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Full Length Research Paper

Microsatellite and mitochondrial DNA analysis of the genetic structure of Chinese horseshoe crab (*Tachypleus tridentatus*) in southeast China coast

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Chinese horseshoe crab (*Tachypleus tridentatus*) is a Xiphosura animal of significant commercial importance and in danger of extinction in China. To better estimate how genetic structure can be used to obtain a conservation perspective of the species, genetic variation was examined in nine locations covering its distributing range in the coast of Chinese mainland using ten nuclear microsatellite DNA loci and mitochondrial DNA control region (CR) sequences. Moderate levels of genetic diversity were detected (expected heterozygosity from microsatellites was 0.635, haplotype diversity from mitochondrial DNA was 0.800) as a whole. Significant genetic differentiation was detected only by mitochondrial DNA (F_{ST} = 0.0693, P < 0.01), while microsatellite markers indicated nuclear genetic homogeneity of these locations. Probably, nuclear genetic homogeneity was caused by outbreeding among different groups due to artificial transporting. Very weak genetic differentiation indicates that reintroduction programs of the movement and mixing of horseshoe crab from different locations will result in minimal negative genetic effects. Upon four management units were inferred from the results of CR analysis, accordingly four or more nature reserves should be established to conserve this endangered animal along the Chinese coast. Haplotype network pattern indicated that *T. tridentatus* population in Chinese coast has undergone historic population expansion and very recent historic population recession. Mismatch distributions analysis also revealed existence of historic demographic expansion.

Key words: *Tachypleus tridentatus*, microsatellites, mitochondrial DNA, population structure, genetic diversity.

INTRODUCTION

The Chinese horseshoe crab (*Tachypleus tridentatus*) distributes mainly along the coast of the East and South China Sea, extending northward to the Pacific coast of Japan and southward to Southeast Asia (Sekiguchi, 1988; Yang et al., 2007). *T. tridentatus* was once abundant in the southeast coast of China, distributed from the estuary of Changjiang River to Beibu Bay (Sekiguchi, 1988; Liao and Li, 2001). At present, its resources have declined rapidly because of excess fishing (Hsieh and Chen, 2009) for seafood and wide use of amebocyte lysate in endotoxin tests (Swan, 2001) and habitat destruction for rapid economic development (Chiu and Morton, 1999; Liao and Li, 2001; Chen et al., 2004). It is a well accepted concept that the genetic diversity is a basic precondition for populations to react to environmental changes, and to keep their reproductive fitness, which will ensure the long term survival of species (Frankham et al., 2002; Allendorf

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and Luikart, 2007). Urgent attention is required to save this species from extinction in the near future, however, so far, little is known about its genetic diversity in Chinese coast in order to determine some effective measures to conserve this species.

Microsatellite has been considered as one of the efficient molecular markers that provide population genetic information, due to its abundance, randomly distributed in the genome, highly polymorphic, its codominant inheritance and ease of scoring (Guo and Gui, 2008; Kaya and Yildiz, 2008). Twenty-two microsatellite markers were developed from *Limulus polyphemus* (King et al., 2004), which revealed significant population difference among two Atlantic coast and one Gulf of Mexico site. Faurby et al. (2010) applied 12 microsatellite loci to analyze population dynamics in 1218 *L. polyphemus* sampled from 28 localities, covering its range along the west coast of the Atlantic. However, our result showed that no polymerase chain reaction (PCR) bands were achieved with these twenty-two pairs microsatellites primer and DNA extracted from *T. tridentatus* in PCR reaction in our previous study (not published), which indicated that those primer pairs from *L. polyphemus* are not suited for the application in *T. tridentatus*.

Currently, a few studies have been reported in the microsatellite determination in *T. tridentatus*, for instance, Nishida and Koike (2009) showed eight microsatellite markers with low diversity (number of allele from 2 to 4). Also, another eight polymorphic microsatellite markers were developed by Li et al. (2009), with rather low diversity. Although microsatellites are hypervariable nuclear markers, they are considered less appropriate for phylogeographical inference because of their tendency towards homoplasious mutations (Bryja et al., 2010; Hewitt, 2004).

In phylogeographical studies, analysis based on a single type of marker would lead to erroneous results and biased conclusions since different markers each have their own unique genealogy, different modes of inheritance, effective population sizes and sex-biased dispersal (Colbert et al., 2001; Hewitt, 2004). Fast-evolving markers, such as the mitochondrial control region has revealed much higher levels of genetic differentiation (Zink, 1997) and often help to discover clades with significant geographical information (Barrowclough et al., 2005). In conservation genetics, the use of nuclear gene information combining with the inferences from mtDNA sequence data is becoming necessary for a complete picture of the evolutionary forces shaping populations and species (Purvis et al., 2005; Shaw, 2002). More and more studies have applied the combination of microsatellites and mtDNA sequences, which is proved to be superior to that obtained through the use of separate sets of markers (Flanders et al., 2009; Bryja et al., 2010).

In this study, ten polymorphic microsatellite loci developed by our lab were used to analyze genetic diversity and structure of *T. tridentatus* from nine locations covering its distribution in Chinese coast. Furthermore, the control region sequence of mtDNA was used to analyze its genetic structure in seven locations. Combination analy-sis with these two sets of markers may give us more population genetic and phylogeographical information, upon which we can propose scientific conservation management strategy.

MATERIALS AND METHODS

Sampling and DNA extraction

The samples of Chinese horseshoe crabs (*T. tridentatus*) were collected from nine localities across its distribution range along the southeast coast of China during 2007 to 2008 (Figure 1, Table 1). The number of samples analyzed per locality varied due to sample availability and analysis success. The muscles from horseshoe crab were kept in 95% ethanol, then in 4°C refrigerator until DNA was extracted using standard proteinase K digestion/phenol-chloroform isolation procedure.

Genotyping in nine locations using ten microsatellite markers

The polymerase chain reaction (PCR) reactions were amplified in 10 µL reactions containing 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM forward primer, 0.2 mM reverse primer, 200 mM dNTPs, 0.1 U/µL Taq DNA polymerase (Invitrogen), and 50 ng of genomic DNA template. The used primers in this study are listed in Table 1. PCR was performed in a BioRad PTC0200 thermocycler (BioRad Lab, Inc., USA) with the following program: 94°C for 2 min, followed by 29 cycles of 94°C for 30 s, the primer specific annealing temperature (Table 1) for 20 s and 72°C for 30 s, with a final extension period of 7 min at 72°C, and maintained at 4°C until further use. The PCR products were separated on a 6% denaturing polyacrylamide gel and visualized by silver-staining. Product sizes were estimated by comparison with a 10 bp DNA ladder (Promega). A total of 371 individuals from nine locations (Figure 1, Table 1) were genotyped by ten polymorphic microsatlellite markers (CHR11, CHR45, CHR49, CHR51, CHR53, CHR57, CHR63, CHR77, CHR87, CHR91), which have been isolated using a fast isolation by Amplified fragment length polymorphisms (AFLP) of sequences containing repeats (FIASCO) protocol (Zane et al., 2002). They were also evaluated for polymorphism and heterozygosity in 39 individuals of Chinese horseshoe crab (Table 2).

Mitochondrial DNA amplifying and sequencing

Primers for PCR were designed using Primer 5.0 software according to mtDNA control region of *L. polphymus*. The primers used were, H-49: 5'-TTCTAGTATAATACATGGAA-3' and H-50: 5'-TTAGTGTAAAGGCACATT-3'. The final volume of 60 µL included 150 ng of template DNA, 200 µM dNTPs, 0.3 µM of each primer, 1.5 unit of Taq polymerase (Takara), 10× PCR buffer with 1.5 mM $MgCl_2$. PCR was performed in a BioRad PTC0200 thermocycler (MJ Research, Waltham, MA, USA) with the following program: 94°C for 5 min, followed by 35 cycles of 45 s at 94°C, 50 s at 40°C, and 1 min at 72°C; with a final extension period of 7 min at 72°C, and maintained at 4°C until further use. The results of PCR were checked in routine 2% agarose gel electrophoresis. The PCR products were purified using QIAGEN PCR Purification kit (50) (Biotools, Madrid, Spain). Samples were sequenced in both directions, using an ABI 310 automated sequencer (Applied Biosystems) of Invitrogen Biotechnology Company, Limited. Total of 116 individuals from seven locations (Table 1) were analyzed.

Data analysis

The frequency of Null alleles was calculated by MICRO-CHECKER

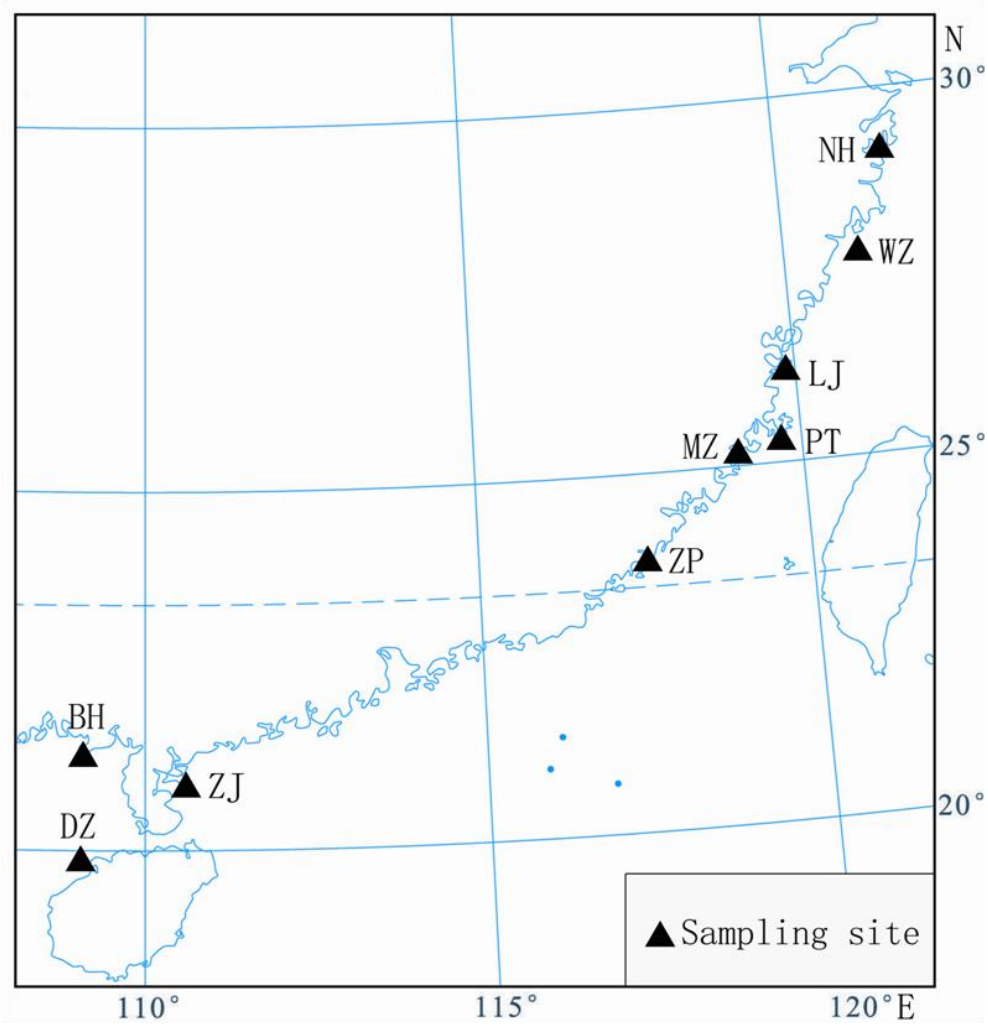

Figure 1. The map of sampling sites of *T. tridentatus* from Southeast coast of China. NH, Ninghai; WZ, Wenzhou; LJ, Lianjiang; PT, Pingtan; MZ, Meizhou Island; ZP, Zhangpu; ZJ, Zhanjiang; BH, Beihai; DZ, Danzhou.

2.2.3 (Oosterhout et al., 2004) using Brookfield algorithms (Brookfield, 1996). Microsatellite genetic diversity was quantified as number of alleles (N_A), average number of alleles per locus (A), effective number of alleles per locus (Ne) (estimates the reciprocal of homozygosity) (Hartl and Clark, 1989), F_{IS} (estimates for each allele) (Weir and Cockerham, 1984), observed heterozygosity (H_0), expected heterozygosity (H_E), which were estimated with GENEPOP version 4.0.1.0 (Rousset, 2008). The polymorphic information content (PIC) value was calculated using CERVUS 3.0 software package (Marshall et al., 1998; Kalinowski et al., 2007). PIC is a measure of informativeness related to expected heterozygosity and is likewise calculated from allele frequencies (Botstein et al., 1980; Hearne et al., 1992). Tests for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were performed using GENEPOP. Genetic differences between locations were evaluated by calculating pairwise F_{ST} values and testing their significance by bootstrapping analysis (10,000 replicates) using Arlequin 3.0 (Excoffier et al., 2005). This program was also used to partition variation within and between locations using the AMOVA (Analysis of Molecular Variance) procedure.

The control region sequences were edited and aligned with MEGA 4.0 (Tamura et al., 2007). The nucleotide composition, numbers of variable sites and population diversity parameters were calculated using DNASP 4.10 (Rozas et al., 2003). A mantel test was performed with 10,000 permutations in Arlequin to assess the statistical significance in correlation between genetic and the geographical distances. Correlation between microsatellites and CR genetic diversity was calculated by SPSS 12.0. Phylogenetic relationships between the identified haplotypes were determined by calculating an unrooted haplotype median network using NETWORK 4.5.1.0 (Bandelt et al., 1999). Demographic history was examined using mismatch distribution (Slatkin and Hudson, 1991), and neutral test was conducted by Tajima`s *D*-test and Fu`s *F*s-test based on mitochondrial DNA variation by Arliquin3.0.

RESULTS

Genetic diversity of *T. tridentatus* from microsatellite and mtDNA analysis

Most of these ten microsatellite loci were high polymorphic

Table 1. Sampling sites of *T. tridentatus* across its range of the east and south coast of China.

Locality	Province	Coordinates	Number of individual	
			Microsatellite analyses	Mitochondrial analyses
Ninghai (NH)	Zhejiang	29º9′29′′N, 121º42′54′′E	12	20
Wenzhou (WZ)	Zhejiang	27º57′04′′N, 121º07′00′′E	25	0
Lianjiang (LJ)	Fujian	26º13′56′′N, 119º42′19′′E	36	19
Pingtan (PT)	Fujian	25º25′33′′N, 119º46′07′′E	50	0
Meizhou Island (MZ)	Fujian	25º03′40′′N, 119º04′39′′E	50	16
Zhangpu (ZP)	Fujian	23º48′12′′N, 117º33′53′′E	50	15
Zhanjiang ((ZJ)	Guangdong	20º55′17′′N, 110º33′32′′E	50	13
Beihai (BH)	Guangxi	21º21′27′′N, 109º05′44′′E	50	16
Danzhou (DZ)	Hainan	19º51′33′′N, 109º07′28′′E	48	17

(PIC > 0.5) and only two loci (CHR53, CHR91) were deviated from Hardy-Weinberg equilibrium, no significant linkage disequilibrium was detected among them (Table 2), thus they were suitable for genetic analysis. Three hundred and seventy-one (371) horseshoe crab individuals from nine locations of *T. tridentatus* covering its range distributed in Chinese coast were genotyped by these polymorphic microsatellite loc. The results showed that nuclear genetic diversity is moderate, with a mean number of alleles over loci across nine locations ranging from 4.714 to 8.571 (7.018 averagely), and the mean effective numbers of alleles ranged from 2.915 to 3.837 (3.697 averagely) (Table 3). The observed heterozygosity (H_o) over all locations was 0.620 and ranged from 0.599 (ZP) to 0.701 (NH), while the H_E was 0.635 and varied from 0.598 (PT) to 0.655 (LJ).

In total, 18 polymorphic sites (accounting for 5.0% of the total number of sites), including nine parsimoniously informative sites, were detected based on 368 bp of full length mitochondrial control region (CR) from 116 individuals (Table 4), which were sampled from seven locations. The polymorphic sites were all transitions, without transversion or insertion/deletion. The average base composition of the control region sequence in *T. tridentatus* was in a strong adenine-thymine bias (43.3% thymine, 40.9% adenine, 6.7% cytosine and 9.1% guanine). The AT content (84.2%) was much higher than GC one (15.8%). Twenty-seven haplotypes were defined (H1 ~ H27) (Table 4) (GeneBank Accession Numbers JX437089-JX437115). The nucleotide diversity (*P*i) over these locations was 0.00549 and ranged from 0.00207 to 0.00667, and haplotype diversity (*h*) was 0.80 and ranged from 0.524 to 0.889, which indicated moderate high levels of genetic diversity of this species. Nucleotide and haplotype diversities for different locations are shown in Table 5.

Four sample-specific haplotypes were in Ninghai locality, while three in Lianjiang, one in Meizhou, one in Zhanjiang, three in Beihai, but none in Zhangpu and Danzhou. H1 was the dominant haplotype in 49 individuals (43%), existing in all locations. The constructed phylogenetic relationships among these 27 haplotypes were constructed, yielding a complex network (Figure 2). Haplotypes such as H1, H20 and H2 were in the center of star-like networks, indicating that they were ancestral types. The star-like network indicated a likely existence of historic population expansion of horseshoe crab population. As shown in Figure 2, H20 was an ancestral type, but there were only two individuals in it, which indicated very recent historic population recession.

Comparison between microsatellites and CR genetic diversity

There was a significant correlation (r = 0.686, $P < 0.05$) between the values of *H*e evaluated from microsatellite markers and *P*i from CR, which stand for nuclear and mitochondrial genetic diversities, respectively (Figure 3). This result suggested that microsatellite data could be used to predict mitochondrial genetic diversity, and vice versa.

Analysis of molecular variance (AMOVA) analysis

The AMOVA analysis by microsatellite data partitioned 99.20% of the total genetic variation within location, and the value of the part among locations was only 0.80 with F_{ST} not significant ($P > 0$), which indicated there was not genetic differentiation among nine locations of *T. tridentatus*. However, the fixations index at the level of inter-locations was significant ($P < 0.01$) when analyzed with mtDNA sequences data. As shown in Table 6, significant genetic variance was discovered among locations, although most molecular variances were observed to occur within location (94.62%).

Pairwise F_{ST} analysis

Pairwise F_{ST} analysis was calculated for each pair of locations over ten microsatellite loci, with values ranging from -0.04084 to 0.02615, and no statistically significant difference existed among these locations ($P > 0$) (Table 7).

Table 2. Characterization of the ten polymorphic microsatellite loci used in this study.

Locus	Primer sequences(5'→3')	Repeat motif	Size range (bp)	T_a (ºC)	N_A	PIC	H_O	H_E	Null allele frequence	F_{IS}	GenBank accession number
CHR11	F: CTCGTATCAAGACAAACAATCA R: AGAAAAGTCTACTGAAAGCACA	$(GT)_8$	146-168	46	9	0.732	0.744	0.768	0.008	0.026	JX443509
CHR45	F: CCACAAGCACCCATTGAAACAC R: ATCGGAACTGTGAACAACTACA	$(TG)_{15}$	195-205	50	7	0.652	0.641	0.702	0.030	0. 087	JX443503
CHR49	F: ATAACATGGCGGATTATTACAT R: GTTGCTGTTTAGTCCTGTTCAT	$(GT)_9(GA)_{10}$	145-197	48	22	0.932	0.974	0.948	0.	-0.030	JX443504
CHR51	F: CAAGCCGGGTCCCATCTTTGAT R:AGCGCTCAGTTAGACTTACCATTTAC	$(TG)_{15}$	160-184	51	11	0.840	0.872	0.868	0	-0.005	JX443506
CHR53	F: GTAGAATTATCTGTTATACACGCA R: TTACCATAACCTGGAGACAATA	$(CA)_{10}(AT)_9$	154-180	47	5	0.402	0.359	0.450	0.059#	0.204*	JX443505
CHR57	F: AAACATCAGCCTTATCTAACGG R: CGACAAGAAAGTAAAAGCAAAA	$(TG)_{14}$	74-90	48	8	0.725	0.769	0.770	0	0.001	JX443507
CHR63	F: TTGAAAGCCTATTCCTTTACGT R: AAGTGCAATGAAGAAGCAGTGT	$(TA)_5(AC)_{17}$	64-110	47	8	0.653	0.769	0.699	0	-0.101	JX443508
CHR77	F:CGAGATAACATAAAAGTATACAT R:TGGGATTCTGTTGTATTGTA	$(AT)_5(TG)_{13}$	118-130	45	7	0.703	0.821	0.749	0	-0.097	JX443510
CHR87	F: TCCAGGACAAACGTTAAAGAAT R: ACAGGTGATCTTTGAGTGTTGC185	$(TG)_{12}$	173-189	48	8	0.747	0.744	0.790	0.019	0.055	JX443511
CHR91	F:AGTCCAGAGGCCAGTTTCATAT R: TGGTTTCACTTTCCCAACAATC	$(GT)_{15}$	162-192	51	8	0.274	0.180	0.283	0.078#	0.370*	JX443512
Average					9.2	0.678	0.709	0.714			

T_a, Annealing temperature; N_A, number of alleles; PIC, polymorphic information content; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, fix index; *indicates significantly deviating from Hardy–Weinberg equilibrium ($P < 0.05$); # indicates null allele existed (greated than 0.05).

As a result, F_{ST} indicated high level of gene flow between these locations, thus weak genetic structure of *T. tridentatus* along Chinese coast was revealed by the analysis with microsatellite markers. However, analysis according to control region of mitochondrion DNA showed significant difference

Table 3. Polymorphic characteristics of nine locations of *T. tridentatus* from ten microsatellite loci analysis.

Location	A	*Ne*	H_O	H_E
NH	4.714±2.215	2.915±1.151	0.701±0.234	0.623±0.212
WZ	6.286±2.828	3.401±1.626	0.660±0.264	0.640±0.222
LJ	6.714±2.812	3.517±1.722	0.647±0.266	0.655±0.201
PT	7.857±2.795	3.388±1.952	0.605±0.267	0.598±0.246
MZ	7. 714±3.039	3.399±1.963	0.609±0.247	0.619±0.214
ZP	7.286±2.690	3.565±2.126	0. 599±0.261	0.613±0.242
ZJ	7.286±2.628	3.511±1.938	0.609±0.251	0.619±0.253
DZ	7.429±2.225	3.633±1.841	0.637±0.236	0.647±0.223
BH	8.571±2.230	3.837±1.764	0.614±0.249	0.646±0.236
Species level	7.018±2.107	3.697±2.122	0.620±0.241	0.635±0.229

A, average number of alleles per locus; N_e, effective number of alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity.

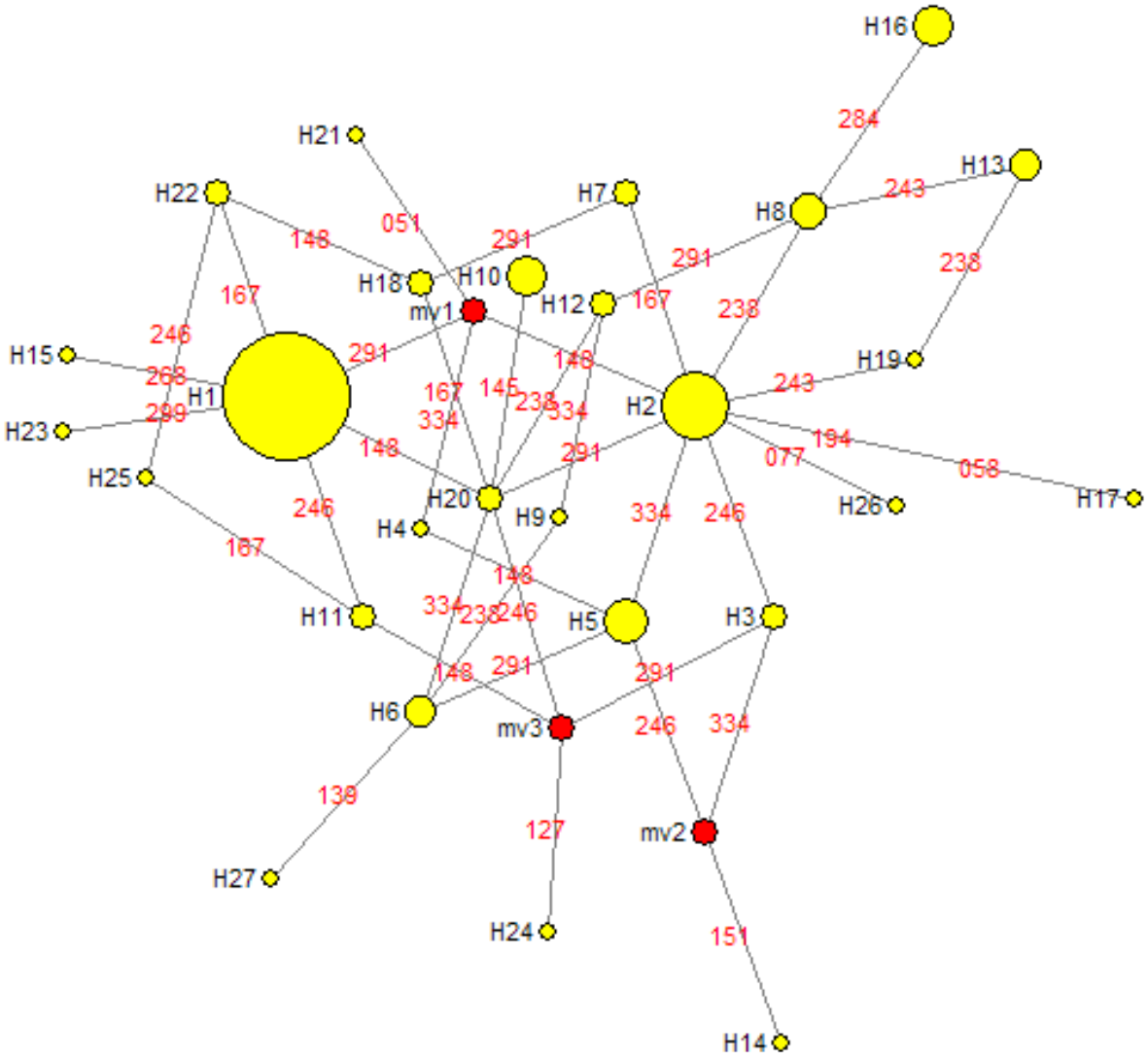

Figure 2. Median-Joining network showing phylogenetic relationships between 27 mtDNA control region haplotypes found in *T.tridentatus*. Each line represents a mutational step, numbers cross the lines represent the sites of nucleotide substitutions, circle areas depict proportions of haplotypes, yellow circles represent existing hyplotypes, red circles represent hypothesized sequences which were required to connect existing sequences within the network.

Table 4. Twenty-seven haplotypes, their mutation sites and their distribution in seven locations.

Haplotype	Variable nucleotide sites																		Distribution of haplotypes							
	51	58	77	127	139	145	148	151	167	194	238	243	246	268	284	291	299	334	NH	LJ	MZ	ZP	ZJ	BH	HN	total
H1	T	T	T	A	A	A	T	A	C	T	A	A	C	T	T	T	A	A	5	6	4	9	9	6	10	49
H2							C									C			2	3	7	1			2	15
H3							C						T			C			1		1					2
H4																C		G	1							1
H5							C									C		G	4	1	1					6
H6							C											G	3							3
H7							C		T							C								2		2
H8							C				G					C					2			1	1	4
H9							C				G							G						1		1
H10						G	C															3			2	5
H11													T							1		1				2
H12							C				G													2		2
H13							C				G	G				C				2					1	3
H14							C	G					T			C		G		1						1
H15														C						1						1
H16							C				G				C	C				1		1	1	1	1	5
H17		C					C			C						C								1		1
H18							C		T														1			1
H19							C					G				C								1		1
H20							C												1	1						2
H21	C															C				1						1
H22									T											1			1			2
H23																	G				1					1
H24				G			C						T						1							1
H25									T				T						1							1
H26			C				C									C			1							1
H27					G		C											G					1	1		2
Total																			20	19	16	15	13	16	17	116

among seven locations. NH population was significantly different from ZJ, two Beibu Bay locations (BH, DZ) and ZP (collected from semienclosed bay of Dongshan Bay) ($P < 0.01$). The significant difference also existed between BH and ZP, MZ and ZP, ZJ and BH (Table 7).

Mantel test

To test the degree of correlation between genetic variation and geographical distances, the Mantel test was used. The results indicate that genetic differentiation for *T. tridentatus* along Chinese mainland coast was not correlated with total geographical distance by the analysis with microsatellite markers ($r^2 = 0.026$, $P > 0.05$). However, converse result revealed by mtDNA sequences ($r^2 = 0.346$, $P < 0.05$), which indicated that the geographical distance is an important fact to restrict

Table 5. Genetic diversity parameters of mitochondrial control region (CR) among seven locations of *T. tridentatus.*

Location	Number of sample	Haplotypes observed	Haplotype diversity (*h*)	polymorphic site (*S*)	Mean number of pairwise difference (*k*)	Nucleotide diversity (*Pi*)
NH	20	10	0.895	7	2.074	0.00564
LJ	19	11	0.889	11	2.456	0.00667
MZ	16	6	0.767	6	1.525	0.00414
ZP	15	5	0.629	6	1.467	0.00399
ZJ	13	5	0.538	7	1.436	0.00390
BH	16	9	0.858	9	2.408	0.00654
HN	17	6	0.654	6	1.721	0.00468
Total	116	27	0.800	18	2.020	0.00549

Table 6. AMOVA analysis of genetic variation of *T. tridentatus.*

Parameter	Microsatellite analysis		Control region analysis	
Source of variation	Percentage of variation	F_{ST}	Percentage of variation	F_{ST}
Among locations	0.80	0.00803[a]	5.38	0.0538[b]
Within locations	99.20		94.62	

The superscript 'a' indicated $P > 0.01$ and 'b' for $P < 0.01$.

gene flow of *T. tridentatus*.

Demographic analysis

*F*u`s *F*s and Tajima`s *D* were used to test for selective neutrality and population equilibrium. Significant values of *F*s and *D* can be due to factors other than selective effects, like population expansion or bottleneck (Fu, 1997; Tajima et al., 2007). The values of Tajima`s *D* were negative except for that of NH locality, but they were not significant ($P > 0.05$) for all locations. Fu (1997) has noticed that the *F*s statistic was very sensitive to population demographic expansion which generally led to large negative *F*s values. The values of *F*s were also negative, furthermore they were significantly negative ($P < 0.02$) for three locations (NH, LJ and BH) and the whole data set, which indicated historic population expansion of horseshoe crab in the coast of China. Mismatch distributions analysis revealed that there was no significant difference between the observed and expected distribution under the expansion model, not only for each locality but also for the whole data set ($\tau = 2.625$; $\theta_0 = 0.005$; $\theta_1 = 9.023$; SSD = 0.025; $P = 0.074$) (Table 8, Figure 4). This indicated that the whole population of *T. tridentatus* in Chinese coast has undergone a historic demographic expansion, which was consistent with the result of star-like network of haplotypes and *F*s test.

DISCUSSION

Genetic diversity of *T. tridentatus* distributed in Chinese coast

In general, genetic variation of nine locations in Chinese coast is still moderate according to allele numbers (7.018 averagely), H_E values (0.635 averagely) from microsatellite loci and *h* values (0.80 averagely) from mtDNA control regions in this study, although the horseshoe crabs are so rare in most of Chinese coast now. Compared with other population of *T. tridentatus*, it was higher than that of the population from Japan (H_E values 0.53 averagely) (Nishida and Koike, 2010), also higher than that of the population from Taiwan Strait (*h* values 0.626, averagely). The Chinese horseshoe crab (*T. tridentatus*) used to distribute mainly along the offshore of the East and South Sea of China, extending northward to estuary of Changjiang River and southward to Beibu Bay. The samples in this study cover the historical distribution region in China. The moderate genetic diversity of *T. tridentatus* may be due to historical abundance

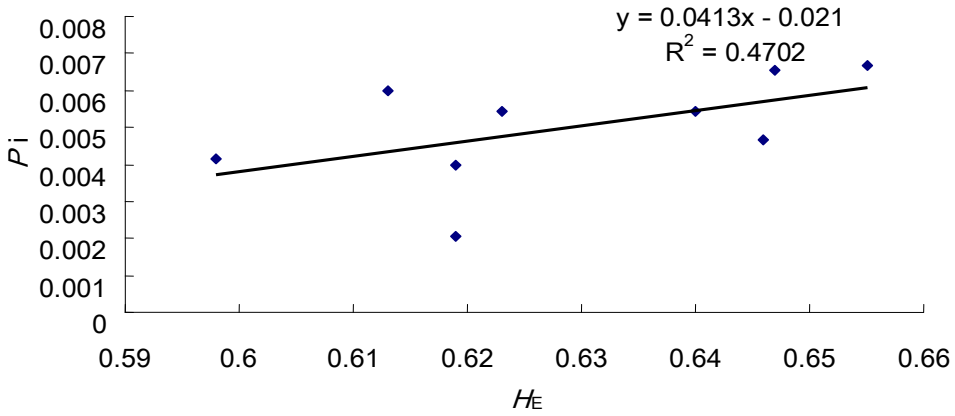

Figure 3. Genetic diversity comparison between microsatellites and mitochondrial loci (r = 0.686, *P* = 0.044). Each location is characterized by its expected heterozygosity (*H*e, microsatellites) and nucleotide diversity (*P*i, mtDNA).

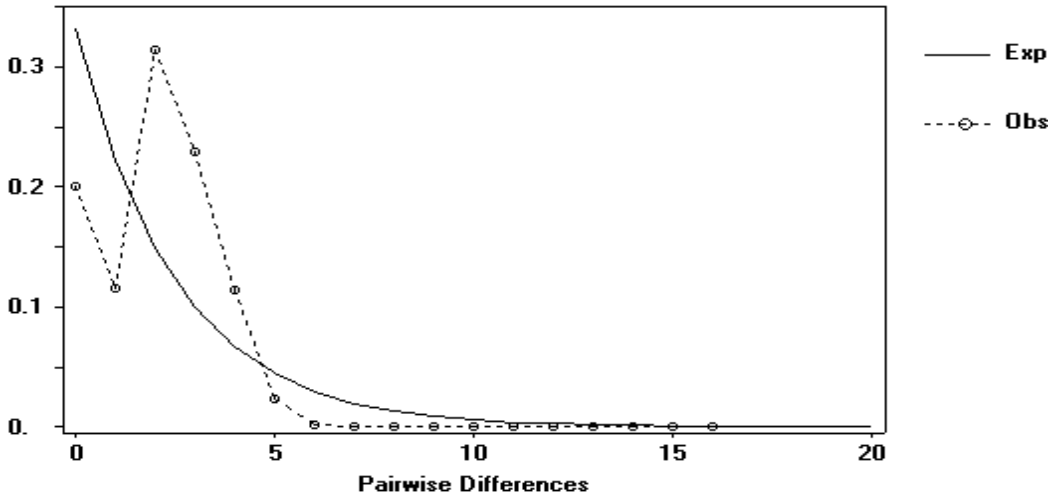

Figure 4. Mismatch distributions of CR haplotyes. Observed frequency distribution (dotted line) for the number of pairwise nucleotide differences among the total individuals of *T. tridentatus*. Solid line shows the expected distribution given a population expansion.

in resources and high in genetic diversity along China's southeast coast.

According to our survey, Chinese horseshoe crabs were still quite abundant in Chinese coast even 30 years ago. During summer, there were once a large number of horseshoe crabs spawning in many beaches several decades ago. But in recent years, the number of Chinese horseshoe crab in China drastically declined, almost disappeared in most sea water (weng et al., 2012). Accordingly, the reduction of the genetic diversity of Chinese horseshoe crab will be inevitable. It is surely necessary to take urgent and effective measures to protect the horseshoe crab from extinction in Chinese sea.

Genetic structure in *T. tridentatus*

The result of population differentiation tests at microsatellite loci indicated no genetic differentiation among different locations in Chinese coast, and none evolution significant unit was found. However, mtDNA markers revealed distinct subpopulation among these locations. Therefore, it is necessary to combine the analysis of microsatellites and mtDNA sequence in order to identify the population genetic and phylogeographical information. Actually, lack of congruence between nuclear and mitochondrial-based variation has also been previously reported, for example in terrestrial mammal the African elephant (Nyakaana and Arctander, 1999), cartilaginous fish, the blacktip shark (Keeney et al., 2005), teleost, the European bitterling (Bryja et al., 2010). This discrepancy between mtDNA and microsatellite data in these studies was thought to be due to male-biased gene flow which would lead to homogenization of nuclear alleles while the maternally inherited such as mitochondrial markers would remain restricted to native

Table 7. Genetic difference (F_{ST}) for mtDNA control region (above diagonal) among seven locations and for microsatellites (below diagonal) among nine locations.

Parameter	NH	WZ	LJ	PT	MZ	ZP	ZJ	DZ	BH
NH			0.02446		0.05343	**0.14898**	**0.1644**	**0.10272**	**0.06519**
WZ	0.0148								
LJ	-0.0034	0.0085			0.00391	0.04625	0.06327	-0.01534	-0.01436
PT	0.0262	-0.0154	0.0347						
MZ	-0.0037	0.0088	-0.0021	-0.0083		**0.20734**	**0.26066**	0.09328	0.0355
ZP	0.0049	-0.0031	-0.0106	-0.0408	0.0039		-0.00723	-0.02673	**0.08133**
ZJ	0.0152	0.0037	0.0139	-0.0312	0.0172	0.0039		0.02111	**0.08399**
DZ	0.0117	0.0065	0.0037	-0.0208	0.0103	0.0039	0.0076		0.00325
BH	0.0038	-0.0035	-0.0052	-0.0393	0.0009	0.0031	0.0063	-0.0089	

F_{ST} values are calculated with the method of Tajima and Nei (1984). Statistically significant values are shown in bold text ($P < 0.05$).

Table 8. Historical demography analysis based on control region variation in seven locations of *T. tridentatus.*

Location	NH	LJ	MZ	ZP	ZJ	DZ	BH	Total
Tajima`s *D*	0.168	-0.789	-0.536	-0.718	-1.378	-0.102	-0.412	-1.125
P_D	0.608	0.257	0.323	0.278	0.060	0.497	0.384	0.190
*F*s	-4.421	-5.204	-1.432	-0.571	-0.863	-0.981	-3.394	-19.849
P_{Fs}	**0.001**	**0.002**	0.134	0.323	0.202	0.245	**0.017**	**0.003**

P_D, P_{Fs}, P_r, refer to *P*-value of Tajima`s *D*, *F*s, Raggedness index, respectively. Statistically significant values are shown in bold text ($P < 0.05$).

localities (Nyakaana and Arctander, 1999). However, for horseshoe crab, the female is larger than male generally, and the male does not seem to be more movable or migrate farther distance than the female. Therefore, it needs more research to find out the reason for the inconformity in horseshoe crab.

According to the result revealed by CR, the NH location was significantly different from those sampling from South sea and Beibu Bay such as ZP, ZJ, BH and DZ. This can be interpreted by the fact that the geographical distance played an important role in genetic differentiation. Interestingly, ZP population was significantly different from MZ, although their distance is only about 200 km. The ZP population was sampled from semien-closed bay, Dongshan Bay (500 km in diameter), and the geographical barriers may play an important role in genetic differentiation. Likewise, Leizhou Peninsula might separate ZJ population from BH for genetic exchange. Similar pattern was also observed on the distribution in *L. polyphemus*. There is a genetic break between the Gulf of Mexico and Atlantic populations, and Florida Peninsula may be the barrier for gene flow (Pierce et al., 2000). Yang et al. (2007) also discovered that geographical barriers played an important role in genetic subdivision of *T. tridentatus*.

However, it was difficult to be interpreted by geographical distance for the LJ locality, since the population was not significantly different from any other locations even BH (1,360 km in distance), DZ (1,310 km in distance) and ZJ (1,130 km in distance) as well. Unlike most migration marine animals, the horseshoe crab has no significant potential for long distance dispersal and gene flow (Pierce et al., 2000; Yang et al., 2007), since adults stay on the offshore sea bottom and migrate to intertidal areas for reproduction, trilobite larvae settle right after hatching, and juveniles spend their life stages at or near the natal beach for feeding (Sekeguchi, 1988; Chen et al., 2004). A factor contributing to the poor structure of Chinese horseshoe

crab at microsatellite markers and confused population structure pattern at CR sequences may be the influence of artificial movements of individuals due to commercial fishing and discharging among the south-eastern coast of China. Due to the fact that the horseshoe crab is popular as delicious seafood in China, and with the sharp drop in resources around Chinese coast, the horseshoe crab was extensively transported from south coast to the north, most from Beibu Gulf to other places for commercial purposes. However, this activity is illegal, a large number of horseshoe crab were confiscated and then discharged to local coast, which may mix with native species or even take the place of the native individual. The phenomenon of mixture of native species may be common in Chinese coast, which have been frequently found in news. However, due to the lack of available data, it is difficult to estimate their exact contribution to the genetic structure of Chinese horseshoe crab.

In this study, the divergence was much more pronounced revealed by mtDNA than microsatellites. This may reflect increased rate of differentiation by genetic drift at the mitochondrial compared with the nuclear genome, which was caused by the smaller effective population size of the former genome (Haavie et al., 2000). As a fast-evolving marker, the mitochondrial control region indicated much higher levels of genetic differentiation in this study, and similar results can be found in other reports (Scribner et al., 2001; Kerth et al., 2002; Clemencet et al., 2005; Brito, 2007).

Conservation implications for *T. tridentatus*

A drastic decline has occurred in the size of the horseshoe crab population in Chinese southeast coast, so it is urgent to carry out effective management strategies, such as reintroducing the species into those coasts where the horseshoe crabs have disappeared, determining management units, establishing nature reserves and so on. The microsatellite survey indicated weak population structure of horseshoe crabs along Chinese coast. Taken alone, these data would not distinguish separated evolution significant units and mandate the species in Chinese coast as a single management unit. The weak genetic differentiation suggests that exchangeability of individuals between locations will make minimal negative genetic impacts, and this may facilitate re-introduction efforts in a region that is suffering the greatest impacts in terms of habitat loss and resources destruction. The economic developed area such as Zhejiang Province, where horseshoe crab resources are extremely scarce is especially necessary for the introduction of Chinese horseshoe crab from other locations for population rejuvenation. Meanwhile, when we introduce horseshoe crab to other locations, we should avoid introgression by mixing differentiated populations which has been revealed by CR marker. Therefore conservation strategies such as *ex situ* conservation should be carried out.

However, mitochondrial DNA survey displayed significant difference among these locations, four management units were implied including locations from Beibu Bay, ZP, MZ and NH. There are only six municipal nature reserves of horseshoe crab established in China mainland nowadays, all merely in Guangdong Province. Upon four management units we proposed above, four or more nature reserves except six existing reserves should be established accordingly. Dongshan Bay and Meizhou Island were expected to be selected as nature reserves of Fujian Province, Shanmen Bay where NH location was sampled as nature reserve of Zhejiang Province, and Beihai coast selected as the representative reserve of Beibu Bay. For the species were just regarded as the province-level of “key protected aquatic wildlife” in zhejiang, Fujian, Guangdong Province and Guangxi Zhuang autonomous region, another important conservation strategy is the need to promote horseshoe crab to be included in wildlife conservation species list in China.

In the future, more conservation strategies such as extensive survey of the population size, artificial propagation should be carried out.

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