Population Subdivision of the Tri-Spine Horseshoe Crab, *Tachypleus tridentatus*, in Taiwan Strait

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The genetic structure of the Asian tri-spine horseshoe crab, *Tachypleus tridentatus*, was investigated in three populations of Taiwan Strait using mitochondrial (mt) AT-rich region DNA sequences. We examined 23 individuals from Kinmen, an island located on the western side of Taiwan Strait, and 12 each from Tiexianwei and Dongwei near Magong Island in the Penghu Archipelago, in the middle of Taiwan Strait. DNA sequence analysis of 369 base pairs (bp) of the mt AT-rich region revealed 10 haplotypes among the 47 individuals, with a mean haplotypic diversity (h) of 0.626 ± 0.075 and nucleotide diversity (π) of 0.0039 ± 0.00055. Pairwise F-statistics (F_{ST}) revealed significantly high gene flow between Kinmen and Dongwei ($F_{ST}=-0.0351$, $p>0.05$, $N_em=\infty$), but marked population subdivision and restricted gene flow between Kinmen and Tiexianwei ($F_{ST}=0.1382$, $p<0.05$, $N_em=3.1176$). Between populations at Magong Island, gene flow was moderate ($F_{ST}=0.0634$, $p>0.05$, $N_em=7.3913$). Mismatch distribution analysis indicated that the relatively low haplotype and nucleotide diversity observed in the Tiexianwei *T. tridentatus* population can be attributed to a recent bottleneck, probably due to isolation of Tiexianwei in semi-closed Magong Bay that prevents gene flow from neighboring populations.

Key words: tri-spine horseshoe crab, *Tachypleus tridentatus*, population genetics, mitochondrial AT-rich region, bottleneck, demographic history

INTRODUCTION

Marine organisms often show strong gene flow due to dispersal across wide geographic ranges (reviewed in Palumbi, 1994). Sampling species with high and intermediate dispersal abilities must be extensive, since gene flow often precludes genetic subdivision (Lessios *et al*., 1998). In contrast, the population structure can be distinct due to genetic drift, strong post-settlement selection (Hedgecock, 1986), and spatial-landscape patterns (Parsons, 1996; Johnson and Black, 1998; Watts and Johnson, 2004), as well as to a restricted dispersal capability (Collin, 2001). Species with a restricted dispersal capability are often composed of highly genetically structured populations with small geographic ranges, thus providing more opportunities to compare the depths and positions of intraspecific genetic divisions with the magnitude and locations of extrinsic factors (Bernardi and Talley, 2000).

Horseshoe crabs are ancient marine arthropods exhibiting life-history and habitat preferences that might indicate a restricted dispersal capability (reviewed in Sekiguchi, 1988). *Limmulus polyphemus*, the only Atlantic species, inhabits the eastern coast of North America from Maine to Mexico (reviewed in Rutecki *et al*., 2004). Three Asian horseshoe crab species, including *Tachypleus tridentatus, T. gigas,* and *Carcinoscorpius rotundicauda,* are distributed sporadically from Southeast Asia to Japan (Sekiguchi, 1988; Morton, 1999). Mature horseshoe crabs migrate from the deeper continental shelf to low-energy, well-oxygenated sandy beaches near the high tide line for egg deposition and external fertilization during the breeding period. The hatched trilobite larvae of *L. polyphemus* swim freely for a short period and settle to the bottom in shallow waters of the intertidal zone near their natal beaches (Shuster, 1982). In contrast, *T. tridentatus* larvae tend to stay in their nests immediately after hatching, where they usually reside over winter, then leave the nests and reside at or near their natal beaches in the following spring or summer. Juveniles of *T. tridentatus* bury themselves in the sandy-mud bottom during high tide and feed during low tide (Sekiguchi, 1988; Chen *et al*., 2004; Chiu and Morton, 2004). Adults of *T. tridentatus* spend the winter on the offshore ocean bottom at depths of 20 m for hibernation, and then migrate from their winter deeper-water habitat to the shallow areas of coves when seawater temperatures rise (Sekiguchi, 1988). These life-history characteristics and habitat preferences suggest that the dispersal capability of horseshoe crabs might be restricted, resulting in substantial population subdivisions on a small geographic scale (Pierce *et al*., 2000).

Population genetic studies of horseshoe crabs have generally focused on *Limulus polyphemus* along the eastern
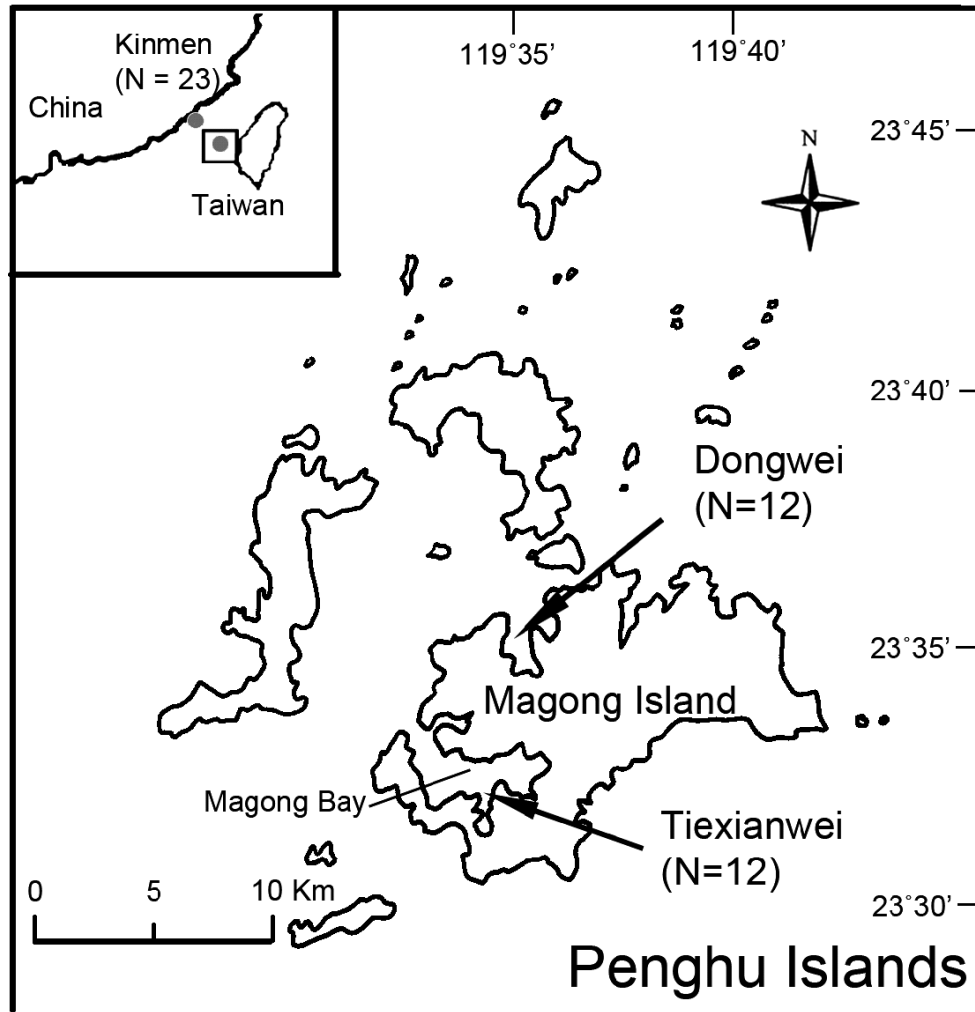
Fig. 1. Map of collection locations in Taiwan Strait. Horseshoe crabs, *Tachypleus tridentatus* were collected at 2 sites, Kinmen and the Penghu Islands, indicated by gray circles. Collections were made from two populations near Magong Island: Dongwei and Tixianwei that are indicated by arrows.

coast of North America (Saunders *et al*. 1986; Orti *et al*. 1997; Pierce *et al*., 2000; King *et al*., 2004). The existence of a genetic division between the Gulf of Mexico and Atlantic populations, a pattern observed for a variety of species (reviewed in Avise, 2004), has been detected using allozymes, restriction fragment length polymorphisms (RFLPs) of the mitochondrial (mt) genome, and microsatellites (Selander *et al*., 1970; Saunders *et al*., 1986; King *et al*., 2004). In contrast, limited gene flow on small scales was reported for sequence variants of the mtDNA cytochrome oxidase I (COI) 5'-end region in the Chesapeake Bay and Delaware Bay populations (Pierce *et al*., 2000).

The Asian tri-spine horseshoe crab, *T. tridentatus*, was formerly abundant in Southeast Asia to Japan (Sekiguchi, 1988). However, *T. tridentatus* in this region is declining, mainly due to loss of suitable spawning grounds because of coastal development and overharvesting for food and biomedical purposes (Itow, 1993; Chiu and Morton, 1999; Botton, 2001; Chen *et al*., 2004). *Tachypleus tridentatus* was once relatively abundant along the western coast of Taiwan and adjacent islands in Taiwan Strait. It is now thought that

population of *T. tridentatus* is locally endangered along the western coast of Taiwan due to overfishing, land reclamation, and water pollution (Chen *et al*., 2004). Residual populations were only found in the Penghu Islands in the middle of Taiwan Strait, and around Kinmen Island off the Chinese mainland (Fig. 1). Conservation programs, including life-history investigations, public education, and translocation, have been promoted (Chen *et al*., 2004). Our study examines population structure and gene flow in the tri-pine horseshoe crab, *T. tridentatus*, from these two island groups in Taiwan Strait, in order to facilitate conservation efforts for this species. We analyzed genetic variation in the mt AT-rich region, which has proven to be a useful marker in intraspecific studies of some other arthropods (*e.g*., *Drosophila subobscura*, Brehm *et al*., 2001).

MATERIALS AND METHODS

Study sites and sample collection

Juveniles of *T. tridentatus* were surveyed and collected intertidally from sandy mudflats at both Kinmen Island and the Penghu Islands in 2002 and 2003 (Fig. 1). At Kinmen, juveniles were

Table 1. Localities and molecular characters in the AT-rich region. Sample size (N), nucleotide content (AT %), number of substitutions (Ti, transition; Tv, transversion), number of haplotypes (N_h), haplotypic diversity (h), and nucleotide diversity (π) (±standard deviation; SD).

Population	Code	N	A+T%	Ti:Tv	N_h	$h\pm$SD	$\pi\pm$SD
Tiexianwei	**T**	12	84.51	5:0	4	0.455±0.17	0.00263±0.00216
Dongwei	**D**	12	84.28	5:0	4	0.561±0.154	0.00435±0.00295
Kinmen	**K**	23	84.28	5:0	6	0.696±0.073	0.00403±0.00282
Total		47	84.34	9:0	10	0.626±0.075	0.0039±0.00055

obtained from Hsia Su (20°58'58" N, 118°35'49" E). At Magong Island, the largest island in Penghu County, collecting was conducted with the guidance of local fishermen. Only two local populations, Dongwei (23°35'00" N, 119°35'40" E) and Tiexianwei (23°32'00" N, 119°35'30" E), were discovered around Magong Island after intensive field surveys. Dongwei is an open bay facing the Inner Sea, whereas Tiexianwei is located in Magong Bay, a semi-closed bay (Fig. 1). All individuals were preserved in 95% ethanol immediately after collection. In total, 47 *T. tridentatus* juveniles were used in this study.

Genomic DNA extraction, PCR, and DNA sequencing

Total genomic DNA was extracted from muscle tissues using the Blood & Tissue Genomic DNA Extraction Miniprep System Kit (VIOGENE, Taipei) following the instructions provided by the supplier. The complete mt AT-rich region was amplified by a pair of primers, Hb-trna (5'-GAGCCCAATAGCTTAAAT TAGCTTA-3') and Hb-12S (5'-GTCTAACCGCGGTAGCTGGCAC-3'), designed from the published mt genome of *L. polyphemus* (Lavrov *et al.*, 2000). Amplification reactions were conducted in 50-μL volumes using the buffer supplied with the enzyme and under the conditions recommended by the manufacturer. Each 50-μL volume contained 50 mM KCl, 10 mM Tris (pH 9.0), 3 mM MgCl$_2$, 0.2 mM each dNTP, 0.04 mM each primer, 0.033 units of *Taq* polymerase (Invitrogen, Karlsruhe, Germany), 1μL DMSO, and 50 ng of genomic DNA. PCR was performed in a PC-9606 thermal sequencer (Corbett Research, Mortlake, Australia) using the following thermal cycle: 1 cycle at 95°C (3 min); 30 cycles at 94°C (30 s), 50°C (1 min), and 72°C (2 min), and one cycle at 72°C (2 min), 25°C (5 min). The PCR products were electrophoresed on a 1% agarose (FMC Bioproduct, Rockland, ME USA) gel in 1X TAE buffer to check the yield. The amplified DNA was extracted using a DNA/RNA gel extraction column (Viogene, Taipei) under conditions recommended by the manufacturer. The nucleotide sequences were determined from both ends by direct sequencing of the PCR products with an ABI 377 Genetic Analyzer. Sequences obtained in this study have been submitted to GenBank under accession numbers DQ516529~ DQ516538.

Sequence and population-genetic analyses

DNA sequences were initially assembled and aligned using the program SeqMan^{TM} II (DNASTAR, Madison, WI), followed by manual editing using SeqApp 1.9 (Gilbert, 1994). All data analyses were implemented using Arlequin 2.0 for a Macintosh platform (Schneider *et al.*, 2000). Unique haplotypes and all transitions and transversions were counted. Haplotype diversity (h), nucleotide diversity (π) (Nei, 1987), and their standard errors were calculated. Pairwise F-statistics (F_{ST}) were calculated as genetic distances based on pairwise differences (Gamma α value=0.248) between populations (Reynolds *et al.*, 1983; Slatkin, 1995). The null distribution of pairwise F_{ST} values under the hypothesis of no difference between populations was obtained by permuting haplotypes between populations in 1,000 permutation simulations. An indirect estimate of gene flow was calculated based on the equation, $N_em=1/2 [(1/F_{ST}) - 1]$, where N_e is the effective number of females and m is the migration rate. The historical demography of *T. tridentatus* was examined

using mismatch distribution analysis (MDA) based on the observed number of differences among all pairs of haplotypes (Rogers and Harpending, 1992). Simulation studies have shown that the distribution is multimodal in samples drawn from populations at demographic equilibrium, a pattern reflecting stochastic lineage loss; a unimodal distribution suggests stationary population growth, and a Poisson distribution indicates a recent bottleneck effect. However, one concern about using MDA for a group of subdivided populations is that the assumption of random mating is violated (Hoffman and Blouin, 2004). Rogers and Harpending (1992) showed that the theory behind MDA is robust and should approximately hold true even when populations are completely isolated. Moreover, when subdivided populations are linked by gene flow, an even better fit to the random mating approximation should be obtained (Hoffman and Blouin, 2004).

RESULTS

Mitochondrial AT-rich region sequence variation

Using primers designed from the homologous regions in the 12S rRNA and tRNA-Met genes of the American horseshoe crab, *L. polyphemus* (Lavrov *et al.*, 2000), we determined the complete nucleotide sequence of the *T. tridentatus* mtAT-rich region, two tRNA genes, and a partial sequence of the flanking region of 12S rRNA. The length of the PCR fragment was 689 base pairs (bp), including 183 bp of 12S rRNA, 67 bp for tRNA-Ile, and 67 bp for tRNA-Gln. The total length of the AT-rich region was 369 bp in *T. tridentatus*. In total, 10 haplotypes of the AT-rich region with nine variable sites were identified from 47 *T. tridentatus* individuals in three populations (Table 1). Overall and population-specific nucle-

Table 2. Variable sites found in a fragment of the AT-rich region of *Tachypleus tridentatus* and their distribution in the populations. Nine variable sites were found in a fragment of the AT-rich region in 47 horseshoe crabs defining 10 haplotypes (H01–H10). K, T, and D represent the populations in Kinmen, Tiexianwei, and Dongwei, respectively. H01 was the most-common haplotype.

	Nucleotide positions									Populations			
		1	1	1	2	2	2	2					
	3	7	2	3	5	2	4	9	9				
	5	8	4	1	1	1	2	2	8	K	T	D	Total
H01	T	A	G	T	C	A	T	A	A	11	9	8	28
H02	•	•	A	•	•	•	•	•	•	1			1
H03	•	G	•	•	•	G	C	•	•			1	1
H04	C	•	•	C	•	G	•	•	•	2			2
H05	•	G	•	•	•	G	•	G	•		1	1	2
H06	•	•	•	•	T	•	•	•	•		1		1
H07	•	•	•	•	T	•	•	•	G		1		1
H08	•	G	•	•	•	G	•	•	•	7			7
H09	C	G	•	•	•	G	•	•	•	1		2	3
H10	•	•	•	•	•	G	•	•	•	1			1

otide composition, number of haplotypes, haplotype diversity, and nucleotide diversity are presented in Table 1. A common haplotype, H01, was shared by 28 individuals (60%) among the three populations in Taiwan Strait (Table 2).

Population structure, gene flow, and mismatch-distribution analyses

For tri-spine horseshoe crabs, geographic distance between the populations of different localities was not reflected in the degree of genetic differentiation between them

Table 3. Pairwise F-statistic (F_{ST}) values of genetic differentiation and female migrants per generation ($N_e m$) values of gene flow among horseshoe crab populations. F_{ST} values are above the diagonal, and $N_e m$ values are below the diagonal.

Population	Tiexianwei	Dongwei	Kinmen
Tiexianwei	–	0.0634[ns]	0.1382*
Dongwei	7.3913	–	–0.0345[ns]
Kinmen	3.1176	Infinite	–

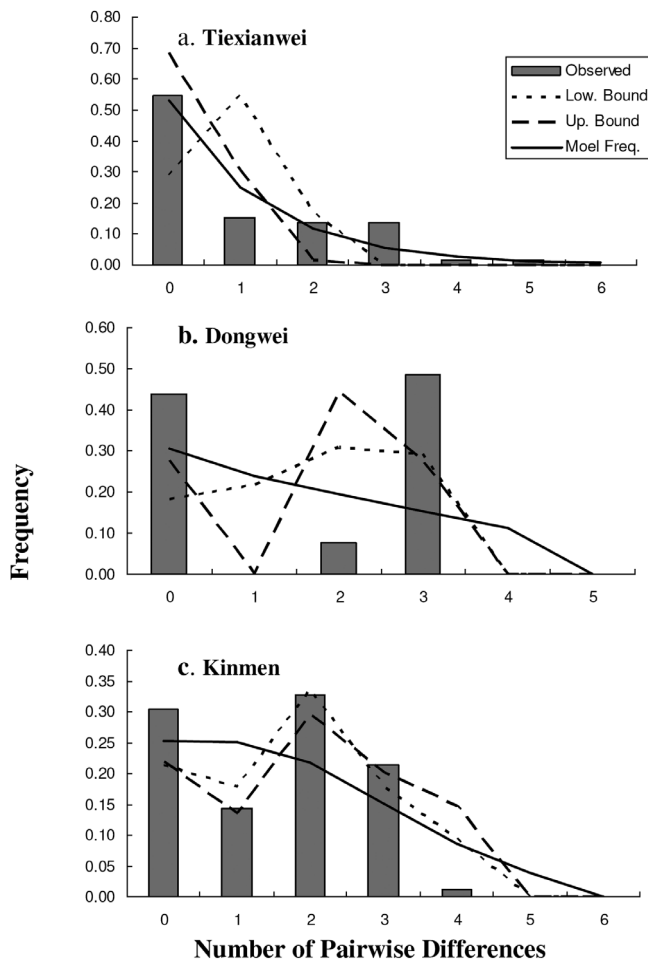*$p<0.05$;   [ns]not significant

Fig. 2. Mismatch distribution analyses of the *Tachypleus tridentatus* AT-rich region pairwise sequence differences as computed by ARLEQUIN 2.0. The observed distributions (bars) are compared to the sudden expansion model illustrated by the overlaid curve (solid line), and dashed lines are the 5 (low boundary) and 95(up boundary) percentile values of 1000 simulated samples.

(Table 3). Population pairwise F_{ST} values were low both between Dongwei and Tiexianwei ($F_{ST}=0.0634$, $p>0.05$) at Magong Island, and between Dongwei and Kinmen ($F_{ST}= -0.0351$, $p>0.05$), indicative of a high degree of gene flow between these pairs of populations; however these values were not statistically significant. A negative F_{ST} value indicates great differences between two random individuals from the same population, rather than between two random individuals from different populations (Arnason and Palsson, 1996). In contrast, the F_{ST} value between Tiexianwei and Kinmen was 0.1382, indicative of restricted gene flow, and this value was statistically significant ($p<0.05$). The number of female migrants per generation ($N_e m$) indirectly estimated from the inverted F_{ST} values was infinite between Dongwei and Kinmen, moderate between Dongwei and Tiexianwei ($N_e m=7.3913$), and relatively low between Tiexianwei and Kinmen ($N_e m=3.1176$). Mismatch distribution analyses and simulations suggested that the tri-spine horseshoe crabs at Dongwei and Kinmen yield a ragged distribution of pairwise differences (Fig. 2b, c), as might be expected from a stable population at equilibrium (Rogers and Harpending, 1992). In contrast, the Tiexianwei population exhibited an approximate Poisson distribution of pairwise differences, suggesting a recent population bottleneck (Fig. 2a).

DISCUSSION

Population genetic analysis of the mitochondrial (mt) AT-rich region demonstrated that the Asian tri-spine horseshoe crab, *Tachypleus tridentatus,* displays genetic subdivision over a relatively small geographic range. Genetic subdivision is anticipated for marine species for which adults are relatively immobile and larvae are non-dispersive, although genetic isolation has also been found in species with a high level of adult migration or with long-lived planktonic larvae (reviewed in Avise, 2004). Adults of *T. tridentatus* stay on the offshore ocean bottom and migrate to shallow areas for reproduction (Sekiguchi, 1988). Trilobite larvae settle right after hatching, and juveniles spend their life stages at or near the natal beach for feeding (Sekiguchi, 1988; Chen *et al.*, 2004). These life-history traits indicate that the tri-spine horseshoe crab has limited adult migratory abilities and limited dispersal of larvae; as a consequence, it should exhibit genetic subdivision between populations and isolation-by-distance. However, our observations do not fulfill these predictions. Despite small sample sizes, two populations at Magong Island, Dongwei and Tiexianwei, separated by 15 km, showed moderate gene flow. However, strikingly different results were observed at the larger geographic distance (>150 km). The tri-spine horseshoe crabs of Kinmen and Dongwei showed a negative F_{ST} value, indicating there to be unrestricted gene flow. In contrast, the F_{ST} value (p<0.05) between Kinmen and Tiexianwei is indicative of restricted gene flow. These contrasting results highlight that life history characteristics alone cannot entirely explain the genetic structure of tri-spine horseshoe crab populations in Taiwan Strait.

The discovery of substantial population subdivision in *T. tridentatus* between Kinmen and Tiexianwei in Taiwan Strait is not surprising. A similar pattern was also observed in a comparison of two populations of American horseshoe crab, *L. polyphemus*, along the east coast of North America using

mt COI DNA sequences (Pierce *et al*., 2000). For *L. polyphemus,* it has long been known that there is a genetic "break" between the Gulf of Mexico and Atlantic populations (Saunder *et al*., 1986; Orti *et al*., 1997; King *et al*., 2004), a pattern also seen for a variety of other coastal and marine species (reviewed in Avise 2001, 2004). In contrast, mtDNA variation in the upper Chesapeake Bay and Delaware Bay demonstrate that gene flow between populations inhabiting the sampled sites is restricted (Pierce *et al*., 2000). This suggests that the American horseshoe crabs in the upper Chesapeake Bay population are resident, separate from the larger shelf-inhabiting population found in Delaware Bay (Pierce *et al*., 2000). A similar explanation might apply to *T. tridentatus* of Tiexianwei. Magong Bay is semi-closed, and as a consequence the population there might have limited genetic exchange with populations outside the bay.

Differences in demographic history also shade the patterns of mtDNA sequence variation and genetic subdivision among the three *T. tridentatus* populations. Low haplotype diversity, low nucleotide diversity, and an approximately Poisson mismatch distribution imply the genetic consequences of a population bottleneck (Nei *et al*., 1975; reviewed in Avise, 2001, 2004). This pattern was observed only for the tri-spine horseshoe crab population at Tiexianwei. For the Dongwei and Kinmen populations, there was an indication of stable populations at equilibrium. The population bottleneck could have resulted from genetic drift in a small population, as if it were neutrally rising to fixation with about the same probability as strictly neutral change (Kimura, 1979); genetic drift with sufficient time is also one of the causes of diversification among populations (Frankham *et al*., 2002; Avise, 2004). A similar demographic difference was also observed in *L. polyphemus* in Delaware and Chesapeake Bays of the East Coast of the US. The population in the former was suggested as being a bottleneck population, whereas the latter was suggested to be a stable population at equilibrium (Pierce *et al*., 2000). The recent population bottleneck in Delaware Bay can be attributed to population reduction by overharvesting for commercial bait early in the last century (Pierce *et al*., 2000). Despite the geographical isolation, loss of habitat for natal grounds in Magong Harbor might also be causing loss of genetic diversity in the Tiexianwei population of *T. tridentatus*. Development of Magong Harbor began over two centuries ago, and modification of habitat by construction with cement continued into the early 20th century. The consequent loss of sandy beaches for spawning and feeding grounds might have strengthened the isolation of the Tiexianwei population of *T. tridentatus*.

The results of this study highlight the importance of population genetic analyses for conservation efforts of *T. tridentatus* in Taiwan as well as Southeast Asia and Japan. *Tachypleus tridentatus* was historically abundant from Southeast Asia to its northern limit in Japan; however, serious depletion of this species has been reported, resulting from pollution, overharvesting for food and biomedical usage, and loss of suitable spawning and feeding grounds by coastal development (Itow, 1993; Chiu and Morton, 1999; Botton, 2001; Chen *et al*., 2004). Population genetic approaches can rapidly identify the population status (or population demography, such as population bottlenecks) and connectivity among local populations. The genetic structures of local populations provide information that can be used for implementing different conservation strategies. For example, the higher genetic diversity and larger census population size (Chen *et al*., 2004) found at Kinmen provide a reservoir of horseshoe crab genetic variation and supports the ongoing implementation of a marine protected area in Kinmen County (Chen *et al*., unpublished data). On the other hand, Tiexianwei with the bottleneck population deserves high priority in the establishment of a conservation and management plan for Penghu County. Translocation of horseshoe crabs from nearby populations, such as Dongwei, might be initiated to increase the genetic variability and to help prevent local extinction of the Tiexianwei population.

In summary, our preliminary study indicates that the Asian tri-spine horseshoe crab, *Tachypleus tridentatus*, exhibits population subdivision over a relatively small geographic range. The genetic data presented here indicate that the gene flow between Tiexianwei of Magong Bay and other populations is limited. It should be noted that the gene flow revealed by the mitochondrial AT-rich region represents the evolutionary history only of female tri-spine horseshoe crabs. Genetic information on male tri-spine horseshoe crabs contributing to the local population was not addressed by our study. Additional approaches using biparentally inherited markers from the nuclear genome (*e.g*., microsatellites) and extension of the geographic range of sampling are currently being undertaken and will be useful in elucidating the population structure and genetic connectivity of *Tachypleus tridentatus* throughout its distribution range from Southeast Asia to Japan.

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