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

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**A B S T R A C T**

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 *Actinocara*) 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 cyt *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 discordant to the morphological species classification. The genetic distances between the *Phalerebus* species were 2–5% in cyt *b* and 10–12% in the control region.

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1. 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 Macroactyla 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. 1a).

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. 1b), 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. Materials and methods

2.1. 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. 1). 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.

![Fig. 1.](image-url) (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).

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Abbr.</th>
<th>No. CR</th>
<th>Accession No. CR</th>
<th>No. cyt b</th>
<th>Accession No. cyt b</th>
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</tr>
</tbody>
</table>

The sequence from the Solomon Islands was obtained from Genebank. CR, control region; cyt b, cytochrome b.
Thirty-two samples of *A. ocellaris* were obtained from five different locations over a range of 1500 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 genebank (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).

### 2.2. 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 10× PCR buffer, 0.075 μmol Mg2+, 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 (AAAAACCCCGGTGTTATCCACTACA; Nelson et al., 2000) and H15149 (AAACCTGACGGCCCTCAGAATGTAATGGTGTCTCA; Kocher et al., 1989) were used. PCR was performed in Eppendorf EP Mastercycler in 25 μl reaction mix, containing 2.5 μl 10× PCR buffer, 0.0625 μmol Mg2+, 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. Phylogenetic analyses

Both strands were assembled and edited with help of the program Sequencher (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 and Hasegawa, 1999) and Kishino–Hasegawa (Kishino and 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 (Schneider et al., 2000). The program Modeltest (Ver. 3.06; Posada and Cranall, 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 (dXY = dST – (πX + πY)/2, Nei 1987 as cited in Campton et al., 2000). The corrected genetic distances were further used for comparison and molecular divergence time calculations, where 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 (Alvaredo 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).

### 3. Results

#### 3.1. 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 invariant 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 invariant sites of 0.67. The latter model is a modified GTR model with equal substitution rates for A-G and C-T (Paraskevis 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. 2a), showing a grouping of all species in two main clades, both of them supported by high bootstrap values.

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. peri-
formed sister clades, and *A. akallopisos* was basal to them (Fig. 2a).

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. 2a).

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. 2b). 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.

### 3.2. Haplotype networks

The parsimonious haplotype networks of the *A. akallopisos*/perideraion/sandaracinos complex revealed three clearly separated clades for both markers (Fig. 3). 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. 4a). 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 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.

### 3.3. 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/sandaracinos/perideraion* complex did not exceed 12.3% in CR and 4.8% in cyt b (Table 2).

### 3.4. 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 million years ago), 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 million years ago). *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).

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 (2–2.8 million years in CR), similar to the split between *A. ocellaris* and *A. percula* (Table 3).

### 4. 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 inconsistency 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 insights 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 Stepiein, 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.

4.1. *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 Amphiprion 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 (1.7–1.9 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).

### 4.2. 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 Toonozuka (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 glacial. 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. 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 glacial. 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.

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