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Strong genetic population structure in the boring giant clam, *Tridacna crocea*, across the Indo-Malay Archipelago: implications related to evolutionary processes and connectivity

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Abstract

Even though the Indo-Malay Archipelago hosts the world's greatest diversity of marine species, studies on the genetic population structure and gene flow of marine organisms within this area are rather rare. Consequently, not much is known about connectivity of marine populations in the Indo-Malay Archipelago, despite the fact that such information is important to understand evolutionary and ecological processes in the centre of marine biodiversity. This study aims to investigate the genetic population structure of the boring giant clam, *Tridacna crocea*. The analysis is based on a 456-bp fragment of the cytochrome oxidase I gene from 300 individuals collected from 15 localities across the Indo-Malay Archipelago. *Tridacna crocea* shows a very strong genetic population structure and isolation by distance, indicating restricted gene flow between almost all sample sites. The observed Φ_{ST} -value of 0.28 is very high compared to other studies on giant clams. According to the pronounced genetic differences, the sample sites can be divided into four groups from West to East: (i) Eastern Indian Ocean, (ii) Java Sea, (iii) South China Sea, Indonesian throughflow, as well as seas in the East of Sulawesi, and (iv) Western Pacific. This complex genetic population structure and pattern of connectivity, characterised by restricted gene flow between some sites and panmixing between others can be attributed to the geological history and prevailing current regimes in the Indo-Malay Archipelago.

Keywords: aquarium trade, conservation, marine protected areas, neutrality test, population expansion, Southeast Asia

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Introduction

Geology, genetic differentiation and evolution in the Indo-Malay Archipelago

The Indo-Malay Archipelago, situated in a triangle between Sumatra, New Guinea, and the Philippines, hosts the world's greatest diversity of marine shallow water species. Several theories are proposed to explain the high diversity. They fall into three main categories: centre of evolutionary radiation from where new species disperse (Briggs 1999),

centre of overlap of the Indian and Pacific Ocean biota (Woodland 1983), and centre of accumulation of species that originated in peripheral areas (Jokiel & Martinelli 1992). Studies on molecular systematics of butterflyfishes (*Chaetodon* spp., McMillan & Palumbi 1995), lionfishes (*Pterois miles* and *P. volitans*, Kochzius *et al.* 2003), and starfishes (*Linckia laevigata* and *Linckia multifora*, Williams 2000) have shown a phylogenetic break between the Indian and Pacific Ocean, supporting the view of speciation in separated ocean basins.

Primary hypothesised triggers for this genetic separation of the two ocean basins are plate tectonic movements in the Indo-Malay Archipelago and global fluctuation of sea level during multiple Pliocene and Pleistocene glaciations. About 30 million years ago, most of the present islands in the Indo-Malay Archipelago did not exist or were not at

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their current position. For instance, the Bird's Head Peninsula was not joined yet with New Guinea, which was still much further south, and Halmahera did not exist. Therefore, the Indian and Pacific Ocean were freely interconnected by the so-called Indonesian seaway that allowed the water masses of the Pacific South Equatorial Current (SEC) to enter the Indian Ocean. Because of the northward movement of Australia, New Guinea, and the Bird's Head Peninsula, as well as the development of Sulawesi by the amalgamation of several fragments, this current pattern started to change about 25 million years ago (Hall 1998). Especially the northward displacement of New Guinea about 5 million years ago had a major impact and 3–4 million years ago, the Indonesian seaway was closed (Cane & Molnar 2001). The SEC was deflected, finally leading to the present situation that the waters of the Indonesian throughflow (ITF), which is the major exchange of water masses between the two oceans, originates from the northern Pacific (Gordon & Fine 1996; Gordon 2005).

Parallel to these tectonic events, multiple glaciations in the Pliocene and Pleistocene caused global fluctuations of the sea level with low stands of up to 120 m below current sea level (Krantz 1991; Rohling *et al.* 1998; Siddall *et al.* 2003). Shallow shelf areas, such as the Sunda shelf (Java Sea) between Java, Sumatra and Borneo, fell dry and separated ocean basins (Voris 2000; Fig. 1), which has been hypothesised to have caused allopatric speciation (McManus 1985; Pandolfi 1992; Benzie 1998, 1999a; Randall 1998). Molecular genetic studies suggest as well that these separations have a Pliocene and Pleistocene origin (Kochzius *et al.* 2003; Barber *et al.* 2006; Timm *et al.* 2008).

A genetic break can also be observed in populations of damselfish (*Chromis viridis*, Froukh & Kochzius 2008; *Stegastes nigricans*, *Chrysiptera biocellata*, *Chrysiptera glauca* and *Chrysiptera leucopoma*, Lacson & Clark 1995), clownfish (*Amphiprion ocellaris*, Nelson *et al.* 2000, Timm *et al.* 2008), seahorse (*Hippocampus spinosissimus*, Lourie *et al.* 2005), barramundi (*Lates calcarifer*, Chenoweth *et al.* 1998), starfish (*Linckia laevigata*, Williams & Benzie 1998; Williams *et al.* 2002; *Acanthaster planci*, Benzie 1999b), mantis shrimp (*Haptosquilla pulchella*, *Haptosquilla glyptocercus*, and *Gonodactylus viridis*, Barber *et al.* 2002, 2006), coconut crab (*Birgus latro*, Lavery *et al.* 1996), tiger prawn (*Penaeus monodon*, Duda & Palumbi 1999; Brooker *et al.* 2000; Sugama *et al.* 2002), mudcrab (*Scylla serrata*, Gopurenko *et al.* 1999), giant clam (*Tridacna squamosa*, Kittiwattanawong *et al.* 2001), and a mangrove tree (*Avicennia marina*, Duke *et al.* 1998). The persistence of this genetic differentiation across the Indo-Malay Archipelago indicates that factors such as restricted dispersal capabilities, ecological requirements, and current patterns prevent panmixing in these species.

However, such a genetic break between the Indian and Pacific Ocean is not a general pattern. For example, mole-

cular phylogenetic studies on marine intertidal snails have revealed that, although there is a phylogenetic break in two species from ecologically continental habitats (*Echinolittorina trochoides* clade A and *Echinolittorina vidua*, Reid *et al.* 2006), a similar species pair from ecologically oceanic habitats (*E. trochoides* clade D and *Echinolittorina reticulata*) does not show such a break. Such a genetic break is also missing in a sea urchin (*Diadema savignyi*, Lessios *et al.* 2001), tasslefish (*Polynemus sheridani*, Chenoweth & Hughes 2003), bigeye tuna (*Thunnus obesus*, Alvarado Bremer *et al.* 1998; Chow *et al.* 2000), and swordfish (*Xiphias gladius*, Chow *et al.* 1997).

Connectivity of populations in the Indo-Malay Archipelago

Studies on the genetic population structure and gene flow of marine organisms within the Indo-Malay Archipelago are rather rare (Benzie 1998). Those few studies which have been conducted have investigated fishes, such as an anemonefish (Nelson *et al.* 2000), jobfish (Ovenden *et al.* 2004), seahorses (Lourie *et al.* 2005) as well as scad mackerels (Arnaud *et al.* 1999; Perrin & Borsa 2001; Borsa 2003; Rohfritsch & Borsa 2005), and a few invertebrates, such as a mushroom coral (*Heliofungia actiniformis*, Knittweis *et al.* 2008), mantis shrimps (Barber *et al.* 2002; Barber *et al.* 2006), and the giant tiger prawn (Sugama *et al.* 2002). Of these species, only mushroom coral, mantis shrimps, and seahorses are well covered, with many populations sampled across the archipelago. Even though several studies have dealt with the population genetics of giant clams (Ayala *et al.* 1973; Campbell *et al.* 1975; Benzie & Williams 1992a, b; Macaranas *et al.* 1992; Benzie & Williams 1995; Benzie & Williams 1997; Kittiwattanawong 1997; Yu *et al.* 2000; Kittiwattanawong *et al.* 2001; Laurent *et al.* 2002; Juinio-Meñez *et al.* 2003; Ravago-Gotanco *et al.* 2007), none of them investigated populations within the Indo-Malay Archipelago on a large scale. Therefore, not much is known about connectivity of marine populations in the Indo-Malay Archipelago in general and especially for giant clams, despite the fact that such information is important to understand evolutionary and ecological processes in the centre of marine biodiversity. Data on connectivity are also urgently required to design effective conservation strategies for these living marine resources.

Studied species and applied molecular marker

The boring or crocus clam *Tridacna crocea* is the smallest species of the giant clams (Tridacnidae). It grows to a length of about 15 cm and is characterised by bright colour pattern. As a sessile organism, it bores into the substrate, such as coral boulders or reef rocks. The distribution of

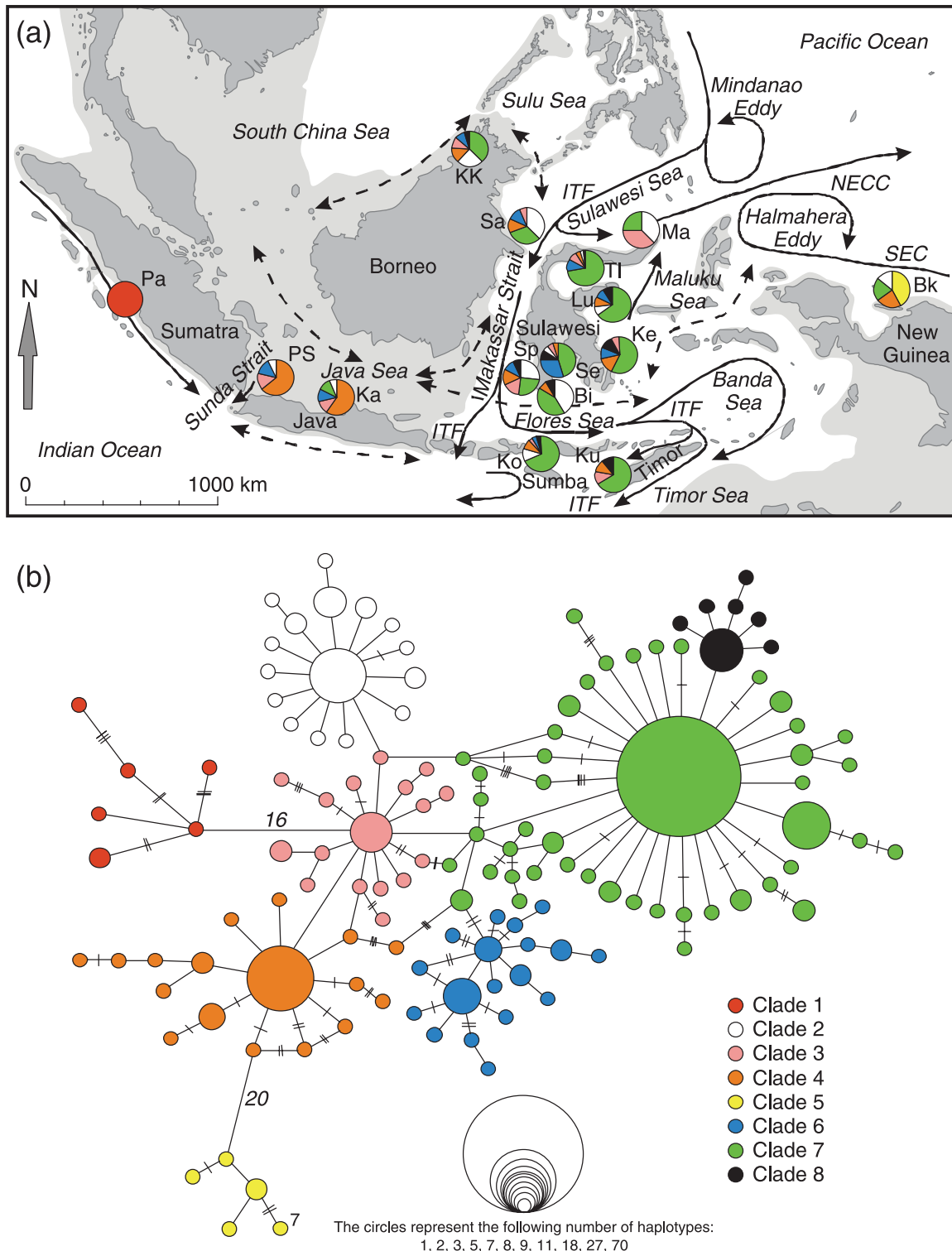


Fig. 1 (a) Map of the Indo-Malay Archipelago showing samples sites (for abbreviations see Table 1) as well as oceanographic patterns with dominant (solid lines) and seasonally changing (dashed lines) currents (Wyrski 1961; Gordon & Fine 1996; Gordon 2005; ITF, Indonesian throughflow; SEC, Southern equatorial current; NECC, Northern Equatorial Counter Current). Pleistocene maximum sea level low stand of 120 m is indicated by the light grey area (Voriss 2000). Pie charts represent the proportion of clades defined in the network at the different sample sites. (b) Network of 132 mitochondrial cytochrome *c* oxidase I haplotypes from 300 individuals of *Tridacna crocea*. Connecting lines between circles represent one mutational step. The hatches and numbers indicate additional mutational steps. The size of the circles is proportional to haplotype frequency.

T. crocea ranges from the Eastern Indian Ocean across the Indo-Malay Archipelago to the Western Pacific (Lucas 1988). Like other giant clams, *T. crocea* hosts photosymbiotic dinoflagellates of the genus *Symbiodinium* (Hirose *et al.* 2006). These zooxanthellae transfer photosynthetically fixed carbon, mainly as glucose, to their host (Ishikura *et al.* 1999).

Connectivity of populations can be inferred by investigating the genetic population structure with molecular markers, such as the cytochrome *c* oxidase I gene (COI). Studies on the population genetics of bivalves have shown that COI is a suitable marker for such studies (e.g. Luttikhuisen *et al.* 2003; Shefer *et al.* 2004).

This study aims to investigate the genetic population structure of the boring giant clam *T. crocea* across the Indo-Malay Archipelago based on COI sequences in order to study gene flow in the centre of marine biodiversity.

Materials and methods

Sampling

Tissue samples from 300 specimens of *Tridacna crocea* were collected during several field trips in 2004 and 2005 at 15 sample sites across the Indo-Malay Archipelago (Fig. 1, Table 1). A small piece of mantle tissue was cut off from each specimen under water. This is a biopsy method that ensures the survival of the animal. Tissue samples were preserved in 96% of ethanol and later stored at 4 °C.

DNA extraction, amplification and DNA sequencing

Genomic DNA was isolated with the Chelex method, following the protocol of Walsh *et al.* (1991). The molecular marker used in this study is the mitochondrial COI gene. A fragment of this gene was amplified using the following tridacnid-specific primers, which were designed based on *T. crocea* sequences obtained in a preliminary analysis using COI primers from Folmer *et al.* (1994): COI-Trico-Frwd 5'-GGGTGATAATTCGAACAGAA-3' and COI-Trico-Rev 5'-TAGTTAAAGCCCCAGCTAAA-3'. The PCR was carried out in a total volume of 50 µL. PCRs contained 1 µL DNA template, 10 mM Tris-HCl (pH 9), 50 mM KCl, 2 mM of MgCl₂, 0.2 µM of each primer, 0.2 mM of each dNTP and 1 U *Taq* polymerase. The following temperature profile was used for the PCR: 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, 1.5 min at 43 °C, and 1 min at 72 °C. Final extension was conducted at 72 °C for 5 min. The PCR products were purified using the QIAquick spin column PCR purification kit (QIAGEN), following the manufacturer's protocol. Both strands of the purified DNA were sequenced using the DyeDeoxy terminator chemistry (PE Biosystem) and an automated sequencer (ABI PRISM 310, Applied Biosystems).

Genetic diversity

All sequences were edited with the program SEQUENCE NAVIGATOR (version 1.0.1; Applied Biosystems) and checked

Table 1 Sample sites, number of sequences (*n*), number of haplotypes (*N*_{hp}), haplotype diversity (*h*), nucleotide diversity (*π*), Tajima's *D*, Fu's *F*_s, sum of square deviation (SSD), and Harpending's raggedness index (HRI) for *Tridacna crocea* in the Indo-Malay Archipelago

| Code | Sample site | <i>n</i> | Genetic diversity | | | Neutrality tests | | Mismatch distribution | |
|------|--------------------------|----------|------------------------|--------------|-----------------------|---------------------|----------------------------|-----------------------|--------------------|
| | | | <i>N</i> _{hp} | <i>h</i> | <i>π</i> (percentage) | Tajima's <i>D</i> | Fu's <i>F</i> _s | SSD | HRI |
| Bi | Bira, Sulawesi | 12 | 8 | 0.91 ± 0.065 | 0.89 ± 0.54 | -0.51 ^{NS} | -1.84 ^{NS} | 0.03 ^{NS} | 0.06 ^{NS} |
| Bk | Biak, New Guinea | 14 | 13 | 0.99 ± 0.031 | 4.34 ± 2.29 | -0.21 ^{NS} | -2.84 ^{NS} | 0.04 ^{NS} | 0.02 ^{NS} |
| Ka | Karimunjawa, Java | 16 | 9 | 0.77 ± 0.113 | 0.69 ± 0.42 | -1.71* | -2.65* | 0.01 ^{NS} | 0.04 ^{NS} |
| Ke | Kendari, Sulawesi | 28 | 16 | 0.83 ± 0.074 | 0.79 ± 0.46 | -1.73* | -7.84*** | 0.00 ^{NS} | 0.01 ^{NS} |
| KK | Kota Kinabalu, Borneo | 21 | 16 | 0.97 ± 0.023 | 1.14 ± 0.64 | -1.39 ^{NS} | -7.98*** | 0.00 ^{NS} | 0.02 ^{NS} |
| Ko | Komodo | 26 | 17 | 0.89 ± 0.059 | 1.01 ± 0.57 | -2.12** | -8.11*** | 0.02 ^{NS} | 0.03 ^{NS} |
| Ku | Kupang, Timor | 9 | 7 | 0.92 ± 0.092 | 0.70 ± 0.45 | -1.52 ^{NS} | -2.55* | 0.02 ^{NS} | 0.08 ^{NS} |
| Lu | Luwuk, Sulawesi | 23 | 13 | 0.82 ± 0.082 | 0.73 ± 0.44 | -1.57* | -4.50** | 0.00 ^{NS} | 0.01 ^{NS} |
| Ma | Manado, Sulawesi | 8 | 8 | 1.00 ± 0.063 | 0.85 ± 0.54 | -0.66 ^{NS} | -4.89** | 0.01 ^{NS} | 0.06 ^{NS} |
| Pa | Padang, Sumatra | 7 | 6 | 0.95 ± 0.096 | 1.03 ± 0.66 | -0.79 ^{NS} | -1.49 ^{NS} | 0.04 ^{NS} | 0.09 ^{NS} |
| PS | Pulau Seribu, Java | 14 | 6 | 0.60 ± 0.150 | 0.68 ± 0.42 | -0.67 ^{NS} | -0.02 ^{NS} | 0.08 ^{NS} | 0.21 ^{NS} |
| Sa | Sangkalaki, Borneo | 16 | 13 | 0.97 ± 0.036 | 1.31 ± 0.74 | -0.89 ^{NS} | -5.35** | 0.01 ^{NS} | 0.02 ^{NS} |
| Se | Sembilan, Sulawesi | 20 | 14 | 0.94 ± 0.043 | 1.28 ± 0.72 | -1.45 ^{NS} | -4.69* | 0.01 ^{NS} | 0.02 ^{NS} |
| Sp | Spermonde, Sulawesi | 40 | 24 | 0.96 ± 0.014 | 1.05 ± 0.58 | -1.43 ^{NS} | -13.83*** | 0.00 ^{NS} | 0.01 ^{NS} |
| TI | Togian Islands, Sulawesi | 46 | 21 | 0.79 ± 0.063 | 0.71 ± 0.41 | -1.88* | -12.16*** | 0.01 ^{NS} | 0.03 ^{NS} |

*0.05 ≥ *P* ≥ 0.01; **0.01 > *P* ≥ 0.001; ****P* < 0.001; NS, not significant.

manually by eye. Orthology to a published sequence available in the European Molecular Biological Laboratory (EMBL) sequence database (*T. crocea*, accession no. AB076920) was also verified. No contamination with DNA from zooxanthellae was observed. The sequences were translated to amino acids with the program SQUINT (www.cebl.auckland.ac.nz/index.php) in order to exclude mistakes in sequencing and to verify if a functional mitochondrial DNA sequence was obtained and not a nuclear pseudogene. A multiple sequences alignment was obtained by using CLUSTAL W (Thompson *et al.* 1994) as implemented in the software BIOEDIT (version 7.0.4.1; Hall 1999). Haplotype diversity h (Nei 1987) and nucleotide diversity π (Nei & Jin 1989) were calculated with the programme ARLEQUIN (version 2.0; Schneider *et al.* 2000).

Historical demography

The null hypothesis of neutral evolution of the marker was tested using Tajima's D test (Tajima 1989) and Fu's F_s test (Fu 1997) with 10 000 permutations as implemented in ARLEQUIN. Mismatch distribution analysis and the model of sudden population expansion (Rogers 1995) were also tested using ARLEQUIN based on 10 000 permutations.

Genetic population structure and connectivity

Significance of population structure was tested with analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) and pairwise Φ_{ST} -values. Both statistical calculations were carried out using the software ARLEQUIN (version 2.0, Schneider *et al.* 2000). A gamma shape parameter of 0.24 and the Tamura–Nei substitution model were used, based on the result obtained with the programmes PAUP (version 4.0b10; Swofford 1998) and MODELTEST (version 3.7; Posada & Crandall 1998). Several groupings were tested in a hierarchical AMOVA, considering the geography of the region. A haplotype network was also calculated with the programme ARLEQUIN. Clades were defined based on the number of mutational steps and abundant haplotypes connected to several singletons, showing a star-like pattern.

The correlation between geographical and genetic distances among populations was investigated using reduced major axis (RMA) regression analysis and significance of the correlation was tested by a Mantel test using the isolation by distance web service (IBDWS 2.6) with 10 000 permutations (Jensen *et al.* 2005; <http://ibdws.sdsu.edu/>). Geographical distances were measured with an electronic world atlas, distances representing the shortest path between two populations by sea.

Results

Genetic diversity

A sequence alignment of 456 bp of the COI fragment from 300 individuals of *Tridacna crocea* collected from 15 localities across the Indo-Malay Archipelago (Table 1) was obtained. Among these 300 individuals, a total number of 132 haplotypes was observed, showing 117 polymorphic sites (25.7%) and 120 substitutions. The number of transitions was much higher than the number of transversions.

Genetic diversity within each population showed high levels of haplotype and nucleotide diversity, except for the populations from the Java Sea (Karimunjawa and Pulau Seribu) and eastern coast of Sulawesi (Togian Islands, Luwuk, and Kendari). Haplotype diversity ranges from 0.60 in the population from Pulau Seribu to 1.00 in Manado, nucleotide diversity from 0.68% in Pulau Seribu to 4.34% in Biak (Table 1). Overall haplotype diversity (h) was 0.93 and nucleotide diversity (π) was 1.5%.

Historical demography

The null hypothesis of neutral evolution of the COI marker could not be rejected for most sample sites based on Tajima's D test, whereas the results of Fu's F_s test rejected the null hypothesis for most of the sites. However, this could indicate population expansion, which is supported by the mismatch distribution analysis and Rogers' test of sudden population expansion (Rogers 1995; Table 1).

Genetic population structure and connectivity

The evolutionary relationships among 132 *T. crocea* haplotypes are presented in the haplotype network (Fig. 1), showing eight different clades. The distribution of clades across the Indo-Malay Archipelago is also presented on the map in Fig. 1. Clade 1 and clade 5 are the most divergent, separated by 16 and 20 substitutions, respectively. Clade 1 occurs exclusively in the Eastern Indian Ocean (Padang) and no other clade could be observed at that sample site, whereas clade 5 was only found in the Western Pacific (Biak). Clade 2 could be found at all sample sites (except Padang), but in higher proportions at some sites along the ITF (Manado, Sangalaki, Spermonde, and Bira). The proportion of clade 3 was rather low at all sites and it was not observed in the Western Pacific (Biak), Bira, Luwuk, and Padang. Clade 4 was present at the majority of the sample sites (except Padang and Manado) and was dominant in the Java Sea (Pulau Seribu and Karimunjawa). Most of the sample sites showed a small proportion of clade 6, but this clade was not observed in Biak, Bira, Kupang, Manado, and Padang. Clade 7 could be found at all sample sites, except the two most western ones in

Table 2 Pairwise Φ_{ST} -values between populations of *Tridacna crocea* in the Indo-Malay Archipelago. For abbreviations of sample sites, see Table 1

| | Pa | PS | Ka | KK | Sa | Sp | Bi | Se | Ke | Lu | TI | Ma | Ko | Ku |
|----|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|----------|-------|---------|-------|
| PS | 0.85*** | | | | | | | | | | | | | |
| Ka | 0.85*** | -0.03NS | | | | | | | | | | | | |
| KK | 0.81*** | 0.05NS | 0.06* | | | | | | | | | | | |
| Sa | 0.80*** | 0.07* | 0.09** | -0.02NS | | | | | | | | | | |
| Sp | 0.82*** | 0.07* | 0.08** | -0.0NS | -0.02NS | | | | | | | | | |
| Bi | 0.84*** | 0.17** | 0.18*** | 0.01NS | -0.01NS | -0.01NS | | | | | | | | |
| Se | 0.80*** | 0.20*** | 0.20*** | 0.05* | 0.07* | 0.09** | 0.13** | | | | | | | |
| Ke | 0.85*** | 0.24*** | 0.21*** | 0.07** | 0.10** | 0.09*** | 0.11** | 0.02NS | | | | | | |
| Lu | 0.86*** | 0.26*** | 0.23*** | 0.06* | 0.08* | 0.07** | 0.08* | 0.04NS | -0.02NS | | | | | |
| TI | 0.87*** | 0.27*** | 0.24*** | 0.08** | 0.12*** | 0.10*** | 0.12*** | 0.04NS | -0.01NS | -0.01NS | | | | |
| Ma | 0.83*** | 0.12* | 0.14** | -0.01NS | -0.02NS | -0.03NS | -0.03NS | 0.15** | 0.18* | 0.16** | 0.19*** | | | |
| Ko | 0.83*** | 0.21*** | 0.19*** | 0.05* | 0.07* | 0.07** | 0.04NS | 0.06* | -0.01NS | -0.02NS | -0.002NS | 0.10* | | |
| Ku | 0.85*** | 0.29*** | 0.24*** | 0.08* | 0.10* | 0.09* | 0.09NS | 0.06NS | -0.02NS | -0.02NS | -0.007NS | 0.18* | -0.03NS | |
| Bk | 0.58*** | 0.29** | 0.30** | 0.30*** | 0.29** | 0.37*** | 0.28** | 0.34*** | 0.39*** | 0.30*** | 0.45*** | 0.22* | 0.35*** | 0.27* |

* $0.05 \geq P \geq 0.01$; ** $0.01 > P \geq 0.001$; *** $P < 0.001$; NS, not significant.

Table 3 Hierarchical analysis of molecular variance (AMOVA) of mtDNA COI sequences in *Tridacna crocea* from the Indo-Malay Archipelago. For abbreviations of sample sites, see Table 1

| Region groupings | Φ_{CT} | Percentage variance among groups |
|---|-------------|----------------------------------|
| (Pa) (PS, Ka) (KK, Sa, Ma, Sp, Bi, Se, Ke, Lu, TI, Ko, Ku) (Bk) | 0.50*** | 50.80 |
| (Pa) (PS, Ka) (KK, Sa, Ma, Sp, Bi) (Se, Ke, Lu, TI, Ko, Ku) (Bk) | 0.37*** | 36.87 |
| (Pa, PS, Ka) (KK, Sa, Ma, Sp, Bi) (Se, Ke, Lu, TI, Ko, Ku) (Bk) | 0.17NS | 17.26 |
| (Pa, PS, Ka, KK) (Sa, Ma, Sp, Bi) (Se, Ke, Lu, TI, Ko, Ku) (Bk) | 0.14NS | 13.83 |
| (Pa) (PS, Ka, KK) (Sa, Ma, Sp, Bi) (Se, Ke, Lu, TI, Ko, Ku) (Bk) | 0.35*** | 35.51 |
| (Pa) (PS, Ka) (KK, Sa, Ma, Sp, Bi) (Se, Ke, Lu, TI) (Ko, Ku) (Bk) | 0.34*** | 33.98 |
| (Pa) (PS, Ka) (KK, Sa, Ma, Sp, Bi) (Se, Ke, Lu, TI, Bk) (Ko, Ku) | 0.22** | 22.37 |
| (Pa) (PS, Ka) (KK, Sa, Ma, Sp, Bi) (Se, Ke, Lu, TI, Ko, Ku, Bk) | 0.26** | 26.46 |
| (Pa, PS, Ka) (KK, Sa, Ma, Sp, Bi) (Se, Ke, Lu, TI, Ko, Ku, Bk) | 0.06* | 5.98 |

* $0.05 \geq P \geq 0.01$; ** $0.01 > P \geq 0.001$; *** $P < 0.001$; NS, not significant.

Padang and Pulau Seribu. The highest proportions (> 50%) of clade 7 were observed along the eastern coast of Sulawesi (Kendari, Luwuk, Togian Islands), as well as in Komodo and Kupang. Clade 8 showed only a distribution in the central part of the Indo-Malay Archipelago (Bira, Kendari, Komodo, Kota Kinabalu, Kupang, Luwuk, Sembilan, and Spermonde), with a generally low proportion at each of these sites.

The most common haplotype from clade 7 was found in 23.3% of all individuals and was unique to the eastern areas of the Indo-Malay Archipelago (from Makassar Strait eastward to New Guinea). The second most common haplotype from clade 4 occurred in a frequency of 9% and had a broader distribution, ranging from the Java Sea, Makassar Strait, and eastern coast of Sulawesi to Biak.

The observed genetic structure among *T. crocea* populations is verified by AMOVA and pairwise Φ_{ST} -values. These analyses considered a gamma shape parameter of 0.24 as well as the

Tamura–Nei substitution model and revealed a highly significant genetic structure among *T. crocea* populations across the Indo-Malay Archipelago ($\Phi_{ST} = 0.28$; $P < 0.001$). However, no significant genetic structure was found between inner and outer shelf populations of the Spermonde Archipelago ($\Phi_{ST} = 0.02$; $P = 0.21$). Pairwise Φ_{ST} -values between sample sites revealed a very complex pattern of significant differences and homogeneity. The majority of pairwise comparisons showed significant Φ_{ST} -values (74%), the remainder indicated panmixing (Table 2). Significant Φ_{ST} -values range from 0.05 to 0.86. The population from Padang (Eastern Indian Ocean) is the most divergent population with Φ_{ST} -values ranging from 0.57 to 0.87, followed by Biak (Western Pacific) with values ranging from 0.22 to 0.57.

Based on the pairwise analysis and geography, a hierarchical AMOVA was carried out with different groupings (Table 3). The highest fixation index ($\Phi_{CT} = 0.5$) and variation

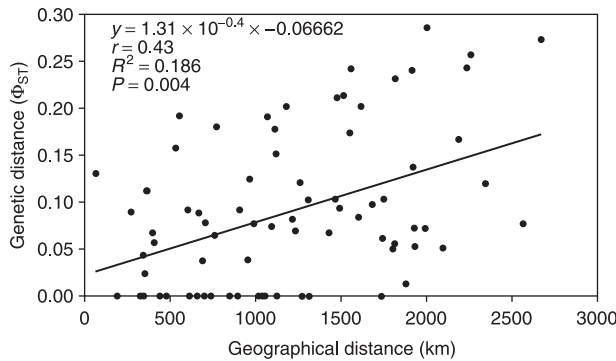


Fig. 2 Relationship between genetic vs. geographical distances in *Tridacna crocea* using reduced major axis (RMA) regression without considering divergent populations from Padang (Eastern Indian Ocean) and Biak (Western Pacific).

(50.80%) in the hierarchical AMOVA among groups showed the assignment of the sample sites to the following four different regions: (i) Eastern Indian Ocean (off Padang = West Sumatra), (ii) Java Sea (Pulau Seribu, Karimunjawa), (iii) South China Sea (off Kota Kinabalu, North Borneo), Indonesian throughflow (off Manado = North Sulawesi, Sangalaki = East Borneo, Spermonde and Bira = South Sulawesi, Komodo, off Kupang = West Timor), as well as seas in the East of Sulawesi (Sembilan, off Kendari, off Luwuk, Togian Islands), and (iv) Western Pacific (Biak = New Guinea). Additionally, we found a positive correlation between genetic (Φ_{ST}) and geographical distances ($r = 0.43$, $P = 0.004$, Fig. 2), clearly showing isolation by distance. The genetically very divergent populations from the sample sites in Padang and Biak were excluded from this analysis.

Discussion

Genetic diversity

The high level of polymorphism in the COI fragment found suggests greatly restricted gene flow between populations of the boring giant clam *Tridacna crocea*. Levels of genetic diversity are comparable with other studies on marine bivalves (e.g. Luttikhuisen *et al.* 2003; Shefer *et al.* 2004).

High genetic diversity in *T. crocea* was found in all populations, except the populations from the Java Sea (Pulau Seribu and Karimunjawa) and sites from the eastern coast of Sulawesi (Kendari, Luwuk, and Togian Islands). Low genetic diversity in giant clam populations could be explained by one of three factors or a combination of the three. In the Java Sea, low genetic diversity could be due to a founder effect, which was caused by the recolonisation of the Sunda Shelf as sea level rose after the last glacial. However, the low genetic diversity at the eastern coast of

Sulawesi is difficult to explain by a founder effect. Due to the very narrow shelf around Sulawesi, only very small areas were exposed during low sea level stands in the last glacial (Voris 2000). Therefore, the low genetic diversity could not be due to recolonisation.

Another factor could be overexploitation; for example, giant clams in the Java Sea have been heavily harvested for their shells. The Javanese shell industry in Jakarta and Jepara received giant clam shells from Pulau Seribu and Karimunjawa, respectively. They have been used among others as material for the production of so-called 'teraso' tiles for interior decoration. However, it is not reported which giant clam species are utilised in the shell industry (Pasaribu 1988; Wells 1997). Another factor could be over-exploitation caused by the marine ornamental trade, but no data are available. It appears that populations of giant clams are also overexploited in the Togian Islands, but *T. crocea* seems to be less affected (Allen & McKenna 2001). Therefore, it is difficult to estimate to which extend exploitation is responsible for the low genetic diversity.

The third factor could be a population bottleneck due to bleaching events in the Java Sea in 1983 (Pulau Seribu; Brown & Suharsono 1990; Hoeksema 1991) and 1998 (Pulau Seribu and Karimunjawa, Wilkinson 2002), as well as at the eastern coast of Sulawesi (Togian and Banggai Islands, Allen & McKenna 2001; Wilkinson 2002). Since giant clams depend nutritionally on their zooxanthellate symbionts (Ishikura *et al.* 1999), the loss of zooxanthellae during bleaching events negatively affects their fitness (Leggat *et al.* 2003).

Historical demography

Based on the results of Tajima's *D* and Fu's *F_s* neutrality tests, the null hypothesis of neutral evolution of the utilised marker was rejected at some sample sites (Table 1). However, these tests cannot distinguish between selection and changes in population size. Mismatch distribution analysis and Rogers' test of sudden population expansion (Rogers & Harpending 1992; Rogers 1995) indicated demographic growth. Changes in population size could be explained by the reduction of habitat during sea level low stands and recolonisation of new habitats after rising of the sea level (Fig. 1). Loss of habitat resulted in population bottlenecks, whereas the availability of new habitats after the rise of the sea level at the end of the last glacial enabled growth of the reduced populations. This hypothesised impact of the quaternary ice ages has been detected in the genetic population structure of Pacific coral reef fish populations (Fauvelot *et al.* 2003). In that study, the authors found that lagoonal species, which were impacted by the loss of their habitat during sea level low stands, show a lower genetic diversity than outer slope species that did not suffer from such a habitat loss.

Genetic population structure and connectivity

Tridacna crocea shows a very strong genetic population structure across the Indo-Malay Archipelago and isolation by distance, indicating restricted gene flow between almost all sample sites. The observed Φ_{ST} -value of 0.28 is very high compared to other studies on giant clams. However, a comparison is difficult, because all other studies used allozymes as a genetic marker and differences in the biology of even closely related species, as well as different oceanographic conditions can have profound effects on the population structure. Studies on *T. crocea* in the Philippines revealed F_{ST} -values of 0.066 (Juinio-Meñez *et al.* 2003) and 0.065 (Ravago-Gotanco *et al.* 2007). Values for other giant clams (*Tridacna derasa*, *T. gigas*, and *T. maxima*) ranged from 0 to 0.156 in the Western Pacific (Benzie & Williams 1992a, b, 1995, 1997; Macaranas *et al.* 1992).

According to the pronounced genetic differences, the sample sites can be divided into four groups from West to East (Table 3): (i) Eastern Indian Ocean; (ii) Java Sea, (iii) South China Sea, Indonesian throughflow, as well as seas in the East of Sulawesi, and (iv) Western Pacific. Such genetic breaks in the transition of the Indian and Pacific Ocean was also found in populations of a starfish (Williams & Benzie 1998), mantis shrimp (Barber *et al.* 2002, 2006), and fishes (Chenoweth *et al.* 1998; Nelson *et al.* 2000; Lourie *et al.* 2005; Timm *et al.* 2008). The differentiation between populations of the Indian and Pacific Ocean is hypothesised to be due to low sea level during glacials, when huge ice shields in higher latitudes bound water and sea level was dropped up to 120 m. During those times, the Java Sea on the Sunda shelf between Java and Borneo was dry land. This created several more or less isolated ocean basins and restricted the exchange between the Indian and Pacific Oceans (McManus 1985; Voris 2000). Persistence of such genetic pattern and additional differentiation could be caused by major prevailing currents.

A detailed comparison of the genetic structure with current patterns and results from other genetic studies in the region is conducted below for the four identified groups in *T. crocea*. However, comparison of the genetic population structure in *T. crocea* with other species is sometimes difficult due to differences in the geographical resolution of sampling.

Eastern Indian Ocean. The extremely high Φ_{ST} -values of up to 0.87 raise the question if the population from Padang represents a cryptic species. However, based on current morphological taxonomy, the specimens from Padang belong to *T. crocea* (Lucas 1988). Further comparative studies with additional giant clam species and an integrated taxonomy approach (Dayrat 2005), including genetics, morphology and ecology, would be needed to clarify if the population from Padang represents a cryptic species.

The genetic isolation of the population in Padang (Fig. 1, Table 2) is most probably caused by the prevailing outflow of water masses from the Java Sea through the Sunda Strait into the Indian Ocean (Wyrski 1961; Hendiarti *et al.* 2004). Even though the main current along the western coast of Sumatra is directed to the Southeast, planktonic larvae of *T. crocea* probably can not enter the Java Sea through the Sunda strait because of this tremendous outflow of water from North to South. Currents parallel along the southern coast of Java are seasonally changing and eastward currents are deflected at Sumba in a westward direction. Larvae from the Java Sea that enter the Indian Ocean through the Sunda Strait cannot reach the western coast of Sumatra because of the southeastern currents (Fig. 1, Wyrski 1961).

A genetic separation of populations in the Eastern Indian Ocean from other sites in the Indo-Malay Archipelago could also be observed in the seahorse *Hippocampus spinosissimus* (Lourie *et al.* 2005), the clownfish *Amphiprion ocellaris* (Nelson *et al.* 2000), the scad mackerel *Decapterus macrosoma* (Arnaud *et al.* 1999), and the mantis shrimps (*Haptosquilla pulchella*, Barber *et al.* 2002, 2006). However, the seahorses *Hippocampus barbouri*, *H. kuda*, and *H. trimaculatus* (Lourie *et al.* 2005) as well as the Indian scad mackerel *Decapterus russelli* (Rohfritsch & Borsa 2005) did not show such a genetic separation of the Eastern Indian Ocean.

Java Sea. Populations from the Java Sea (Pulau Seribu and Karimunjawa) were not statistically different from each other, indicating high gene flow and panmixing (Table 2). This could be due to seasonal changing currents in the Java Sea (Fig. 1), which connect these populations that are more than 400 km apart. Furthermore, the pelagic larval duration of about nine days (Lucas 1988) seems to be sufficient for *T. crocea* to travel such distance mediated by currents. However, both populations showed different patterns of genetic differentiation to Kota Kinabalu in the South China Sea. The population from Pulau Seribu was not statistically different from Kota Kinabalu, while the differentiation of the population from Karimunjawa to Kota Kinabalu was significant. Connectivity between the South China Sea and Java Sea could be facilitated by southward currents along the northwestern coast of Borneo, which enter the Java Sea in February (Wyrski 1961). The different patterns of genetic differentiation of the two populations from the Java Sea to the South China Sea can be explained by isolation by distance (Fig. 2). Both populations in the Java Sea are significantly different to all the other sites from the Makassar Strait to the East (Table 2). Even though seasonal easterly currents could connect the Java Sea to the Makassar Strait and Flores Sea, isolation by distance seems to play a role.

Genetically distinct populations in the Java Sea were also present in the mushroom coral *Heliofungia actiniformis* (Knittweis *et al.* 2008) and mantis shrimp *Gonodactylus viridis* (Barber *et al.* 2006), but could not be detected in the

seahorses *H. kuda*, *H. spinosissimus*, and *H. trimaculatus* (Lourie *et al.* 2005), as well as the Indian scad mackerel *D. russelli* (Rohfritsch & Borsa 2005). However, the genetic break observed in *T. crocea* in the eastern Java Sea (Fig. 1, Table 2) also occurred in the seahorse *H. trimaculatus* (Lourie *et al.* 2005) and Indian scad mackerel *D. russelli* (Rohfritsch & Borsa 2005).

South China Sea, Indonesian throughflow, and seas in the East of Sulawesi. Pairwise comparisons of populations from the South China Sea at the northwestern coast of Borneo (Kota Kinabalu), Sulawesi Sea at the northeastern coast of Borneo (Sangalaki) and northern coast of Sulawesi (Manado), as well as Makassar Strait (Spermonde) and Flores Sea (Bira) at the southwestern tip of Sulawesi did not show significant Φ_{ST} -values, indicating panmixing. Connectivity of the South China Sea to the Sulawesi Sea could be facilitated by northeasterly currents around the northern tip of Borneo into the Sulu Sea and southward currents from the Sulu Sea into the Sulawesi Sea in October (Wyrтки 1961).

Sites in the Sulawesi Sea and Makassar Strait down to the southeastern tip of Sulawesi in the Flores Sea are well connected by the ITF, which transports about 10 million m³ of water per second (= 10 sverdrup; Godfrey 1996; Gordon & Fine 1996; Godfrey & Masumoto 1999; Gordon 2005; Susanto & Gordon 2005), reaching surface currents of up to 36 nautical miles per day (Wyrтки 1961). Connectivity of populations facilitated by the ITF can also be observed among sites bordering the Flores Sea (Bira and Komodo) as well as Kupang (Timor), which show no significant genetic differentiation (Table 2). However, Komodo and Kupang were significantly different from sites upstream the ITF (Spermonde, Sangalaki, and Manado). This pattern of genetic differentiation along the ITF is most probably due to isolation by distance (Fig. 2). One branch of the ITF flows from the Makassar Strait eastward through the Flores Sea into the Banda Sea, where it changes its direction and flows to the Southwest, reaching Kupang and Komodo (Fig. 1, Gordon 2005). The genetic structure suggests that the populations in Kupang and Komodo are connected along this route to populations upstream the ITF, rather than being directly connected to the southwestern tip of Sulawesi (Spermonde and Bira) across the Flores Sea. This is supported by the significant difference of Kupang and Komodo to Spermonde (Table 2).

A hierarchical AMOVA did not support a separate grouping of the sites in Bone Bay (Sembilan) and at the eastern coast of Sulawesi (Kendari, Luwuk, and Togian Islands). These sites are not significantly different from each other. However, pairwise comparisons showed low, but significant Φ_{ST} -values to all other sites, except Komodo and Kupang that are also not significantly different from each other (Table 2). It seems that sites in the seas East of Sulawesi (Kendari, Luwuk, and Togian Islands) are well connected

and linked via Kendari at the southeastern tip of Sulawesi to the ITF, which facilitates connectivity to Kupang and Komodo further downstream (Fig. 1).

A genetic break between the western and eastern coast of Sulawesi was observed in Bone Bay. Populations in Bira and Sembilan are genetically significantly different (Φ_{ST} = 0.13), even though the geographical distance is only 67 km. Oceanographic conditions and the influence of different current regimes obviously prevent an exchange between the western and eastern coast of the island. Selayar, an island south of Bira at the southwestern tip of Sulawesi, stretches along a North–South axis and is separated from Sulawesi only by a small strait. Therefore, the ITF coming from the Makassar Strait is deflected further south into the Flores Sea. However, lack of significant genetic differentiation suggests connectivity of the site in Bone Bay to the eastern coast of Sulawesi by currents.

Connectivity of sites at the northwestern coast of Borneo in the South China Sea to sites along the ITF and eastern Sulawesi was also revealed in the seahorses *H. barbouri* and *H. trimaculatus* (Lourie *et al.* 2005). Panmixing at sites in the Sulawesi Sea and Makassar Strait along the ITF was also found in the mushroom coral *H. actiniformis* (Knittweis *et al.* 2008), Indian scad mackerel *D. russelli* (Rohfritsch & Borsa 2005), the clownfish *Amphiprion ocellaris* (Nelson *et al.* 2000), as well as the mantis shrimps *H. pulchella*, *H. glyptocercus*, and *G. viridis* (Barber *et al.* 2006). Tomini Bay was also not isolated in a study on the scad mackerel *Decapterus macarellus*, which showed no genetic heterogeneity to the Moluccas (Arnaud *et al.* 1999). However, a study on the mushroom coral *H. actiniformis* (Knittweis *et al.* 2008) and mantis shrimp *H. pulchella* (Barber *et al.* 2002) revealed a genetic isolation of the population from Tomini Bay. No separation of the eastern and western coast of Sulawesi was observed in the two mantis shrimp species *Haptosquilla glyptocercus* and *G. viridis* (Barber *et al.* 2006). These differences between species could be attributed to different dispersal capabilities of early life stages and adults.

Western Pacific. The population from Biak in the Western Pacific showed restricted gene flow to all other sites, with a genetic break somewhere in the Moluccas. The strong separation of Biak off the northwestern coast of New Guinea could be caused by the Halmahera eddy, which redirects the westward SEC into the North Equatorial Counter Current (NECC) to the East (Fig. 1, Wyrтки 1961, Gordon & Fine 1996). The strong genetic separation also raise the question if this population represents a cryptic species. Further studies are needed to clarify this. A genetic separation of Western Pacific sites was also found in the above-mentioned three species of mantis shrimps, with a genetic break occurring in the Moluccas (Barber *et al.* 2006).

Conservation

Giant clams are exploited for food, shells and aquarium trade on a local and international scale. According to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) data, international trade in noncaptive bred giant clams increased from about 40 000 specimens in 1993 to about 100 000 in 2001 (Wabnitz *et al.* 2003). Especially in the Indo-Malay Archipelago, stocks have been severely overexploited, including most parts of Indonesia. All tridacnid species are listed in Appendix II of CITES, allowing international trade only with appropriate export permits. Even though giant clams are fully protected in Indonesia and collection, sale and export of wild specimens is banned, enforcement of this legislation is poor (Wells 1997). Clam meat has been consumed extensively by the coastal population in Indonesia and shells were used for different purposes, such as basins, ornaments, and production of tiles (Pasaribu 1988; Wells 1997). Because of its bright colouration pattern, *T. crocea* is a popular species in the aquarium trade (Wabnitz *et al.* 2003). In the 1990s, an Indonesian company was reported to export over 100 000 wild caught, live specimens of *T. crocea* to the international market. It is known from the Philippines that collection causes severe reef damage, because corals are destroyed to extract the clams (Wells 1997). Main importers for giant clams in the marine ornamental trade are the European Union and the USA with about 48 000 and 45 000 specimens imported in 2001, respectively (Wabnitz *et al.* 2003).

Marine protected areas (MPA) are considered an important tool to prevent overexploitation and ensure the sustainable use of living marine resources (Palumbi 2001). Therefore, MPAs are strongly recommended to manage Indonesia's marine fisheries (Mous *et al.* 2005). It is proposed that MPAs should be arranged in a network and that the spatial distribution should match the dispersal capabilities of the species to be protected (Palumbi 2003).

The strong genetic population structure of *T. crocea* across the Indo-Malay Archipelago has important implications for the conservation of this species. The four defined groups should be managed separately, because exchange between the regions is limited. Detected small-scale genetic differentiations in some regions suggest limited larval dispersal, which should be considered in the spatial arrangement of MPAs. Therefore, the Indonesian national system of about 51 MPAs covering 58 000 km² (Spalding *et al.* 2001) seems to be not sufficient to protect species with limited larval dispersal, because these MPAs only cover about 1% of the total marine area of the Indonesian archipelago. While further studies on other invertebrates and fishes are needed, the results of the present study and others cited herein strongly underscore the need to carefully consider connectivity in designing a functional network of MPAs in the Indo-Malay Archipelago and provide preliminary

guidance to the authorities on regions that should be considered as separate management units.

Summary

The complex genetic population structure and pattern of connectivity, characterised by restricted gene flow between some sites and panmixing between others can be attributed to the geological history and prevailing current regimes in the Indo-Malay Archipelago. The major observed genetic differentiation between the Indian Ocean and Western Pacific is most probably due to historical isolation by sea level changes, whereas current oceanographic conditions facilitate connectivity along the ITF and separation at sometimes very small scales, such as in Bone Bay. Prevailing current regimes at the western coast of Sumatra as well as the Halmahera eddy off northwestern New Guinea prevent connectivity of populations in the Eastern Indian Ocean and Western Pacific to the central Indo-Malay Archipelago. These factors cause vicariance between populations, which can lead to allopatric speciation, suggesting that the Indo-Malay Archipelago is a centre of evolutionary radiation (Briggs 1999).

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