998, Duda & Palumbi 1999, Nelson et al. 2000, Barber et al. 2002, 2006, Kochzius et al. 2003, Froukh & Kochzius 2008, Kochzius & Nuryanto 2008, Timm et al. 2008), as well as pelagic species (Perrin & Borsa 2001). Other pelagic fish (Alvarado Bremer et al. 1998, Chow et al. 1997, 2000, Borsa 2003) and marine organism like the sea urchin *Diadema savignyi* (Lessions et al. 2001) did not show this kind of separation.

Interestingly, the CR shows a high potential for population genetic studies in anemonefish, although mitochondrial markers were criticised not to be suitable because of possible selective sweeps. Therefore, it was suggested that results based on mitochondrial markers should be interpreted with caution (Bazin et al. 2006). The structure revealed by the previous study based on CR was mostly supported by the microsatellite study in this paper. Even more, the CR showed stronger population differentiations in many cases, maybe reflecting the traces of former isolations of lineages in different sea areas during sea level low stands.

In order to reach a higher resolution from microsatellites, especially for small scale studies, more loci should be tested for *A. ocellaris* and additionally it would be important to have a larger sample size in order to increase the statistical power. Generally, the comparison of different genetic markers or marker systems is very important to draw correct conclusions and get additional information about demographic conditions on historic and recent time scales.

The study has also shown that at least in some cases mitochondrial markers are powerful tools for population genetic analyses.

4.6. Acknowledgements

We would like to thank the German Federal Ministry of Education and Research (BMBF, grant no. 03F0390B) for funding the project "Molecular Genetics as a Tool for the Management of Marine Ornamentals in Sulawesi (Indonesia)" in the framework of the SPICE project (Science for the Protection of Indonesian Coastal Marine Ecosystems); the colleagues from the University of Bremen, especially A. Nuryanto for help during field work, and D. Blohm; the colleagues from the University of Perpignan (France) especially C. Hervet and E. Rochel for support during lab work; U. John, T. Alpermann and A. Müller from the Alfred-Wegener-Institute Bremerhaven (Germany) for providing technical facilities, analytical support and help during lab work; colleagues from Universitas Hasanuddin (Makassar, Indonesia) for logistical support during field work in Spermonde Archipelago, especially J. Jompa.

4.7. References

Allen GR, Werner TB (2002) Coral reef fish assessment in the "coral triangle" of southeastern Asia. *Environmental Biology of Fishes*, 65, 209-214.

Alvarado Bremer JR, Stequert B, Robertson NW, Ely B (1998) Genetic evidence for inter-oceanic subdivision in bigeye tuna (*Thunnus obesus*) populations. *Marine Biology*, 132, 547-575.

Árnason E (2004) Mitochondrial cytochrome *b* DNA variation in the high-fecundity Atlantic cod: trans-Atlantic clines and shallow gene genealogy. *Genetics*, 166, 1871-1885.

Arnaud S, Bonhomme F, Borsa P (1999) Mitochondrial DNA analysis of the genetic relationships among populations of scad mackerel (*Decapterus macarellus, D. macrosoma, D. russelli*) in South-East Asia. *Marine Biology*, 135, 699-707

Barber PH, Palumbi SR, Erdmann MV, Moosa MK (2002) Sharp genetic breaks among populations of *Haptosqulla pulchella* (Stomatopoda) indicate limits to larval transport patterns, causes and consequences. *Molecular Ecology*, 11, 659-674.

Barber PH, Erdmann MV, Palumbi SR (2006) Comparative phylogeography of three codistributed stomatopods: origins and timing of regional lineage diversification in the coral triangle. *Evolution*, 60, 1825-1839.

Barrière A, Félix M (2007) Temporal dynamics and linkage disequilibrium in natural *Caenorhbditis elegans* populations. *Genetics*, 176, 999-1011.

Bazin E, Glémin S, Galtier N (2006) Population size does not influence mitochondrial genetic diversity in animals. *Science*, 312, 570-572.

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society*, Series B, 57, 289-300.

Bentzen P, Taggart CT, Ruzzante DE, Cook D (1996) Microsatellite polymorphism and the population structure of Atlantic cod (*Gadus morhua*) in the northwest Atlantic. *Canadian Journal of Fishery and Aquatic Science*, 53, 2706-2721.

Benzie JAH, Ballment E, Forbes AT, et al. (2002) Mitochondrial DNA variation in Indo-Pacific populations of the giant tiger prawn, *Penaeus monodon. Molecular Ecology*, 11, 2553–2569.

Benzie JAH, Smith C, Sugama K (2003) Mitochondrial DNA reveals genetic differentiation between Australian and Indonesian pearl oyster *Pinctada maxima* (Jameson 1901) populations. *Journal of Shellfish Research*, 22, 781-787.

Bernardi G, Holbrook SJ, Schmitt RJ (2001) Gene flow at three spatial scales in a coral reef fish, the three- spot dascyllus, *Dascyllus trimaculatus*. *Marine Biology*, 138, 457-465.

Berry OF (2006) Mitochondrial DNA and population size. *Science*, 314, 1388.

Borsa P (2003) Genetic structure of the round scad mackerel *Decapterus macrosoma* (Carangidae) in the Indo-Malay archipelago. *Marine Biology*, 142, 575-581.

Bradbury IR, Snelgrove PVR (2001) Contrasting larval transport in demersal fish and benthic invertebrates: the role of behaviour and advective processes in determining spatial pattern. *Canadian Journal of Fisheries and Aquatic Sciences*, 58, 811-823.

Briggs JC (2000) Centrifugal speciation and centres of origin. *Journal of Biogeography*, 27, 1183-1188.

Carlsson J, McDowell JR, Díaz-Jaimes P, Carlsoon JEL, Boles SB, Gold JR, Graves JE (2004) Microsatellite and mitochondrial DNA analyses of atlantic bluefin tuna (*Thunnus thynnus thynnus*) population structure in the Mediterranean Sea. *Molecular Ecology*, 13, 3345-3356.

Casey SP, Hall HJ, Stanley HF, Vincent ACJ (2004) The origin and evolution of sea horses (genus *Hippocampus*): a phylogenetic study using the cytochrome *b* gene of mitochondrial DNA. *Molecular Phylogenetics and Evolution*, 30, 261-272.

Chakraborty R, Kimmel M, Stivers DN, Davison LJ, Deka R (1997) Relative mutation rate at di., tri-, and tetranucleotide microsatellite loci. *Proceedings of the National Academy of Sciences USA*, 94, 1041-1046.

Chenoweth SF, Hughes JM, Connolly RC (2002) Phylogeography of the pipefish, *Urocampus carinirostris*, suggests secondary intergradation of ancient lineages. *Marine Biology*, 141, 541-547.

Chow S, Okamoto H, Uozumi Y, Takeuchi Y, Takeyama H (1997) Genetic stock structure of swordfish (*Xiphias gladius*) inferred by PCR-RFLP analysis of the mitochondrial control region. *Marine Biology*, 127, 359-367.

Chow S, Okamoto H, Miyabe N, Hiramatsu K, Barut N (2000) Genetic divergence between Atlantic and Indo-Pacific stocks of bigeye tuna (*Thunnus obesus*) and admixture around South Africa. *Molecular Ecology*, 9, 221-227.

Concheiro Pérez GA, Říčan O, Ortí G, Bermingham E, Doadrio I, Zardoya R (2007) Phylogeny and biogeography of 91 species of heroine cichlids (Teleostei: Cichlidae) based on sequences of the cytochrome *b* gene. *Molecular Phylogenetics and Evolution,* 43, 91-110.

DeInnocentiis S, Sola L, Cataudella S, Bentzen P (2001) Allozyme and microsatellite loci provide discordant estimates of population differentiation in the endangered dusky grouper (*Epinephelus marginatus*) within the Mediterranean Sea. *Molecular Ecology*, 10, 2163-2175.

Duda TF, Palumbi SR (1999) Population structure of the black tiger prawn, *Penaeus monodon*, among western Indian Ocean and western Pacific populations. *Marine Biology*, 134, 705-710.

Ellegren H (2000) Heterogeneous mutation processes in human microsatellite DNA sequences. *Nature*, 24, 400-402.

Eyre-Walker A (2006) Size does not matter for mitochondrial DNA. *Science*, 312, 537-538.

Excoffier L, Laval LG, Schneider S (2005) Arlequin vers. 3.1: an integrated software package for population genetic data analysis. *Evolutionary Bioinformatics online*, 1, 47-50.

Farias IP, Ortí G, Sampaio I, Schneider H, Meyer A (2001). The cytochrome b gene as a phylogenetic marker: The Limits of resolution for analysing relationships among cichlid fishes. *Journal of Molecular Evolution*. 53, 89-103.

Fautin DG & Allen GR (1994) Anemonenfische und ihre Wirte. Tetra-Verlag, Melle, 1-168.

Froukh T, Kochzius M (2007) Genetic population structure of the endemic fourline wrasse (*Larabicus quadrilineatus*) suggests limited larval dispersal distances in the Red Sea. *Molecular Ecology*, 16, 1359-1367.

Froukh T, Kochzius M (2008) Species boundaries and evolutionary lineages in the blue green damselfishes *Chromis viridis* and *Chromis atripectoralis* (Pomacentridae). *Journal of Fish Biology*, 72, 451-457.

Garcia de Leon FJ, Chikhi L, Bonhomme F (1997) Microsatellite polymorphism and population subdivision in natural populations of European sea bass *Dicentrarchus labrax* (Linnaeus, 1758). *Molecular Ecology*, 6, 51-62.

Garza JC, Slatkin M, Freimer NB (1995) Microsatellite allele frequencies in humans and chimpanzees, with implications for constrints on allele size. *Molecular Biology and Evolution*, 12, 594-603.

Glaubitz JC (2004) CONVERT: A user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages. *Molecular Ecology Notes*, 4, 309-310.

Gordon AL, Fine RA (1996) Pathways of water between the Pacific and Indian oceans in the Indonesian seas. *Nature*, 379, 146-149.

Goudet J (1995). Fstat version 1.2: a computer program to calculate F-statistics. *Journal of Heredity*, 86, 485-486.

Goudet J, Raymond M, de Meenüs T, Rousset F (1996) Testing differentiation in diploid populations. *Genetics*, 144, 1933-1940.

Györffy B, Kocsis I, Vásárhelyi B (2004) Biallelic genotype distributions in papers published in *Gut* between 1998 and 2003: altered conclusions after recalculating the Hardy-Weinberg equilibrium. *International Journal of Gastroenterology and Hepatology*, 53, 614-616.

Hall R (1996) Reconstructing Cenozoic SE Asia. In: Hall R, Blundell D (eds) Tectonic Evolution of Southeast Asia. Geological Society of London Special Publication, No. 106, pp. 153-184.

Hauser L, Adcock GJ, Smith PJ, Bernal Ramírez JH, Carvalho GR (2002) Loss of microsatellite diversity and low effective population size in an overexploited population of New Zealand snapper (*Pagus auratus*). *Proceedings of the National Academy of Sciences USA*, 99, 11742-11747.

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

Hoeksema BW (2007) Delineation of the Indo-Malayan centre of maximum biodiversity: The Coral Triangle. In: Renema W (ed) Biogeography, time and place: distributions, barriers and islands. Topics in Geobiology 29: 117-178.

Jensen JL, Bohonak AJ, Kelley ST (2005) Isolation by distance, web service. BMC *Genetics*, 6(13), 1-6.

Jones AG, Ardren WR (2003) Methods of parentage analysis in natural populations. *Molecular Ecology*, 12, 2511-2523.

Kellogg KA, Markert JA, Stauffer jr. JR, Kocher TD (1995) Microsatellite variation demonstrates multiple paternity in lekking cichlid fishes from Lake Malawi, Africa. *Proceedings of the Royal Society of London*, Series B, 260, 79-84.

Kochzius M, Blohm D (2005) Genetic population structure of the lionfish *Pterois miles* (Scorpaenidae, Pteroinae) in the Gulf of Aqaba and northern Red Sea. *Gene*, 347, 295-301.

Kochzius M, Nuryanto A (2008) Strong genetic population structure in the boring giant clam *Tridacna crocea* across the Indo-Malay Archipelago: implications related to evolutionary processes and connectivity. *Molecular Ecology*, 17, 3775-3787

Kochzius M, Söller R, Khalaf MA, Blohm D (2003) Molecular phylogeny of the lionfish genera *Dendrochirus* and *Pterois* (Scorpaenidae, Pteroinae) based on mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, 28, 396-403.

Kuhnt W, Holbourn A, Hall R, Zuvela M, Käse R (2004) Neogene history of the Indonesian throughflow. In: Clift P, Hayes D, Kuhnt W, Wang P (eds) Continent-ocean interactions in the East Asian marginal seas, AGU Monograph, 149, 299-320.

Laan M, Pääbo S (1997) Demographic history and linkage disequilibrium in human populations. *Nature Genetics*, 17, 435-438.

Lessions HA, Kessing BD, Pearse JS (2001) Population structure and speciation in tropical seas: global phylogeography of the sea urchin *Diadema*. *Evolution*, 55, 955-975.

Lourie SA, Green DM, Vincent ACJ (2005) Dispersal, habitat differences, and comparative phylogeography of Southeast Asia seahorses (Syngnathidae: *Hippocampus*). *Molecular Ecology*, 14, 1073-1094.

Mann HB, Whitney DR (1947) On a test of whether one of two random variables is stochastically larger than the other. *Annals of Mathematical Statistics*, 18, 50-60.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*, 27, 209-220.

McMillan WO, Palumbi SR (1995) Concordant evolutionary patterns among Indo-West Pacific butterflyfishes. *Proceedings of the Royal Society of London*, 260, 229-236.

Moll H (1983) Zonation and diversity of *Scleractinia* on reef off S.W. Sulawesi, Indonesia. PhD Thesis, University of Leiden, The Netherlands.

Mulligan CJ, Kitchen A, Miyamoto MM (2006) Comment on "populatin size does not influence mitochondrial genetic diversity in animals". *Science*, 314, 1390a.

Nauta MJ, Weissing FJ (1996) Constraints on allele size at microsatellite loci: implications for genetic differentiation. *Genetics*, 143, 1021-1032.

Nelson JS, Hoddell RJ, Chou LM, Chan WK, Phang VPE (2000) Phylogeographic structure of false clownfish, *Amphiprion ocellaris*, explained by sea level changes on the Sunda shelf. *Marine Biology*, 137, 727-736.

Norris AT, Bradley DG, Cunningham EP (2000) Parentage and relatedness determination in farmed Atlantic salmon (*Salmon salar*) using microsatellite markers. *Aquaculture*, 182, 73-83.

Nugroho E, Takagi M, Sugama K, Taniguchi N (1998) Detection of GT repeats microsatellite loci and their polymorphism for grouper of the genus Epinephelus. *Fisheries Science*, 64, 836-837.

Parker PG, Snow AA, Schug MD, Booton GC, Fuerst PA (1998) What molecules can tell us about populations: choosing and using a molecular marker. *Ecology*, 79, 361-382

Perrin C, Borsa P (2001) Mitochondrial DNA analysis of the geographic structure of Indian scad mackerel in the Indo-Malay archipelago. *Journal of Fish Biology*, 59, 1421-1426.

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

Quenouille B, Bouchenak-Khelladi Y, Hervet C, Planes S (2004) Eleven microsatellite loci for the saddleback clownfish *Amphiprion polymnus*. *Molecular Ecology Notes*, 4, 291-293.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, 86, 248-249.

Reilly A, Elliott NG, Grewe PM, Clabby C, Powell R, Ward RD (1999) Genetic differentiation between Tasmanian cultured Atlantic salmon (*Salmon salar* L.) and their ancestral Canadian population: comparison of microsatellite DNA and allozyme and mitochondrial DNA variation. *Aquaculture*, 173, 459-469.

Renema W, Troelstra SR (2001) Larger foraminifera distribution on a mesotrophic carbonate shelf in SW Sulawesi (Indonesia). *Palaeogeography Palaeoclimatology Palaeoecology,* 175, 125-146.

Rohfritsch A, Borsa P (2005) Genetic structure of Indian scad mackerel *Decapterus russelli*: Pleistocene vicariance and secondary contact in the central Indo-West Pacific seas. *Journal of Heredity*, 95, 315-326.

Rousset F, Raymond M (1995) Testing heterozygote excess and deficiency. *Genetics*, 140, 1413-1419.

Ruzzante DE (1998) A comparison of several measures of genetic distance and population structure with microsatellite data: bias and sampling variance. *Canadian Journal of Fisheries and Aquatic Sciences*, 55, 1-14.

Santini S, Polacco G (2006). Finding Nemo: Molecular phylogeny and evolution of the unususal life style of anemonefish. *Gene* 385, 19-27.

Shaw PW, Pierce GJ, Boyle PR (1999) Subtle population structuring within a highly vagile marine invertebrate, the veined squid, Loligo forbesi, demonstrated with microsatellite DNA markers. *Molecular Ecology*, 8, 407-417.

Sokal RR, Rohlfs FJ (1982) Biometry: the principles and practice of statistics in biological research. San Francisco: W.H. Freeman, 1969, p.776.

Tautz D (1989) Hypervariabflity of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research*, 17, 6463-6471.

Timm J, Kochzius M (2008) Geological history and oceanography of the Indo-Malay Archipelago shape the population structure in the false clown anemonefish (*Amphiprion ocellaris*). *Molecular Ecology*, 17, 3999-4014.

Timm J, Figiel M, Kochzius M (2008) Contrasting patterns in species boundaries and evolution of anemonefishes (Amphiprioninae, Pomacentridae) in the centre of marine biodiversity. *Molecular Phylogenetics and Evolution*, 49, 268-276.

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.

Vázquez JF, Pérez T, Albornoz J, Dominguez A (2000) Estimation of microsatellite mutation rates in *Drosophila melanogaster*. *Genetic Research*, 76, 323-326.

Voris HK (2000) Maps of Pleistocene sea levels in Southeast Asia: shorelines, river systems and durations. *Journal of Biogeography*, 27, 1153- 1167.

Wabnitz C, Taylor M, Green E, Razak T (2003) From ocean to aquarium. UNEP-WCMC. Cambridge, UK.

Wares JP, Barber PH, Ross-Ibarra J, Sotka EE, Toonen RJ (2006) Mitochondrial DNA and population size. *Science*, 314, 1388-1389.

Weber JL, Wong C (1993) Mutation of short tandem repeats. *Human Molecular Genetics*, 2, 1123-1128.

Whitten T, Mustafa M, Henderson GS (2002) The ecology of Sulawesi. In: The ecology of Indonesia series, Vol.IV. Periplus Editions Ltd. Pp. 105, 212-255.

Williams ST (2000) Species boundaries in the starfish genus *Linckia*. *Marine Biology*, 136, 137-148.

Williams ST, Benzie JAH (1998) Evidence of a biogeographic break between populations of a high dispersal starfish: congruent regions within the Indo-West Pacific defined by color morphs, mtDNA, and allozyme data. *Evolution*, 52, 87-99.

Wyrtki K (1961) Physical oceanography of the Southeast Asien waters. NAGA Report Vol.2, the University of California, Scripps Institution of Oceanography, LaJolla, California.

Zavattari P, Deidda E, Whalen M, Lampis R, Mulargia A, Loddo M, Eaves L, Mastio G, Todd, JA, Cucca F (2000) Major factors influencing linkage disequilibrium by analysis of different chromosome regions in distinct populations: demography, chromosome recombination frequency and selection. *Human Molecular Genetics*, 9, 2947-2957.

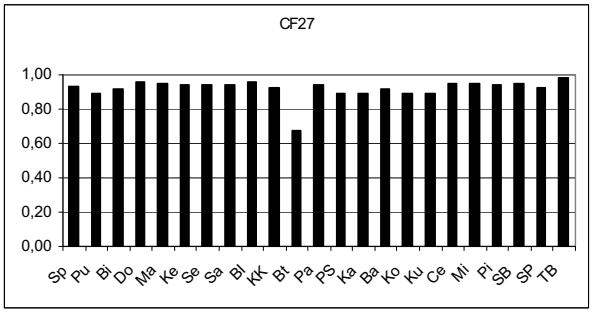
Appendix

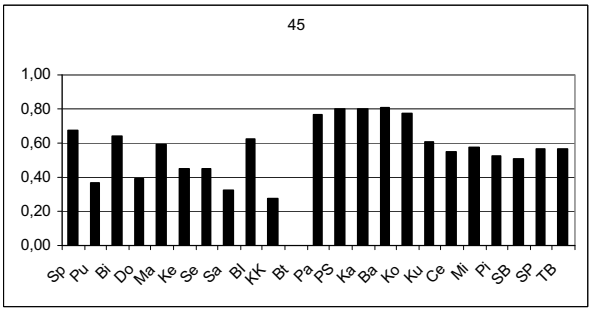
5. Appendix

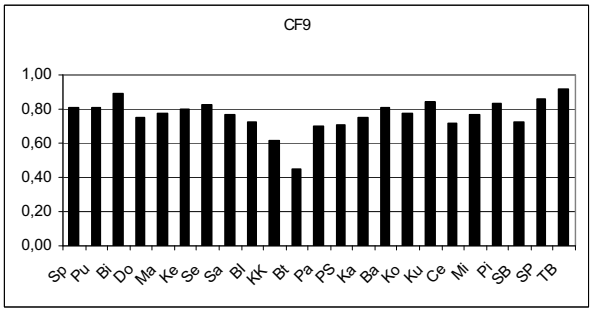
5.1. Appendix of "Microsatellite analysis revealed population patterns similar to findings of mitochondrial control region analysis in the false clown anemonefish (*Amphiprion ocellaris*)"

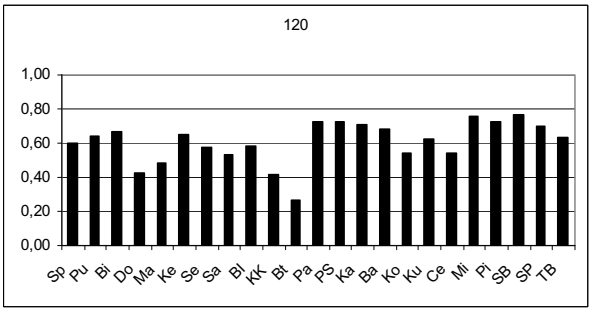
5.1.1. Appendix 1

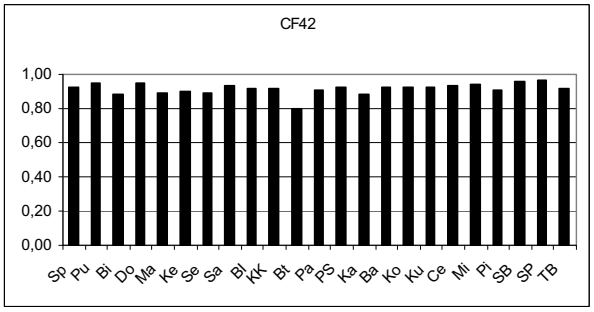
5.1.1.1. Gene diversity per microsatellite locus and population of *A. ocellaris*

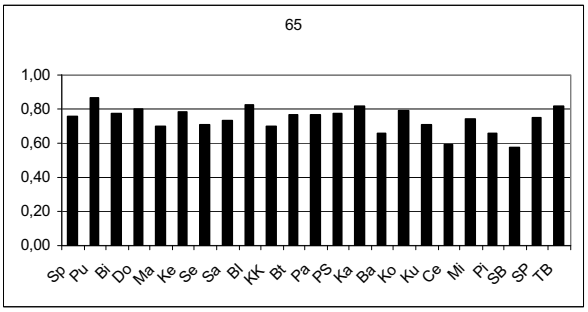




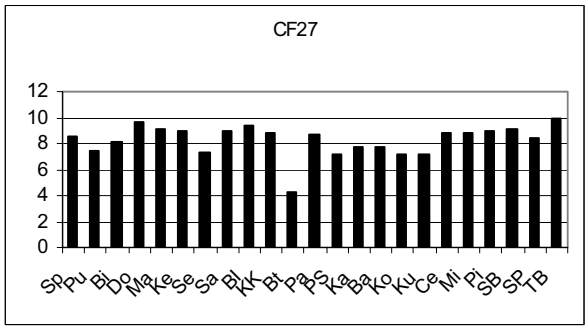


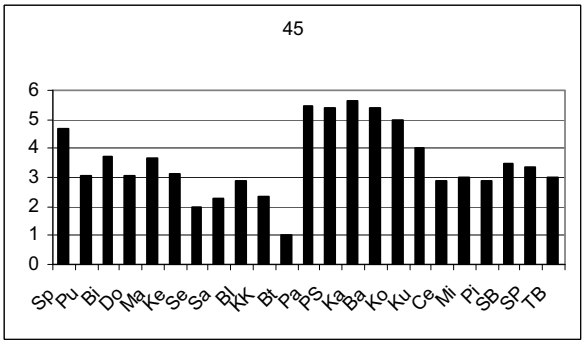


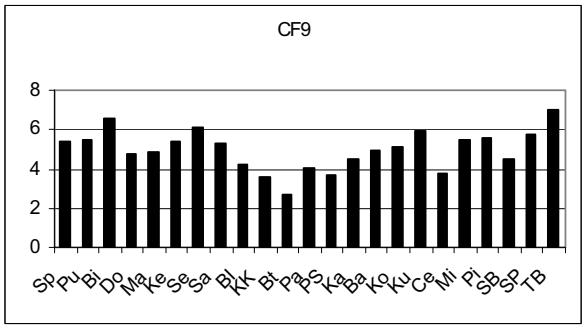


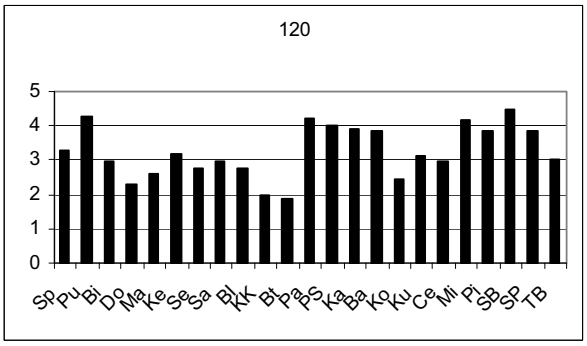


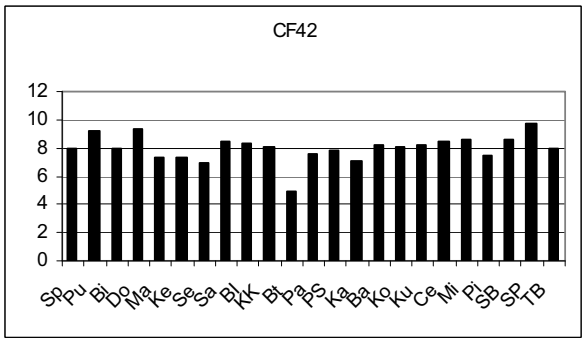
5.1.1.2. Allelic richness per microsatellite locus and population of *A. ocellaris*

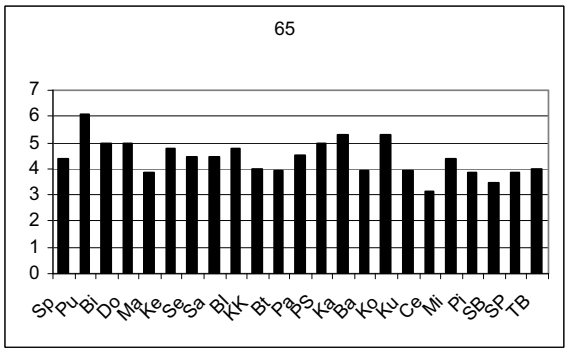












5.1.2. Appendix 2: Genic (below diagonal) and genotypic (above diagonal) differentiation of *A. ocellaris* populations, calculated with the χ^2-test (Genepop). Significant values (p= 0.05- 0.001) are indicated by * and highly significant values ($p < 0.001$) by **.

	Sp	Pu	Bi	Do	Ma	Ke	Se	Sa	BI	KK	Bt	Pa	PS	Ka	Ba	Ko	Ku	Ce	Mi	Pi	SB	SP	TB
Sp		33.21*	30.29*	Inf**	40.20**	59.00**	19.66	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	32.20*	25.89*	61.97**	44.09**	Inf**	Inf**	61.98**	47.21**	47.26**
Pu	34.70*		15.24	22.06*	20.85	32.31*	21.12*	22.81*	22.84*	35.24**	64.83**	Inf**	48.57**	Inf**	24.23*	16.23	31.47*	30.97*	27.17*	24.23*	33.42**	21.15*	13.61
Bi	28.86*	17.93		25.14*	18.61	18.78	7.76	24.88*	19.30	47.91**	Inf**	60.72**	Inf**	38.36**	12.12	11.31	17.91	28.67*	18.91	12.25	23.62*	13.04	7.69
Do	Inf**	23.54*	27.75*		19.09	45.63**	17.82	14.92	23.28*	19.35	Inf**	Inf**	Inf**	Inf**	28.38*	18.87	49.38**	19.95	35.81**	27.93*	29.58*	27.74*	20.85
Ma	37.95**	25.99*	23.43*	22.59*		26.33*	6.66	15.32	15.86	15.27	Inf**	Inf**	Inf**	Inf**	36.61**	24.48*	27.10*	11.87	31.63*	32.50*	29.90*	28.42*	25.28*
Ke	55.73**	35.40**	19.16	Inf**	31.56*		8.16	32.88*	26.14*	53.15**	Inf**	Inf**	Inf**	Inf**	42.45**	34.08*	17.20	37.32**	17.02	33.21*	38.89**	18.42	15.72
Se	13.75	20.11	12.74	18.89	8.14	7.54		11.18	12.92	23.44*	67.88**	Inf**	48.09**	Inf**	20.89	14.23	11.15	12.70	12.20	18.56*	27.92*	5.36	11.19
Sa	Inf**	25.37*	25.58*	15.32	16.29	35.98**	9.89		23.70*	14.89	Inf**	Inf**	Inf**	Inf**	Inf**	29.22*	Inf**	16.71	41.16**	34.94**	39.20**	30.49*	18.69
BI	Inf**	25.00*	19.29	23.33*	18.31	27.26*	11.44	24.43*		24.94*	Inf**	Inf**	Inf**	Inf**	31.38*	26.11*	30.01*	19.94	31.19*	25.65*	29.89*	16.29	20.03*
KK	49.40**	36.34**	55.54**	21.23*	18.62	Inf**	23.64*	14.13	30.43*		45.27**	Inf**	Inf**	Inf**	Inf**	37.01**	Inf**	28.91*	55.58**	47.12**	41.75**	47.34**	39.83**
Bt	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	49.72**		Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	83.87**
Pa	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**		27.86*	33.93*	42.48**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**
PS	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	47.35**	Inf**	Inf**	Inf**	Inf**	29.35*		8.61	12.52	26.05*	Inf**	Inf**	Inf**	48.29**	Inf**	48.41**	55.85**
Ka	Inf**	Inf**	42.11**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	36.48**	9.42		15.86	28.44*	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**	Inf**
Ba	35.67**	25.58*	14.28	33.27*	40.86**	46.64**	20.73	Inf**	31.33*	Inf**	Inf**	47.79**	17.80	20.26		5.63	35.80**	29.02*	45.37**	29.33*	41.59**	25.55*	22.94*
Ko	25.04*	22.79*	17.26	22.97*	27.88*	38.64**	17.06	31.07*	28.03*	42.06**	Inf**	Inf**	29.79*	34.22*	5.37		24.76*	20.36	34.60*	30.73*	28.31*	22.57*	14.91
Ku	61.83**	35.96**	20.02	Inf**	32.80*	17.86	11.02	Inf**	32.38*	Inf**	Inf**	Inf**	Inf**	Inf**	42.27**	27.98*		40.82**	19.53	Inf**	38.53**	25.53*	20.78
Ce	35.76**	34.79*	28.68*	21.91*	13.30	38.63**	14.70	16.24	19.23	31.75*	Inf**	Inf**	Inf**	Inf**	32.13*	24.72*	45.68**		32.14*	16.72	25.72*	27.91*	22.49*
Mi	Inf**	33.57*	17.60	42.29**	35.91**	18.92	14.70	47.13**	35.31**	Inf**	Inf**	Inf**	Inf**	Inf**	50.85**	42.33**	23.22*	37.01**		11.98	14.01	7.67	12.17
Pi	39.31**	31.53*	27.44*	30.65*	36.23**	31.90*	28.23*	35.82**	26.51*	52.58**	Inf**	Inf**	55.11**	70.65**	33.70*	35.53**	Inf**	27.96*	13.15		7.63	7.54	7.30
SB	53.29**	31.59*	26.63*	29.24*	30.70*	35.79**	23.62*	34.49*	34.70*	45.73**	Inf**	Inf**	66.23**	Inf**	40.78**	33.31*	33.87*	27.47*	10.88	9.83		15.56	14.08
SP	Inf**	26.35*	12.53	28.82*	28.86*	18.55	9.23	32.33*	28.38*	53.88**	Inf**	Inf**	52.28**	70.67**	29.87*	27.02*	28.76*	31.11*	8.39	12.34	13.26		11.05
TB	45.89**	20.26	14.46	28.04*	32.61*	16.12	15.45	25.54*	23.81*	49.23**	Inf**	Inf**	66.01**	Inf**	25.43*	23.11*	25.19*	32.75*	13.11	14.41	19.63	15.19	

5.2. Declaration

Gemäß §6 der Promotionsordnung der Universität Bremen für die mathematischen, naturwissenschaftlichen und ingenieurwissenschaftlichen Fachbereiche vom 14. März 2007 versichere ich, dass (1) die Arbeit ohne unerlaubte fremde Hilfe angefertigt wurde, (2) keine anderen als die angegebenen Quellen und Hilfsmittel benutzt wurden und (3) die den benutzten Werken wörtlich oder innhaltlich entnommenen Stellen kenntlich gemacht wurden.

Bremen, den 16.10.2008