ORIGINAL ARTICLE

Concordance of microsatellite and mitochondrial DNA markers in detecting genetic population structure in the boring giant clam *Tridacna crocea* across the Indo-Malay Archipelago

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Abstract

Mitochondrial DNA (mtDNA) is a single, usually non-recombining locus, and often uniparentally inherited. Therefore, its ability to reveal recent gene flow among populations is usually questioned. In this study, the genetic population structure of 16 populations of *Tridacna crocea* (n = 366) from the Indo-Malay Archipelago (IMA) was examined with 10 microsatellite markers and compared to previous studies using mtDNA, in order to test if the revealed population structure was congruent between the two marker systems. The results showed that the genetic population structure revealed by the two marker systems was mostly congruent, with a high correlation between cytochrome c oxidase subunit I (COI) and microsatellites. The studied populations could be divided by both marker systems as follows: (i) Eastern Indian Ocean, (ii) Central IMA, and (iii) Western Pacific. Populations in the Central IMA showed high gene flow. However, populations in the Java Sea (Karimunjava, Pulau Seribu) were grouped into a separate cluster by mtDNA analysis, while this grouping was not detected by microsatellites. It was also noteworthy that there is obvious heterozygosity deficiency in most of the populations, which may be caused by null alleles, inbreeding or population expansion. Overall, these results indicate that the mitochondrial COI gene is applicable for population genetic analysis and precise recovery of connectivity patterns of giant clams. Therefore, the combination of mtDNA and nuclear DNA markers can lead to a more complete understanding of population genetics. Moreover, this study is expected to facilitate fully displaying the population genetic structure of giant clams combining with other researchers' results.

Introduction

Understanding the current distribution of and connectivity among populations of a species is important to illuminate historic and contemporary processes, as well as to provide baseline data for conservation. In recent years, different molecular marker systems have been developed for population genetic studies. Mitochondrial DNA (mtDNA) is widely used in this context and has many advantages. First, it usually evolves fast and thus shows high variability (Brown 1985). Second, due to the availability of universal primers, mtDNA fragments are also relatively easy to amplify and to sequence. Third, in phylogeographic studies, the maternal inheritance reduces

the effective population size and shortens the amount of time required for lineage sorting to reveal phylogeographic patterns (Avise 2000). Lastly, many coral reef organisms, such as corals, sea anemones and giant clams harbor symbiotic algae (zooxanthellae) in their tissues, which needs to be considered during genome marker isolation and application. Therefore, it is much easier to apply mtDNA sequence markers than nuclear markers, as primers are available that do not amplify DNA from zooxanthellae. The population genetic structures of many marine species in the Indo-Malay Archipelago (IMA), one of the main marine evolutionary centers in the world, have been studied using mtDNA, such as giant clams (*Tridacna crocea*, DeBoer *et al.* 2008; Kochzius & Nuryanto 2008; *Tridacna maxima*, Nuryanto & Kochzius 2009), the blue starfish *Linckia laevigata* and its ectoparasite *Thyca crystallina* (Crandall *et al.* 2008a; Kochzius *et al.* 2009), gastropods (*Nerita albicilla* and *Nerita plicata*, Crandall *et al.* 2008b), the tiger prawn *Penaeus monodon* (Benzie *et al.* 2002), the anemonefish *Amphiprion ocellaris* (Nelson *et al.* 2000; Timm & Kochzius 2008) and the scad mackerel *Decapterus russelli* (Perrin & Borsa 2001).

However, mtDNA solely represents one locus, is usually maternally inherited and may be under strong selection (Bazin *et al.* 2006; Lee & Edwards 2008; Zink & Barrowclough 2008; Barrowclough & Zink 2009; Edwards & Bensch 2009). Therefore its ability to reveal recent gene flow among populations is questioned. Nuclear microsatellite markers, which are characterized as codominant and highly polymorphic, are expected to provide greater resolution for population genetic studies and are widely used in studies on genetic population structure of marine species, such as the shrimp *Penaeus monodon* (You *et al.* 2008) and the anemonefish *Amphiprion ocellaris* (Timm *et al.* 2012), as well as the butterflyfishes *Chaetodon meyeri* and *Chaetodon ornatissimus* (DiBattista *et al.* 2012). Many studies have also shown discordant genetic structures in the same species when applying nuclear DNA (nuDNA) and mtDNA (Toews & Brelsford 2012). These findings indicate that caution should be taken when interpreting results from different molecular markers, and multi-locus studies should focus on revealing the driver of this discordance.

The boring giant clam, *Tridacna crocea*, which is distributed around the IMA, has a pelagic larval duration of around 10 days with the adults attached to the substrate (Lucas 1988), and therefore its dispersal capability is limited to some extent. Moreover, it lives in symbiosis with zooxanthellae. It is worth noting that all tridacnid species are listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), allowing international trade

only with appropriate export permits. Special concerns have been raised on the conservation of giant clams, indicating the need for urgent management based on a good understanding of the biology of these species. Population genetic data are already available for giant clams in some areas (Campbell *et al.* 1975; Benzie & Williams 1992a,b, 1995, 1997; Macranas *et al.* 1992; Kittiwattanawong 1997; Yu *et al.* 2000; Kittiwattanawong *et al.* 2001; Laurent *et al.* 2002; Juinio-Meñez *et al.* 2003; Kochzius & Nuryanto 2008; Nuryanto & Kochzius 2009), but only one study has utilized microsatellites (DeBoer *et al.* 2014).

In this study, dense sampling was conducted in Central Indonesia (Borneo and Sulawesi), which was complementary to the study by DeBoer *et al.* (2014). The aims of this study were (i) to investigate the genetic population structure of *T. crocea* in the whole IMA by using microsatellites (DeBoer & Barber 2010; Hui *et al.* 2011), and (ii) to compare the results with a previous investigation utilizing the mitochondrial COI gene (Kochzius & Nuryanto 2008), in order to assess if the revealed genetic population structure is congruent between the two marker systems.

Material and Methods

Sample collection

Small pieces of mantle tissue were collected from 366 specimens of *Tridacna crocea* from 16 sites across the IMA by SCUBA diving (Table 1; Fig. 1). Tissue samples were preserved in 96% ethanol.

DNA extraction, PCR amplification and fragment analysis

Genomic DNA was extracted using the Chelex method (Walsh *et al.* 1991). The population structure was investigated with 10 polymorphic microsatellite markers (DeBoer & Barber 2010; Hui *et al.* 2011; Table 2), which were labelled with 6-Fluorescein amidite or Hexachloro fluorescein. The PCRs were performed in a volume of 15 μl, containing $1 \times$ PCR buffer, 0.5 U *Taq* polymerase (Takara), 1.5 mM of Mg^{2+}, 200 μM of each deoxy-ribonucleotide triphosphate, 0.2 μM of each primer and about 40 ng of genomic DNA. The thermoprofile for all loci was as follows: an initial denaturation of 95 °C for 5 min; 95 °C for 30 s, locus-specific annealing temperature (Table 2) for 45 s, 72 °C for 45 s repeated 35 times, and ending with 72 °C for 5 min. The fragment analysis was conducted with an ABI 3730 Automated Sequencer (Applied Biosystems) using the Liz size standard. The lengths of the fragments were analysed using the software GENEMARKER v. 1.91 (SoftGenetics, LLC).

Table 1. Variation in microsatellite loci of 16 *Tridacna crocea* populations in the Indo-Malay Archipelago.

site	abbreviation	n	locus TC1	TC3	TC5	TC6	TC8	Tc059	Tc074	Tc092	Tc160	Tc161	mean
Padang	Pa	7											
N_a			3	3	5	7	4	3	3	4	3	5	4
H_o			0.429	1.000	1.000	0.857	0.333	0.333	0.000	0.667	0.333	0.667	0.562
H_e			0.582	0.625	0.778	0.827	0.722	0.611	0.667	0.667	0.500	0.778	0.676
P			0.0722	1.0000	1.0000	0.0830	0.0663	0.1984	0.0667	0.6083	0.1945	0.1950	
Pulau Seribu	PS	17											
N_a			5	14	15	16	13	9	10	17	9	14	12.2
H_o			0.167	0.625	0.882	0.529	0.364	1.000	0.765	0.588	0.647	0.824	0.639
H_e			0.764	0.904	0.913	0.905	0.913	0.834	0.848	0.907	0.846	0.901	0.874
P			0.0007	0.0047	0.0031	**0.0000**	**0.0000**	0.0177	0.2608	**0.0000**	0.0560	0.1482	
Karimunjava	Ka	14											
N_a			9	8	13	18	8	7	9	11	7	11	10.1
H_o			0.231	0.636	0.583	0.692	0.571	0.750	0.583	0.600	0.545	0.900	0.609
H_e			0.867	0.810	0.865	0.929	0.857	0.778	0.816	0.875	0.831	0.885	0.851
P			**0.0000**	0.0213	0.0006	0.0079	0.0418	0.1452	0.0068	0.0147	0.0462	0.7049	
Komodo	Ko	27											
N_a			16	11	19	27	18	10	14	24	12	13	16.4
H_o			0.091	0.542	0.731	0.556	0.481	0.778	0.667	0.769	0.815	0.815	0.624
H_e			0.897	0.841	0.920	0.954	0.914	0.802	0.894	0.933	0.885	0.901	0.894
P			**0.0000**	**0.0000**	0.0032	**0.0000**	**0.0000**	0.1031	**0.0002**	**0.0000**	0.3952	0.1325	
Kupang	Ku	9											
N_a			9	11	9	12	10	7	10	12	7	8	9.5
H_o			0.429	0.625	1.000	0.444	0.444	0.889	0.667	0.667	0.889	0.778	0.683
H_e			0.867	0.883	0.858	0.907	0.833	0.772	0.858	0.895	0.846	0.815	0.853
P			**0.0000**	0.0008	1.0000	**0.0000**	**0.0000**	0.4547	0.1068	0.0051	0.8615	0.1074	
Spermonde	Sp	49	49										
N_a			21	14	22	31	26	12	16	25	11	18	19.6
H_o			0.118	0.653	0.625	0.510	0.487	0.776	0.652	0.326	0.625	0.958	0.573
H_e			0.939	0.911	0.928	0.937	0.937	0.832	0.886	0.945	0.900	0.920	0.913
P			**0.0000**	**0.0000**	0.0027	**0.0000**	**0.0000**	0.0199	0.0025	**0.0000**	**0.0000**	0.8890	
Bira	Bi	13											
N_a			6	12	15	15	11	7	11	11	10	12	11
H_o			0.000	0.750	0.923	0.462	0.273	0.818	0.583	0.444	0.700	0.500	0.545
H_e			0.833	0.892	0.908	0.923	0.888	0.781	0.882	0.901	0.840	0.889	0.874
P			**0.0002**	0.1006	0.0456	**0.0000**	**0.0000**	0.2662	**0.0000**	**0.0000**	0.0982	**0.0000**	
Sembilan Islands	Se	30											
N_a			11	17	13	28	17	12	12	21	11	18	16
H_o			0.067	0.655	0.552	0.690	0.520	0.800	0.571	0.433	0.345	0.800	0.543
H_e			0.882	0.898	0.825	0.951	0.913	0.857	0.885	0.914	0.856	0.915	0.889
P			**0.0000**	**0.0000**	**0.0000**	0.0165	**0.0000**	0.1233	**0.0000**	**0.0000**	**0.0000**	**0.0000**	
Kendari	Ke	28											
N_a			19	12	14	23	21	8	11	21	14	17	16
H_o			0.333	0.893	0.680	0.571	0.536	0.840	0.857	0.654	0.679	0.857	0.690
H_e			0.931	0.866	0.874	0.934	0.943	0.816	0.855	0.927	0.882	0.880	0.891
P			**0.0000**	0.0355	**0.0000**	**0.0000**	**0.0000**	0.6049	0.2767	0.0041	**0.0000**	0.5814	
Luwuk	Lu	28											
N_a			11	14	19	18	16	11	14	19	13	16	15.1
H_o			0.214	0.704	0.538	0.357	0.240	0.679	0.821	0.750	0.680	0.815	0.580
H_e			0.880	0.897	0.914	0.908	0.894	0.865	0.879	0.908	0.845	0.901	0.889
P			**0.0000**	0.0143	**0.0000**	**0.0000**	**0.0000**	0.1790	0.4611	0.0122	0.0195	0.0040	

Table 1. Continued

site	abbreviation	n	locus TC1	TC3	TC5	TC6	TC8	Tc059	Tc074	Tc092	Tc160	Tc161	mean
Togian Islands	TI	63											
N_a			27	18	24	32	28	11	19	30	17	20	22.6
H_o			0.538	0.726	0.695	0.435	0.500	0.762	0.754	0.635	0.444	0.903	0.639
H_e			0.945	0.910	0.939	0.948	0.950	0.865	0.853	0.920	0.895	0.903	0.913
P			**0.0000**	0.0009	**0.0000**	**0.0000**	**0.0000**	**0.0000**	0.0168	**0.0000**	**0.0000**	0.0007	
Manado	Ma	10											
N_a			6	6	13	12	7	7	10	10	10	9	9
H_o			0.125	0.500	0.800	0.300	0.429	0.800	0.889	0.444	0.800	0.900	0.599
H_e			0.820	0.800	0.905	0.895	0.786	0.825	0.877	0.858	0.845	0.855	0.847
P			**0.0000**	0.0160	0.1545	**0.0000**	0.0043	0.0061	0.2589	**0.0000**	0.2453	0.9079	
Sangalaki	Sa	17											
N_a			10	12	15	19	16	8	10	19	10	12	13.1
H_o			0.636	0.706	0.647	0.471	0.706	0.765	0.824	0.471	0.765	1.000	0.699
H_e			0.851	0.893	0.882	0.929	0.915	0.768	0.860	0.931	0.843	0.875	0.875
P			0.0293	0.0383	**0.0000**	0.0028	0.0164	0.6118	0.5317	**0.0000**	0.1332	0.5761	
Kota Kinabalu	KK	23											
N_a			7	13	13	23	3	9	12	18	10	15	12.3
H_o			0.100	0.636	0.762	0.762	0.333	0.778	0.762	0.400	0.800	0.818	0.615
H_e			0.805	0.896	0.848	0.940	0.611	0.795	0.838	0.921	0.840	0.914	0.841
P			**0.0000**	**0.0000**	0.0008	**0.0000**	0.1981	0.3930	0.5337	**0.0000**	0.0150	**0.0000**	
Misool	Mi	11											
N_a			9	8	10	17	8	8	8	13	9	10	10
H_o			0.375	0.818	1.000	0.818	0.500	0.818	0.818	0.545	0.545	0.818	0.706
H_e			0.859	0.839	0.885	0.934	0.830	0.773	0.810	0.901	0.806	0.860	0.850
P			**0.0000**	0.0727	0.2876	0.0697	0.0085	0.9206	0.9792	**0.0000**	0.0517	0.2861	
Biak	Bk	20											
N_a			12	11	18	19	2	8	12	20	9	13	12.4
H_o			0.111	0.450	0.950	0.350	0.000	0.750	0.500	0.700	0.600	0.650	0.506
H_e			0.887	0.860	0.925	0.935	0.500	0.850	0.850	0.935	0.808	0.883	0.843
P			**0.0000**	**0.0000**	0.0007	**0.0000**	0.3336	0.0079	**0.0000**	0.0008	0.0274	**0.0000**	

n = number of individuals; N_a = number of alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; P = P-value for deviations from Hardy–Weinberg proportions (HWP; The values in bold, $P < 0.00052$, mean in these loci, the populations are departed from HWP).

Data analysis

The genetic indices, including the number of alleles (N_a), the observed heterozygosity (H_o) and expected heterozygosity (H_e), for each population were calculated with the program GenAlEx (v. 6.41; Peakall & Smouse 2006). Deviations from Hardy–Weinberg proportions (HWP) for each locus in each population as well as all populations and all loci were performed using the Markov chain randomization test (Guo & Thompson 1992) to estimate the exact two-tailed P-values, as implemented in the program GEN-EPOP WEB (v. 4.0.10; Raymond & Rousset 1995; Rousset 2008). The significance level was adjusted by applying the sequential Bonferroni correction (Rice 1989).

Population structure was examined in several ways. First, pairwise genetic differentiation index (F_{st}) estimates were estimated to assess the magnitude of differentiation among sample sites. An unbiased estimate of the significance of the probability test was calculated by using ARLEQUIN (v. 3.5;

Excoffier & Lischer 2010) with 1000 iterations. The significance of the pairwise comparisons was evaluated after a sequential Bonferroni adjustment of the significance level. Second, in order to directly compare the results from both marker systems, correlations among population pairwise F_{st} values obtained from microsatellites and mtDNA were analysed using a Mantel test with 1000 permutations (negative F_{st} values were set to zero). Genetic structure among populations was investigated by conducting a Bayesian analysis with the software STRUCTURE (v. 2.3.1; Pritchard *et al.* 2000), testing for different numbers of clusters (K) in the data. The admixture model was used, with no allele frequencies correlated between populations and no prior information on population origin. The individuals were assigned to clusters, even to two or more clusters, if their genotypes indicated that they were admixed. The assignment settings were 200,000 iterations and the first 20,000 as burn-in. K-values ranged from 1 to 8 and each test was run three times. The Delta K method ($\Delta K = m (|L(K + 1) - 2L$
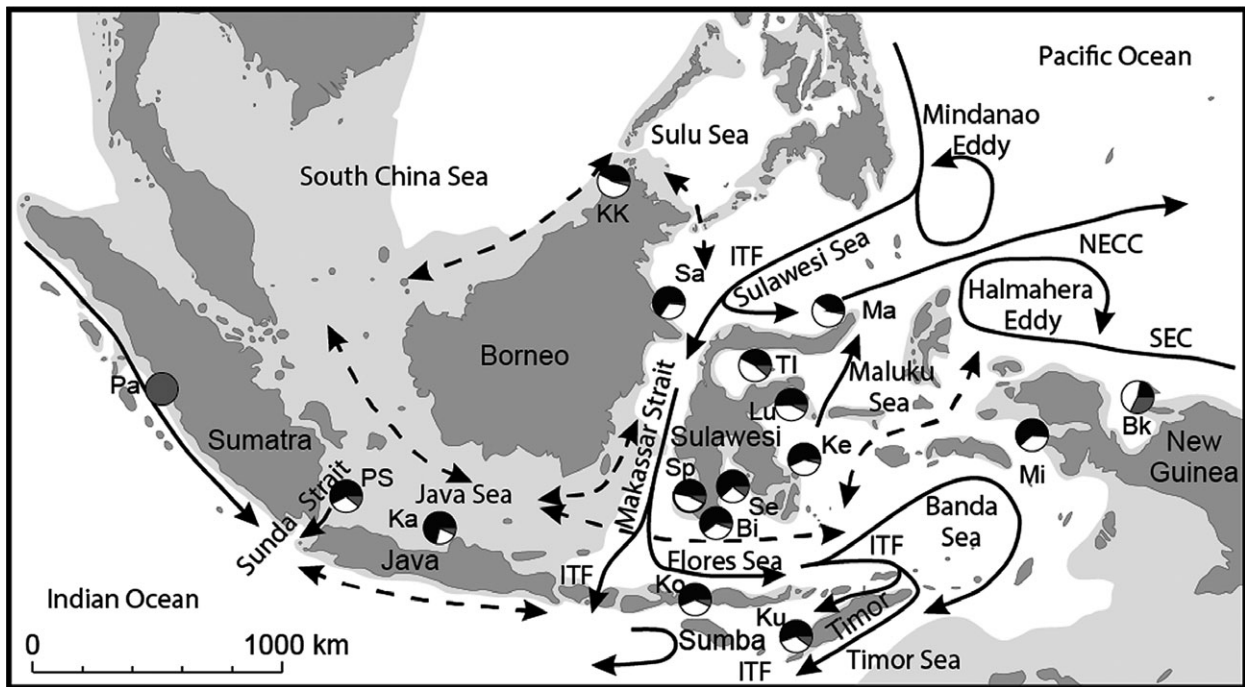
Fig. 1. Map of the Indo-Malay Archipelago with sample sites indicated (see Table 1 for definitions of abbreviations). Surface currents with dominant (solid lines) and seasonally changing currents (dashed lines) (Gordon 2005; Gordon & Fine 1996; Wyrtki 1961), including the Indonesia Throughflow (ITF), South Equatorial Current (SEC) and North Equatorial Counter Current (NECC), are shown. The Pleistocene sea-level low stand of 120 m (light gray area) is also shown (Voris 2000). Pie charts represent the proportions of different genetic clusters (black, gray and white) in each population defined by STRUCTURE 2.3.1. See main text for explanations of the colours.

(K)+L(K−1)|)/s[L(K)]) was used to determine the exact K-value (Evanno *et al.* 2005). Then 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 as pie charts to visualize the distribution of the clusters. Third, an analysis of molecular variance (AMOVA) was conducted to find the spatial groups of populations that best explained the partitioning of genetic variance.

An isolation-by-distance (IBD) analysis was performed to test for a correlation between genetic distances (F_{st}) and geographic distances by reduced major axis regression analysis. Later a Mantel test was conducted to check the significance of the correlation. All calculations were performed using the IBD web service (IBDWS 3.23, http://ibdws.sdsu.edu), applying 30,000 permutations (Jensen *et al.* 2005). Geographic distances were taken as the shortest way between two sample sites by sea, measured with an electronic world atlas (www.earthol.com).

Results

Genetic diversity of different populations

All 10 microsatellite loci showed high polymorphism. Across all loci, the mean number of alleles per population

ranged from 4.0 (Padang, Pa) to 22.6 (Togian Islands) (Table 1). The high within-population diversity was also detected in the expected and observed heterozygosity values (Table 1). The population in Misool had the highest H_o value (0.913), while the population from Biak, located in the West Pacific Ocean, showed the lowest (0.506).

In the HWP test, 94 observed genotype distributions generally conformed to HWP, but in 66 cases a significant deviation from HWP after adjustment of P-values with the sequential Bonferroni method was detected (Table 1). All global estimates of HWP for populations and loci showed significant heterozygote deficiency in each population and loci (P < 0.0034).

Genetic population structure based on microsatellites and comparison with mtDNA

The overall AMOVA showed significant genetic structure ($F_{st} = 0.023$, P < 0.001). When a pairwise F_{st} analysis (P < 0.00054 after sequential Bonferroni correction) was performed, the population from the Eastern Indian Ocean (EIO; Pa), showed significant differentiation from all other populations (F_{st}: 0.158–0.190), while the population from the Western Pacific (WP; Biak) also differed from most of the populations in the Central IMA (F_{st}:

Table 2. Characteristics of microsatellite loci for *Tridacna crocea*.

locus	primer sequence (5′–3′)	T_a (°C)	repeat motif	dye	expected size range (bp)
TC1	F: GCTTTGTGGCTATTGGAGAA	56	$(AG)_{22}...(AG)_{16}...(AG)_6$	6-FAM	275–339
	R: GTCTGTCCCACCCGTCCATA				
TC3	F: ACCATACCCCTGCCATACAGT	56	$(AG)_{19}$	HEX	227–249
	R: AGTCGCGTCACTCTGGATAG				
TC5	F: AGAAATGACGTAACACCCAC	56	$(CT)_{24}$	6-FAM	135–165
	R: ATACTATGCAGAGGAAGGAG				
TC6	F: CATGGTGGACGATGCCAAGT	56	$(AG)_{18}$	6-FAM	160–206
	R: CTACGAAAATCACTGACTCTG				
TC8	F: CAAAGTTCAAAATACACTCG	50	$(ATGG)_6...(ATGG)_8...(ATGG)_8$	HEX	274–358
	R: TACCGTACCAGAGGCAACTA				
Tc059	F: AGGTGACTTGAAGGTTAATGTTG	57.9	$(AAC)15$	HEX	110–140
	R: GGGTTTAAAACAACACGGTGA				
Tc074	F: CCAAAAACAGTCTTCCTTGACA	57.9	$(AGTG)10$	6-FAM	202–266
	R: AGACTGCTCGCTGACCTTTT				
Tc092	F: GCAACCCACTGAGTTCCCTA	50	$(TC)13TTAA(AC)18$	HEX	176–234
	R: TGCAGAGCGATAACATACAGG				
Tc161	F: TGAAAATATGTTAACCCTCATCCTG	57.9	$(AC)8$	6-FAM	92–122
	R: TTTGCAGACTGGTTTAGTCAACA				

F = forward; 6-FAM, fluorescein amidite; HEX, hexachloro fluorescein; R = reverse; T_a = optimal annealing temperature.
Markers TC1, 3, 5, 6 and 8 were developed by Hui *et al.* (2011) and Tc059, 074, 092, 160 and 161 are from DeBoer & Barber (2010).

Table 3. Pairwise genetic differentiation index (F_{st}) values between populations of *Tridacna crocea* in the Indo-Malay Archipelago.

sites	Pa	PS	Ka	Ko	Ku	Sp	Bi	Se	Ke	Lu	Tl	Ma	Sa	KK	Mi
PS	**0.165**														
Ka	**0.178**	0.005													
Ko	**0.173**	0.017	0.006												
Ku	**0.190**	0.017	0.023	0.006											
Sp	**0.158**	0.009	0.013	0.007	0.007										
Bi	**0.174**	0.009	0.020	−0.003	−0.001	0.001									
Se	**0.162**	0.023	0.014	0.009	0.020	0.010	0.006								
Ke	**0.180**	0.015	0.017	0.003	0.009	0.007	0.012	0.010							
Lu	**0.177**	0.020	0.026	0.010	0.014	0.014	0.003	0.018	0.010						
Tl	**0.159**	0.018	**0.027**	**0.014**	0.018	**0.009**	0.008	**0.019**	0.010	0.012					
Ma	**0.188**	0.024	0.029	0.017	0.032	0.016	0.023	0.025	0.027	0.019	0.025				
Sa	**0.174**	0.014	0.009	0.007	0.003	0.005	−0.008	0.009	0.010	0.014	0.014	0.023			
KK	**0.186**	0.013	0.021	0.011	0.017	0.007	0.015	0.021	0.021	0.018	0.016	0.029	0.014		
Mi	**0.186**	0.018	0.028	0.009	0.019	0.014	0.002	0.018	0.017	0.020	0.021	0.033	0.004	0.010	
Bk	**0.161**	0.039	0.048	**0.042**	0.048	**0.036**	0.030	**0.040**	**0.041**	**0.038**	**0.029**	0.039	0.035	**0.039**	0.043

Values in bold mean populations are significantly differentiated after sequential Bonferroni correction with $P < 0.00054$.
See Table 1 for definitions of site name abbreviations.

0.029-0.048) (Table 3). In the Central IMA, the population in the Togian Islands was different from the populations in Karimunjava (Ka), Komodo, Spermonde and Sembilan Islands (Se) (F_{st}: 0.009–0.027), while the other populations showed no significant difference between each other.

A Mantel test revealed a significant correlation of pairwise F_{st} values (r = 0.946, P < 0.001) based on microsatellites (Table 3) and cytochrome c oxidase subunit I (COI)

sequences, suggesting that the two data sets have a similar genetic population structure.

The cluster analysis with STRUCTURE revealed the presence of three rather distinct populations. The ancestry of each individual from the three inferred clusters was then calculated, and the cluster frequencies for each population are presented in Fig. 1. As shown, the dominant cluster in the EIO (Pa) was gray and individuals were all assigned to this cluster. In the Central IMA [Java Sea,

Table 4. Results of hierarchical analysis of molecular variance of microsatellite markers in *Tridacna crocea* from the Indo-Malay Archipelago.

groupings	F_{ct}	percentage of variation (%)
(Pa) (PS, Ka, Ko, Ku, Sp, Bi, Se, Ke, Lu, Tl, Ma, Sa, KK, Mi) (Bk)	0.063*	6.27
(Pa) (PS, Ka, Ko, Ku, Sp, Bi, Se, Ke, Lu, Tl, Ma, Sa, KK) (Mi, Bk)	0.041**	4.10
(Pa) (PS, Ka, Ko, Ku, Sp, Bi, Se, Ke, Lu, Tl, KK, Mi) (Ma, Sa) (Bk)	0.033**	3.35
(Pa) (PS, Ka, Ko, Ku, Sp, Bi, Se, Ke, Lu, Tl, Ma, Mi) (Sa, KK) (Bk)	0.028*	2.82
(Pa) (PS, Ka, Ko, Ku) (Sp, Bi, Se, Ke, Lu, Tl, Ma, Sa, KK, Mi) (Bk)	0.023***	2.32
(Pa) (PS, Ka) (Ko, Ku, Sp, Bi, Se, Ke, Lu, Tl, Ma, Sa, KK, Mi) (Bk)	0.016*	1.60

* $= 0.05 \geq P \geq 0.01$; ** $= 0.01 > P > 0.001$; *** $= P < 0.001$; F_{ct}, genetic differences among groups.
See Table 1 for definitions of site name abbreviations.
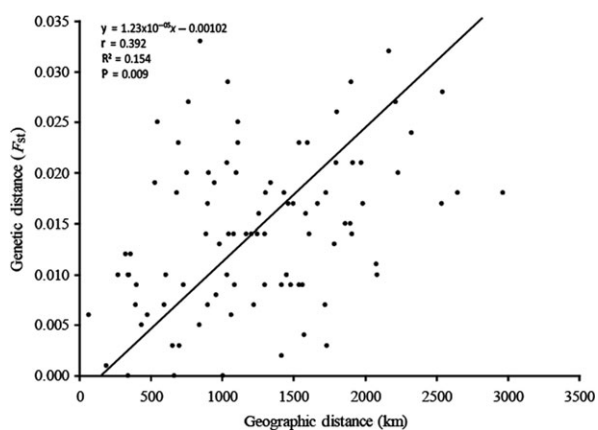
Fig. 2. Relationships between genetic and geographic distances for *Tridacna crocea* by reduced major axis regression without the populations from Padang and Biak.

South China Sea, Indonesian Throughflow (ITF), as well as seas to the east of Sulawesi], the dominant clusters were black and white, while in the WP (Biak), the dominant clusters were gray and white.

Based on geography and oceanography, a hierarchical AMOVA was carried out with different groupings (Table 4) for the 16 sample sites. The AMOVA revealed the highest fixation index ($F_{ct} = 0.063$, P < 0.05) and variation (6.27%) among groups when the populations were grouped into the following regions: (i) EIO, (ii) Central IMA and (iii) WP. Compared with the results of the previous study based on COI (Kochzius & Nuryanto 2008), the structure was highly similar. However, populations in the Java Sea [Ka, Pulau Seribu (PS)] were grouped into one

separate cluster in the study using COI sequences (Kochzius & Nuryanto 2008), while this divergence was not detected by microsatellites in the present study.

Isolation-by-distance showed a significant positive correlation between genetic (F_{st}) and geographic distance in the Central IMA (r = 0.392, P = 0.009) (Fig. 2). Divergent populations from Padang and Biak were excluded from this analysis. This correlation was also detected by the previous study based on COI (r = 0.430, P = 0.004) (Kochzius & Nuryanto 2008).

Discussion

The results based on 10 microsatellite loci were generally concordant with the results obtained using COI sequences, supporting the applicability of this mtDNA marker in connectivity studies for giant clams.

Genetic diversity and HWP

The genetic diversity in all populations based on microsatellites was very high, with the average N_a ranging from 4 to 22.6. The H_o values ranged from 0.506 to 0.706. Similar results were found in the COI analysis, as illustrated by the high overall haplotype diversity (0.93). Molecular markers have also revealed high levels of genetic diversity in some other marine bivalves, such as *Crassostrea plicatula* and *Crassostrea gigas* (microsatellites; Yu *et al.* 2008), *Chlamys farreri* (microsatellites; Zhan *et al.* 2009) and *Tridacna maxima* (COI; Nuryanto & Kochzius 2009). These findings may be due to the large population sizes and high nucleotide mutation rates in this taxon (Launey & Hedgecock 2001; Hedgecock *et al.* 2004). Compared with other populations, the one in the EIO, *i.e.* population Pa, showed a much lower genetic diversity than the others, possibly due to its small population size. Populations from the Java Sea (Ka, PS) showed the lowest genetic diversity in the COI analysis, possibly as a result of over-exploitation or the re-colonization of the Sunda Shelf as sea levels rose after the last glacial (Kochzius & Nuryanto 2008). However, the genetic diversity levels detected by microsatellite markers were moderate in these two populations. This suggests that different markers might detect different levels of genetic diversity (Hoffman *et al.* 2009). Comparing mtDNA and nuDNA, the effective population size of mtDNA is much smaller than that of nuDNA (about a quarter, Avise 1994). Therefore, mtDNA might be more sensitive to genetic drift than nuDNA, which partly induced the difference of genetic diversity revealed by the two marker systems.

Global tests of HWP for populations and loci found significant heterozygote deficiency in all populations, possibly caused by inbreeding or population expansion.

Further, significant departure from HWP in the form of heterozygote deficit may also be ascribed to a Wahlund effect caused by subpopulation structure. It could also be a consequence of the relationship between sample size and microsatellite variability: considering the high allele numbers in the different populations, HWP testing could have been influenced by small sample size (Györffy *et al.* 2004). However, the smallest population (Pa) showed no deviation from HWP.

Genetic population structure

Three distinct groups were found in the IMA based on the microsatellite analysis, corresponding to the (i) EIO, (ii) Central IMA and (iii) WP. This result was similar to previous studies using COI as a genetic marker and the study of DeBoer *et al.* (2014). However, in the study of Kochzius & Nuryanto (2008), populations in the Java Sea were assigned to a separate group. In addition, the highly divergent mtDNA clades in the previous study (Kochzius & Nuryanto 2008) suggested that there might be cryptic species, while the genetic structure revealed by microsatellites in this study was much lower. Given that *Tridacna crocea* is hermaphroditic, sex-biased gene flow in mtDNA cannot be the reason for this difference. One reason might be the homoplasy of microsatellite alleles. Alleles of the same size might have variation in the non-repeated flanking sequences (Grimaldi & Crouau-Roy 1997), which might lead to underestimation of genetic differentiation. Again, random genetic drift due to severe bottlenecks, especially for mtDNA, which is more sensitive to effective population size changes, may have created divergent genetic groups during the sea-level fluctuations. Moreover, the higher mutation rate in microsatellite DNA (average 1.2×10^{-3} per generation; Weber & Wong 1993) compared with mtDNA could be another factor inducing the difference (Fauvelot *et al.* 2003; Timm *et al.* 2012). Therefore, claims of a generally higher resolving power for either marker type are not correct (Larsson *et al.* 2009). However, the pairwise F_{st} values based on microsatellite data obtained here are highly correlated with the values obtained from the COI data ($r = 0.946$, $P < 0.001$), which indicates that the two marker systems generally revealed concordant genetic structures. In the Central IMA, the results from microsatellites also revealed that the populations in this area showed high gene flow (low pairwise F_{st} values that were not significant). There was also a positive correlation between genetic and geographic distances, which was similar to the results revealed by mtDNA.

Similar genetic structures have been found in two other giant clam species (*Tridacna maxima*, Nuryanto & Kochzius 2009; *Tridacna squamosa*, Hui *et al.* 2016). Vicariance due to Plio-Pleistocene sea-level fluctuation is suggested to have been a major force driving the genetic divergence between Indian and Pacific Ocean populations (*e.g.* Mcmillan & Palumbi 1995; Williams & Benzie 1998; Kochzius *et al.* 2003, 2009; Timm & Kochzius 2008; Knittweis *et al.* 2009). The oceanography of the Indo-West Pacific, such as the ITF and Halmahera eddy (Fig. 1), and the somewhat limited larval dispersal capability are possible factors shaping the genetic structure and connectivity of giant clam populations.

Collectively, the concordant genetic structures illustrated by the two marker systems confirmed the reliability of the results presented here. Moreover, the mtDNA revealed much stronger population structure than the nuDNA, which may be because of the characteristics of mtDNA, such as lower effective population sizes and short coalescence times. The combination of mtDNA and nuDNA markers is expected to provide a more complete understanding of population genetics in future studies.

Acknowledgements

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