

GENETIC PATTERNS OF *Tridacna crocea* IN THE BOHOL SEA

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ABSTRACT

*The genetic patterns of six populations of the giant clam, *Tridacna crocea* from reefs in the Bohol Sea was determined using mitochondrial cytochrome oxidase 1 (CO1) primers. Samples from the Spratly group of islands were included for comparison. Results showed a remarkably high level of haplotypic diversity but low nucleotide diversity. Hierarchical Analysis of Molecular Variance (AMOVA) revealed fine scale genetic differentiation among geographical areas, however, within group analysis showed homogeneity in the Bohol Sea which indicates gene flow among the populations. This translates to MPA connectivity and highlights the importance of each MPA sampled as both source and sink of *T. crocea* in the Bohol sea and adjacent areas.*

Keywords: mitochondrial, genetic structure, giant clam, *tridacna crocea*

Introduction

Spatially distributed adult populations of demersal and benthic marine species possessing pelagic larvae have the potential for considerable levels of connectivity among local populations. Connectivity is simply defined as the exchange of individuals among marine populations. It is potentially of considerable importance, particularly those mediated by larval dispersal, for determining demography of local populations (Sale & Kritzer, 2003; Caley et al., 1996, Sale, 1998; Mora & Sale, 2002). Knowledge of the extent of connectivity, and its demographic consequences, is particularly important for population management for fisheries or conservation (Sale & Kritzer, 2003; Roberts, 1997; Kinlan et al, 2005; Sale et al., 2006). Understanding the patterns of connectivity is important for any form of spatially specific management of marine populations, especially if management tools include use of marine protected areas (MPAs).

Tridacna crocea (Bivalvia: Mollusca) is the smallest among the giant clams. Just like any other bivalve, it has planktonic larval stage in its life cycle. It takes 8-10 days for this species to settle and begin its sessile life on massive coral heads and limestone (Alcazar, 1988) Thus, its dispersal is influenced greatly by currents. Among the giant clams, *T. crocea* is the most abundant in Philippine reefs despite the existence of commercial harvesting (Gomez & Alcala, 1988; Alcala, 1986) particularly in south Tubbataha and Pamilacan Island. Exports of *T. crocea* in vast amounts have been known, thus, efforts were made to protect its population (Gomez & Alcala, 1988).

Assessing gene variation within and between giant clam populations is a basic part of a giant clam conservation plan. Earlier studies on giant clam genetic structure in the west Pacific region (Benzie, & Williams, 1995, Benzie, J.A.H. and Williams, S.T. 1997) French Polynesia (Laurent & Salvat. 2002) and eastern Philippine seaboard (Ravago-Gotanco *et al.*, 2007) utilized allozymes with protein electrophoresis. More recently, Kochzius and Nuryanto (2008) and DeBoer *et al.* (2014) determined patterns of genetic connectivity in *Tridacna crocea* in the Indo-Malay Archipelago using the mitochondrial DNA sequence data. Molecular analysis of genetic variability and phylogeny of bivalves using mitochondrial genes (mtDNA), *cytochrome c oxidase I* (COI) have shown that COI is a suitable marker to infer connectivity in bivalve populations (Luttikhuisen *et al.* 2007 ; Ward, 2000). Molecular markers at the DNA-level could justify and reveal better gene flow patterns since the variations at this level come from the changes of a base that may be found in both coding and noncoding regions of the genome (Yu *et al.*, 2000).

Earlier study on genetic connectivity within the Bohol Sea was on two fish species, *Dascyllus trimaculatus* and *Pterocaesio pisang* by Ablan [3] using microsatellites. However, the use of microsatellites requires longer time, more complicated equipment and expertise to develop them compared to mitochondrial DNA and allozyme markers (Ward, 2000). In addition, even two closely related and co-occurring species can exhibit substantially different connectivity patterns (Becker, *et al.*, 2007). Local differences in life-history parameters interacting with ocean currents may cause differences in connectivity patterns. Variations in larval duration, delay of metamorphosis, initiation of spawning, local mortality, and vertical position of the larvae within the water column may affect connectivities. The challenge remains to integrate our understanding of the different connectivity patterns of key species as basis for the formulation of a more effective

management scheme of MPA network in the region. Hence, further studies should be undertaken on other groups of marine organisms with passive larval dispersal like the giant clams to enhance and deepen our understanding on the connectivity patterns in the Bohol Sea and adjacent seas.

For many marine species, population connectivity is determined mainly by tides and ocean currents transporting eggs, larvae and juveniles between distant patches of suitable habitat (Palumbi, S.R., 2001; Treml, 2008; Shanks, 2003; Scheltema, 1986; Scheltema, 1988). Bohol Sea is connected to the Philippine Sea and the Pacific Ocean through the Surigao Strait at the northeast and to the Sulu Sea at the southwest (Fig. 1). Meñez *et al.* (2006) inferred a southwest movement of surface ocean currents in the Bohol Sea which occurs during the northeast monsoon. With this direction of water current, reefs at the entrance of the Bohol Sea can be possible sources of propagules. Whereas, during the SW monsoon water enters the Bohol Sea via the Dipolog Strait from the Sulu Sea which in turn receives currents from the South China Sea. The U.S.NLOM (2008) showed similar current patterns in the Bohol Sea, however, have found the occurrence of eddies and gyres on certain times of the year (Fig. 1). Furthermore, Bohol Sea water enters into the Tañon Strait over the shelf and into the strait driven by water density variations maintained by turbulence generated by the strong constricted flow at the southern opening (2002). Within Tañon Strait, typically, flood tides move surface currents northward while ebb tides move surface currents southward. During the NE monsoon, surface currents during flood phase showed a predominantly strong northward flow at the eastern side (close to the Cebu coast) of the strait which reversed during ebb tides. The Commission found that during SW monsoon, surface currents in the northern boundary did not seem to undergo complete reversals but showed cross strait transport just off the northern tip of Negros Island. Unlike the northern boundary, the flow through the southern boundary underwent complete reversals with the tides.

The Visayan Sea, where Carbin Reef is situated, is located in the midst of the Visayan Islands that comprise the central portion of the Philippines (Fig. 1). It is connected to the Samar Sea to the northeast, the Camotes Sea to the southeast, the Bohol Sea to the southwest via the Tañon Strait, and the Panay Gulf to the southwest via the Guimaras Strait. On the other hand, the Southeastern Samar site (Fig. 1) was hypothesized in this study to be a possible source of propagules of *T. crocea* and other broadcast spawning marine invertebrates, driven by the Mindanao current flowing southward

from the bifurcation of the North Equatorial Current (NEC) from the Pacific Ocean. Mindanao Current has a maximum speed of approximately 1 m s^{-1} and 200 km width (Wijffels, 1995; Takaomi et al., 2003; Ravago-Gotanco *et al.*, 2007). According to Nitani (1982), Qu (2002) and Ravago *et al.* (2007) the bifurcation latitude is estimated to lie between 11 and 14.5 N which exhibits seasonal meridional shifting from 14.8 in summer up to 17 N during winter (Qiu & Lukas, 1996). Aliño and Gomez (Aliño & Gomez, 1993) believed that the divergent current pattern generated by the NEC bifurcation is one of the primary determinants in the north-south geographical differentiation of reef species composition along the eastern Philippines where Southeastern Samar is located.

This study therefore tests the hypothesis that the prevailing water currents in the Bohol Sea, Tañon Strait, Visayan Sea and Southeastern Samar influence the geneflow among *Tridacna crocea* populations in those areas.

Methods

Sampling, DNA Extraction, Amplification and Sequencing

Tridacna crocea populations from the reefs along Tañon Strait, Bohol Sea, Visayan Sea, Southeastern Samar and an outgroup, the Spratly Islands, were sampled (Fig 1). A small piece of tissue from the mantle margin was snipped using a pair of surgical scissors. The tissue was placed in small vials with 95% ethanol and stored at -20°C . Genomic DNA was extracted from approximately 1 mm^2 *Tridacna crocea* mantle tissue. This was done using TNES-urea digestion buffer (6 M urea, 1M Tris-HCl pH 7.5, 5 M NaCl, 0.5 M EDTA, and 1% SDS or sodium dodecyl sulfate) as described in Wasko *et al.* (2003) and Proteinase K treatment followed by standard phenol-chloroform extraction method. Partial sequences (500 bp) of the mitochondrial *cytochrome c oxidase 1* (COI) gene were amplified with a specific primer for *Tridacna crocea* (Tridacna 1F 5'- ACC CTT TAY TTT TTA TTA GCA Y- 3'; Tridacna 3R 5'- CAA TGC TGT AAT CGC CAA TGA C-3'). PCR reactions were performed into a 50 μL reaction volume consisting of 1x PCR buffer, 2 mM MgCl_2 , 10mM dNTP, 0.2 μM of forward and reverse primers, 0.5 U of Promega *Taq* polymerase products and 20-25 ng extracted genomic DNA. Cycling times were 1 min at 94°C followed by

6 cycles of 30 s at 94 °C denaturation, 1 min 30 s at 45 °C annealing and 1 min at 72 °C elongation, 36 cycles of 30 sec at 94 °C, 1 min 30 sec at 51 °C, 1 min at 72 °C plus a final 5 min extension at 72 °C. The PCR products were visualized using 1% (w/v) agarose gel electrophoresis and were sequenced on an ABI 377 or an ABI 3730 automated sequencer using Big Dye (Applied Biosystems, Foster City, CA) terminator chemistry.

Genetic diversity and structure

DNA sequences were subjected to NCBI's BLAST sequence similarity search (1990) to confirm sample identification. These were then aligned and edited using the Geneious v.3 [46]. Standard indices of haplotypes and nucleotide diversity were computed using DnaSP v. 4 [47] and Arlequin 3.1 [48], which was also used to conduct Fu's FS and Tajima's D tests of selective neutrality. Variation among and within collection sites was partitioned by hierarchical Analysis of Molecular Variance (AMOVA) using ARLEQUIN version 3.1 (Excoffier, 2006). Cluster analysis was conducted by constructing a phylogram on Kimura 2-P genetic distance using the neighbor joining (NJ) method. Pairwise comparisons of F-statistics were made to look at significant genetic differences within and among populations. Finally, isolation-by-distance effects on population genetic structure was investigated using Reduced Major Axis (RMA) regression analysis and significance of the correlation was tested by Mantel Test utilizing the Isolation by Distance Web Service (IBDWS Version 3.15). The statistical significance of correlations between distance matrices was obtained from 10,000 random permutations of matrix elements.

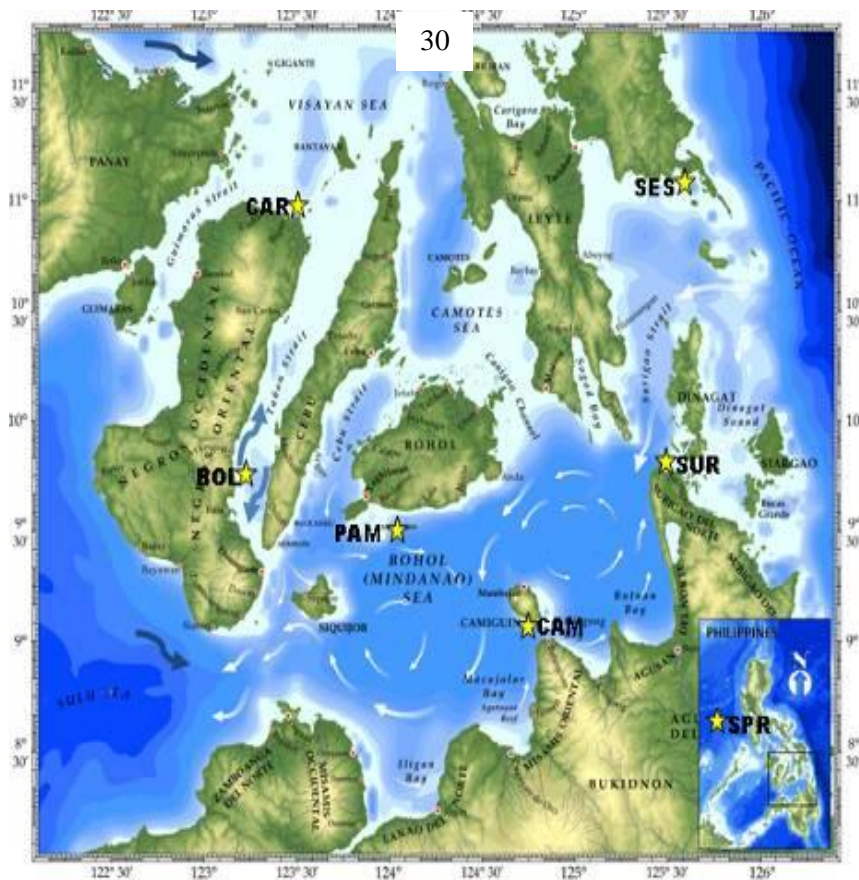


Figure 1. The study sites (★) and current profiles of the Bohol Sea, Tanon Strait and neighboring seas. Ocean currents based on the US-NLOM May 2006 (white arrows), Menez *et al.* (2006) (dark arrows) and Tañon Strait Commission (2002) (light arrows). Site codes found in Table 1. *Basemap modified by J. Maypa from Reefbase GIS.*

Results

Genetic Diversity

A total of 133 *Tridacna crocea* were screened for variation from a 487-bp partial mitochondrial COI sequence and detected 54 haplotypes, 41 of which were unique to single individuals. Of the 487 bases, 46 (9.45%) were polymorphic sites and 248 monomorphic sites. Neighbor-joining analysis of the 54 haplotypes identified ten clades (not shown in this paper). As indicated in Table 1, haplotype diversity was high in all populations (range:

0.735 - 0.957) with populations from Pamilacan having the lowest and Camiguin as the highest while nucleotide diversity was generally small in all populations. Pamilacan exhibited the smallest nucleotide diversity (0.0063) while SE Samar, the highest (0.0114). The overall haplotype diversity (Hd) was 0.889 and nucleotide diversity (PiT, π) was 0.01. The general pattern of relatively high haplotypic diversity and low nucleotide diversity could be due to population expansion following bottleneck (Grant and Bowen, 1998).

Genetic Structure and Relationships Among Populations

Unstructured AMOVA results based on Kimura 2-parameter revealed a highly significant structure ($F_{ST} = 0.197$, $p < 0.05$) with 80.32% of the variance within populations but only 19.68% among populations (Table 2). Hierarchical AMOVA separating the populations into five geographical subdivisions - Bohol Sea, Tañon Strait, Visayan Sea, Southeastern Samar and South China Sea - revealed a very high significant genetic differentiation among the five geographical locations ($F_{CT} = 0.220$, $p = 0.039$) and within populations ($F_{ST} = 0.232$, $p = 0.00$), with 22.02% of the variance due to differences among the five groups. The 1.22% of the variation was among populations within those groups. Among populations within groups, variation was low ($F_{SC} = 0.016$, $p = 0.11$), but, not significant. P-values calculated from 1023 random permutations represent the probability of obtaining a more extreme variance component and F-statistic than the observed values by chance alone (Excoffier and Schneider, 2005).

A neighbor-joining tree was computed from the matrix of corrected genetic distances in PHYLIP v. 3.6 (Fig. 2). The Bohol Sea populations clustered together, particularly Pamilacan and Surigao, while Camiguin branched out separately. Populations from Spratly (South China Sea), southeastern Samar, Bolisong (Tañon Strait) and Carbin Reef (Visayan Sea) clustered successively.

AMOVA analysis of the Bohol Sea group showed no significant structuring ($F_{ST} = 0.018$, $p = 0.123$) (not shown in this paper). This value is lower than the value obtained by Ablan (2005) with *Pterocaesion pisang* utilizing microsatellites, where the overall $F_{ST} = 0.0263$. According to Hauser and Ward (1998), this value is the typical differentiation level detected by mtDNA and microsatellites for small pelagic fishes. Pairwise comparison of the F_{ST} values at 1000 permutations revealed genetic

relatedness between Camiguin and Pamilacan ($F_{ST} = 0.017$, $p= 0.156$), as well as Pamilacan and Surigao ($F_{ST} = -0.006$, $p=0.530$) and Camiguin and Surigao ($F_{ST} = 0.03$, $p = 0.075$). This result conforms to the previous study of Ablan (2005) in the Bohol Sea using microsatellites in *Pterocaesio pisang* where all populations were homogeneous except for Mantigue in Camiguin Island. Although her study sampled different areas from the present study, still it gives a general description of the Bohol Sea in terms of demographic connectivity. Fig. 2 shows that Camiguin branched out from the Pamilacan and Surigao cluster.

Table 1. Mitochondrial cytochrome oxidase I(C01) genetic variation at each site. N – number of gene copies; Hd – Haplotype diversity; Pi, π - nucleotide diversities; θ_s - Theta s , calculated in Arlequin v. 3.11 (Excoffier, 2006) and DnaSP v. 4 (Rozas and Rozas, 1999). BOL – Bolisong; CAM- Camiguin; CAR- Carbin; PAM- Pamilacan; SPR- Spratlys; SUR- Surigao; SES- SE Samar.

Population	N	No. of Polymorphic Sites	No. of Haplotypes	Hd	Pi, π	θ_s
BOL	13	20	10	0.92 ± 0.069	0.009 ± 0.005	6.44 ± 2.76
CAM	26	32	18	0.96 ± 0.027	0.010 ± 0.006	8.39 \pm 2.99
CAR	26	18	11	0.78 ± 0.079	0.006 ± 0.004	4.72 ± 1.82
PAM	23	21	11	0.74 ± 0.100	0.006 ± 0.004	5.69 ± 2.19
SPR	15	24	11	0.95 ± 0.040	0.010 ± 0.006	7.38 ± 2.88
SUR	17	20	9	0.89 ± 0.054	0.008 ± 0.005	5.92 ± 2.41
SES	10	17	7	0.91 ± 0.077	0.011 ± 0.007	6.01 ± 2.76

Table 2. AMOVA results. (A) Unstructured; (B) five groups: Bohol Sea, Tanon Strait, Visayan sea, Leyte Gulf and South China Sea.*= significant at $p \leq 0.5$.

	%variation	F-statistic	P
A. Unstructured			
Among populations	19.7		
Within populations	80.3	F _{ST} = 0.197	0.0000*
B. Five groups ((Bohol Sea, Tañon Strait, Visayan Sea, Leyte Gulf & South China Sea)			
Among groups	22.02	F _{CT} = 0.220	0.039*
Among populations within groups	1.22	F _{SC} = 0.016	0.110
Within populations	76.77	F _{ST} = 0.232	0.000*

Population of *T. crocea* from Southeastern Samar was the most isolated. Pairwise comparison of F_{ST} s revealed Southeastern Samar population to have consistently higher and significant F_{ST} from Bohol Sea populations (Pamilacan, $F_{ST} = 0.53$, $p < 0.05$; 305.2 Km distance; Camiguin, $F_{ST} = 0.44$, $p < 0.05$; 244.8 Km distance,; and Surigao, $F_{ST} = 0.55$, $p < 0.05$; 147.68 Km distance), Tañon Strait (Bolisong, $F_{ST} = 0.46$ $p < 0.05$; 419.2 Km distance), and Visayan Sea (Carbin, $F_{ST} = 0.49$, $p < 0.05$; 566.4 Km distance) populations (Table 3, Fig. 1).

South China Sea (Spratly) which is about 1163.2 km from Tañon Strait, has a weak but significant genetic difference ($F_{ST} = 0.11$, $p < 0.05$). It also showed genetic differentiation from the Visayan Sea ($F_{ST} = 0.12$, $p < 0.05$; 1326.4 km distance) and all populations from Bohol Sea (Pamilacan, $F_{ST} = 0.16$, $p < 0.05$, 1153.6 Km distance; Camiguin, $F_{ST} = 0.05$, $p < 0.05$, 1187.2 Km; Surigao, $F_{ST} = 0.15$, $p < 0.05$, 1102.4 km) (Table 3). A weak but significant genetic variation was also found between Tañon Strait and Visayan Sea ($F_{ST} = 0.07$, $p = 0.03$) about 161.6 km apart. As shown in Table 3, two populations from Bohol Sea also showed moderate but significant genetic variation from Tañon Strait (Pamilacan, $F_{ST} = 0.15$, $p < 0.05$ and Surigao, $F_{ST} = 0.11$, $p = 0.00$). On the other hand, Tañon Strait *T. crocea* population has genetic affinity with Camiguin population ($F_{ST} = 0.03$, $p = 0.11$,

203.2 km distance) (Table 3). Significant levels of genetic heterogeneity between the Visayan Sea (Carbin) and the islands of Pamilacan ($F_{ST}= 0.17$, $p=0.00$) and Surigao ($F_{ST}= 0.16$, $p=0.00$) were also detected. The Visayan Sea, however, showed genetic homogeneity with Camiguin ($F_{ST} = 0.01$, $p=0.20$) (Table 3).

F_{ST} values from pairwise comparisons of the seven individual populations showed a wide scatter and detected no significant relationship to geographical distance (Mantel Test, $r = 0.046$, $p = 0.203$ from 1000 randomizations) (not shown). However, significant patterns of isolation by distance was seen when the Southeastern Samar group was excluded (due to its consistent high F_{ST} values from all sites) ($r = 0.416$, $p = 0.020$ from 1000 randomizations) (Fig 3).

Discussion

The genetic relatedness of the three populations within the Bohol Sea can be explained by the ocean currents inferred by Meñez *et al.* (2006) and US-NLOM (2007) which moved southwest in the Bohol Sea during the NE monsoon driven by the rising of the water level at the Pacific side (Fig.1) which reverses during the SW monsoon. Sulu Sea water which receives currents from the South China Sea and enters the Bohol Sea via the Dipolog Strait during the SW monsoon (Fig.1). It should be observed that the farthest distance within the Bohol Sea was 180.8 km, Pamilacan and Surigao. However, differentiation between the two populations was the lowest. This is probably due to the strong velocity of ocean current moving southwest which forms a counter-clockwise gyre (approx. 20 cm/s) at 124° E section of the Bohol Sea.

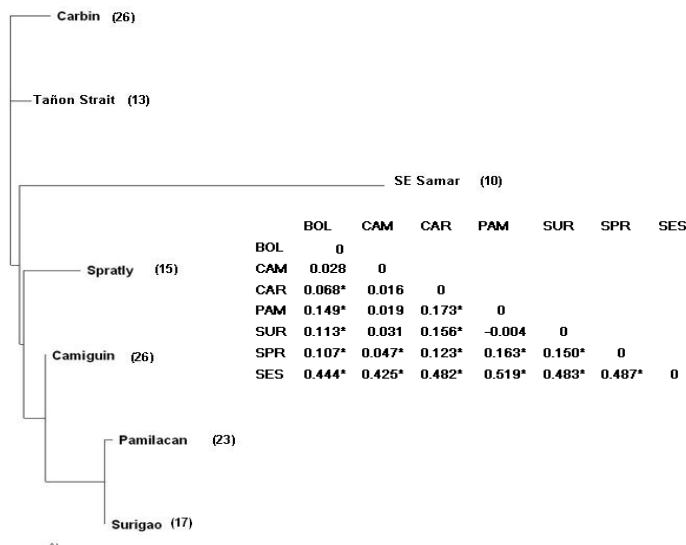


Figure 2. Phylogram showing the genetic relationship of seven *Tridacna crocea* populations from Central Philippines. Pairwise differences of F_{ST} derived from Kimura-2 P (1000 permutations) were clustered using Neighbor joining implemented in PHYLIP v. 3.65 and TreeView. * significant, $p \leq 0.05$. Values in parenthesis pertains to the number of individuals.

Despite the proximity of Bolisong to Carbin (161.6 km) reef as compared with the other populations, it showed significant differentiation. This maybe correlated with the existing current flow within Tañon Strait, particularly in the northern tip (opening) of the Strait. During the NE monsoon, flood tides bring predominantly strong northward surface current flow to the eastern side of the strait instead of flowing directly into the northern end. This pattern reverses during ebb tides. The water flow outwards at the southern and northwestern end of the strait. An outward flow from the Strait in the northern end is predominant regardless of the tides during the NE monsoon, so that even when the current direction reverses during ebb tide, it does not receive water from Carbin Reef. During SW monsoon, surface currents in the northern boundary do not undergo complete reversals but cross the strait just off the northern tip of Negros Island. This probably limits the flow of propagules from Cabin reef to Bolisong and vice versa. On the other hand, the affinity of Bolisong with Camiguin can be correlated with the complete reversal of the current flow at the southern boundary as tides changed, bringing influx from the Bohol Sea (Fig. 1). The

non-significant genetic differentiation between Camiguin and Carbin ($F_{ST} = 0.02$) could not be explained except for the ebb current pattern brought about by the southwest monsoon (Fig. 3.). This observation, however, is not unique in this study. Benzie and Williams (1997) obtained a non-significant genetic differentiation between Bantayan Island (which is located at the northern tip of Tañon Strait) and Tawi-Tawi ($F_{ST} = -0.002$) which are separated by greater geographic distance compared to Camiguin and Carbin.

It was expected for SE Samar population to have high genetic affinity with Surigao populations and consequently with the rest of the populations in the Bohol Sea because of the homogenizing effect of the strong, persistent current flows downstream of the North Equatorial Current, particularly the Mindanao current, to the dispersal of pelagic larvae (Fig.1). However, a high genetic differentiation and a very limited gene flow were obtained. These results were consistent with the observation obtained by Ravago *et al.* (2007) where they found fine-scale genetic differentiation for *Tridacna crocea* populations within Kuroshio and Mindanao current regions. The reef from which the samples are collected was located at the inner side of Camanga, Salcedo (Fig. 3.9) which is about $11^{\circ} 06' N$ and $125^{\circ} 36' E$. The presence of a land barrier which encloses the bay prevents the influence of the NEC into it and consequently from flowing with the Mindanao current southward. Previous study conducted by Ravago *et al.* (2007) using allozymes of *Tridacna crocea* observed significant genetic differentiation between Homonhon and Dinagat Island (70 to 90 km apart, $F_{ST} = 0.135$) and between the two islands of Dinagat (20 km apart, $F_{ST} = 0.069$). Ravago *et al.* (2007) correlated this variation to the strong tidal currents flowing through the Surigao Strait, coupled with the high rugosity of coastlines in the area, which may affect larval dispersal, entrainment potential and recruitment.

The genetic structure of *Tridacna crocea* in Central Philippines seems to be consistent with other studies correlating genetic variation with hydrographic barriers to dispersal. There is connectivity within reefs in Central Philippines particularly in the Bohol Sea, and Tanon Strait. Surigao appears to be the source of *T. crocea* propagules for Pamilacan and Camiguin and probably other nearby reefs. South China Sea can be both a sink and a source of propagules of the Bohol sea populations. Southeastern Samar appears to be an outlier population, having consistently very high F_{ST} values (genetic distances) from the rest of the sites. To strengthen the existing MPAs around Bohol Sea and the neighboring seas and straits,

management should be based on the connectivity of these sites, as well as, the ecological role that a particular site provides either as a sink or source of propagules. Lastly, the mitochondrial DNA *cytochrome c oxidase 1* (CO1) can be used to detect fine-scale genetic variation.

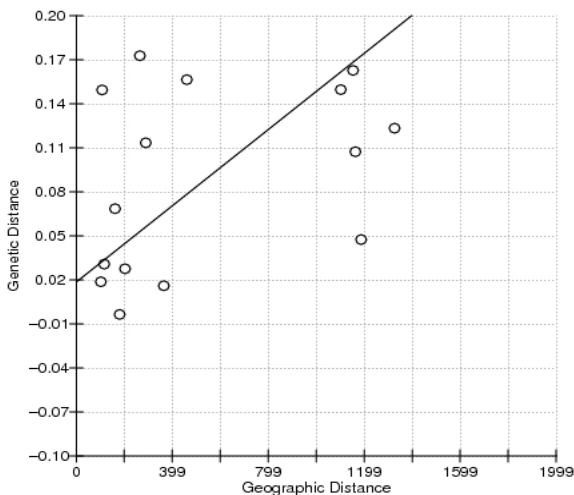


Figure 3. Relationship between F_{ST} values and geographic distance. SE Samar was omitted ($r = 0.3810$, 0.0280 from 1000 per randomizations).

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