

Full Length Research Paper

Genetic diversity and historical demography of kuruma shrimp (*Penaeus japonicus*) species complex off China based on mitochondrial DNA analysis

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Two varieties (I and II) of kuruma shrimp (*Penaeus japonicus*) were found in the north of South China Sea (SCS) and Taiwan Strait (TS). To estimate the demographic history and genetic diversity of this species complex off China, 141 individuals were collected from the East China Sea (ECS), TS and SCS and 27 variety 2 specimens from SCS were also sampled for comparison. Sequence analyses on fragments of 454-bp at 5' end of mitochondrial DNA control region were conducted. Neighbor-joining tree and network of all populations yielded two clades; one included variety I individuals, the other comprised variety II. The variety II could also be found in ECS. The haplotype diversity (h) for variety I was high for all populations (99.9%), with values from 99.3% (ECS) to 1 (SCS). Nucleotide diversity (π) for variety I was low for all populations (0.0321), with values from 0.0285 (TS) to 0.0361 (ECS). The h and π for variety II were 1 and 0.0446, respectively. Analyses of molecular variance and F_{ST} revealed no significant genetic structure for variety I populations. Neutrality tests and mismatch distribution analyses suggested a late Pleistocene population expansion for both variety I (62,132 to 86,605 years ago) and variety II (94,464 to 146,655 years ago) of kuruma shrimp off China.

Key words: Mitochondrial DNA, *Penaeus japonicus*, historical demography, control region.

INTRODUCTION

The kuruma shrimp (*Penaeus japonicus*) is a widely distributed species throughout the Indo-west Pacific, ranging from eastern and southern Africa into the Red Sea through the entire Malay Archipelago to Taiwan,

Korea, Japan and northern Australia, and they have moved through the Suez Canal into the Mediterranean (Dall et al., 1990). This species is of economic importance in fisheries and aquaculture in Japan, China, Australia and many Southeast Asian countries (Rosenberry, 2001). Two morphologically similar but genetically distinct varieties of this species, named varieties I and II, in the north of south China Sea (SCS) were found (Tsoi et al., 2005). These two varieties were characterized by diagnostic color banding patterns on the carapace. Variety I is mainly distributed along the coast of Japan and mainland China, while variety II is spread over Southeast Asia (the Vietnam, Singapore and Philippines), Australia and the

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Abbreviations: SCS, South China sea; TS, Taiwan strait; ECS, East China sea; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; AMOVA, Analysis of molecular variance.

Mediterranean. These two varieties occur simultaneously in the northern coast of the SCS and Taiwan Strait (TS) (Tsoi et al., 2005, 2007). However, sequence comparison showed that no variety II individual was found in the East China Sea (ECS) (Tzeng et al., 2004; Tsoi et al., 2007). Whether these two varieties also coexist in ECS should be further surveyed.

The present population genetic structure of a marine organism may be fully interpreted if the influence of historical events and the complex interactions of biology, geography and climatic shifts were considered (Hewitt, 2000). Stern climatic shifts can create great changes in species' geographical distribution and abundance, which can be expected to have genetic consequences and the advent of DNA technology provides proper markers to examine the genetic effects of these changes (Avise, 2000; Hewitt, 2000).

It is known that, during late quaternary glacial cycles, there were drastic changes in areas and configurations in marginal seas of the Western Pacific (Wang, 1999). For example, during the last glacial maximum, when the sea level was at its lowest, about 130 m below the present sea level, an extensive area of the continental shelf of the ECS was exposed. Therefore, the present genetic structures of populations in the marginal seas of the Western Pacific have been greatly influenced by Pleistocene ice ages.

Mitochondrial DNA (mtDNA) sequences are appropriate for assessing population genetic structure, phylogeography and in making inferences about underlying historical demographic processes (Avise, 2000). The additional advantage for using mtDNA is that its effective population size is four times smaller than nuclear DNA due to its haploid nature and generally maternal inheritance (Hoelzel et al., 1991). Thus, the effect of genetic drift is stronger and a higher level of population differentiation can be observed with mtDNA than with nuclear DNA. This can be of great importance in populations that do not reach migration/genetic drift equilibrium, as in the ECS, TS, or SCS where recolonization history is recent.

In two previous studies on the population genetic structure for variety I of kuruma shrimp in the East Asia, two different conclusions were made. One showed that the variety I from East Asia appeared genetically homogeneous inferred from mtDNA sequences (control region and COI sequences) and two microsatellite loci (Tsoi et al., 2007), the other indicated that, the population from ECS was genetically distinct from the TS and SCS based on mtDNA control region (Tzeng et al., 2004).

Therefore, the population structure of variety I of China ought to be further verified. Although, the population genetic structure of variety I were examined, the information on historical demography of this species was still unknown. In this present paper, sequence analyses on a 454-base segment of the mitochondrial DNA control region were conducted to elucidate the genetic diversity, historical demography and distribution of these two varieties in the ECS, TS and SCS.

MATERIALS AND METHODS

Sample collection

Three populations including 141 individuals were collected during February and May 2006 (Figure 1; Table 1). They were separately sampled from the north of ECS, TS and SCS (SCS-VI). Twenty-seven variety II specimens from SCS were also sampled (SCS-VII). Unfortunately, the specimens from ECS and TS were not differentiated between variety I and variety II beforehand. The abbreviations for the sampling locations are defined in Table 1. Specimens were iced or frozen immediately after capture and later kept at -75°C until extracted.

DNA extraction, amplification and sequencing

Total DNA was extracted from frozen muscle tissue using a standard DNA proteinase K digestion/phenol-chloroform extraction procedure. A segment of the mitochondrial DNA including control region was amplified using the primers P30 (5'-GATCTTTAGGGGAATGGTGT AATTCATTG-3') and P24 (5'-GTGTAACAGGGTATCTAATCCT GG-3'). Thermal cycling was performed in a GeneAmp 2400 thermal cycler (Perkin-Elmer, Norwalk, CT, USA) and polymerase chain reaction (PCR) conditions consisted of 39 cycles of denaturation at 95°C for 50 s, annealing at 50°C for 1 min and extension at 72°C for 1.5 min. An initial denaturation step at 95°C for 5 min and a final extension holding at 72°C for 10 min were respectively included in the 1st and last cycles. Amplified DNA was separated through electrophoresis on 1.5% agarose gels and purified with the Gene Clean II kit (Bio101, Vista, CA, USA). Double-stranded DNA was sequenced on an ABI 377 DNA sequencer (Applied Biosystems, Inc.; Foster City, CA, USA) with the P30 primer.

Data analyses

DNA sequences were aligned by ClustalX, version 1.83 (Thompson et al., 1997), then, subsequently optimized by eye in BioEdit, version 7.0.5.3 (Hall, 1999). The control region sequences were confirmed by comparing them with the complete published mtDNA sequence of *P. japonicus* (Tzeng et al., 2004). Phylogeographic analysis was carried out on nucleotide distances by the neighbor-joining (NJ) method implemented in MEGA 3 (Kumar et al., 2004). Nucleotide distances were estimated with the Tamura-Nei model which assumes unequal nucleotide frequencies and different nucleotide substitution between all four nucleotides (Tamura and Nei, 1993). We used bootstrap analyses with 1,000 replicates to evaluate support for phylogenetic relationships. A haplotype network was also constructed using the median-joining method (Bandelt et al., 1999) in Network version 4.2.0.1. Nucleotide composition and numbers of variable sites were assessed with Arlequin version 3.01 (Excoffier et al., 2005). The genetic (h) and nucleotide diversity (π) (Nei, 1987) in each population were calculated using DnaSP Version 4.10 (Rozas et al., 2003).

To examine whether any two of the populations genetically differed from each other, pair-wise F_{ST} statistics among the 4 populations were estimated and tested using the program, ProSeq (Filatov, 2002). Analysis of molecular variance (AMOVA) implemented in Arlequin was performed to test the geographic divisions among variety I populations. The significance of these Φ statistics was evaluated by 1000 random permutations of sequences among populations.

To check for deviations from neutrality, Tajima's D (Tajima, 1989) and Fu's F_s statistical tests (Fu, 1997) were carried out to assess evidence for population expansion using DnaSP. Meanwhile, the concordance of data with the distribution underlying the expansion model was assessed. Historical demographic expansion was invest-

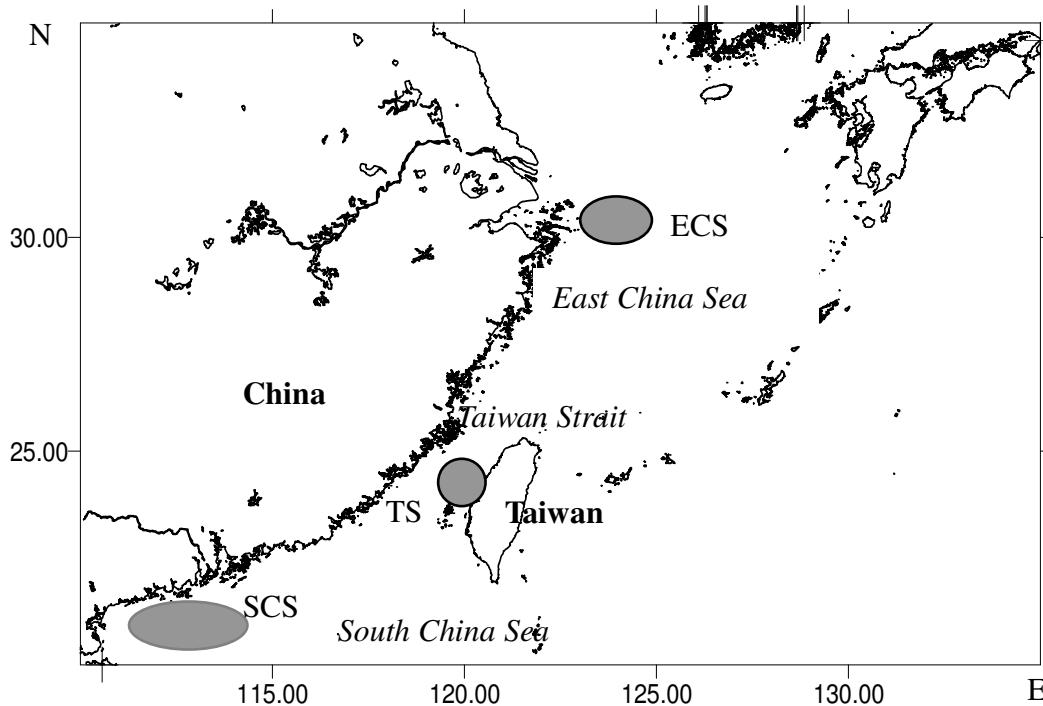


Figure 1. Sampling localities of kuruma shrimp (*P. japonicus*) species complex of China.

Table 1. Code of sampling site, sample size (n), number of variety I and variety II, number of haplotypes, gene diversity (h) and nucleotide diversity (π) with 95% confidence interval in kuruma shrimp (*P. japonicus*) species complex of China.

Code	Sampling site	n	No. of variety I	No. of variety II	No. of haplotype	h	π
ECS	East China Sea	39	35	4 ^a	31	0.993±0.009	0.0361±0.0037
TS	Taiwan Strait	72	71	1 ^a	69	0.999±0.003	0.0285±0.0010
SCS-VI	South China Sea	30	30	---	30	1±0.009	0.0355±0.0037
SCS-VII	South China Sea	27	---	27	27	1±0.01	0.0446±0.0019
Total Variety I		136	136	---	130	0.999±0.001	0.0321±0.0015

a, the variety II individuals from ECS and TS were excluded in following analyses.

tigated by examining the frequency distributions of pair-wise differences between sequences (mismatched distribution) with Arlequin. Rough dates of population expansion were estimated with the formula $\tau = 2 \mu T$ (Rogers and Harpending, 1992), where, T is the time since expansion, τ is the expansion time and 2μ is μ (the mutation rate) \times generation time \times number of bases sequenced.

RESULTS

Amplification of kuruma shrimp mtDNA with P30 and P24 primers produced a PCR product of approximately 1500 nucleotide bases in length. We were able to obtain a 454-base segment of the control region for each specimen. Neighbor-joining tree (Figure 2) and network (Figure 3) of all populations yielded two clades; one included variety I individuals, the other comprised variety II. Shal-

low and no significant genealogical branches were found within clades. The variety II individuals could also be found in ECS. Among the 168 individuals studied, 163 haplotypes were defined. Five haplotypes were separately shared by 2 individuals from single population. All others occurred in only 1 individual. Five variety II specimens from ECS (4) and TS (1) were excluded in following analyses.

The nucleotide composition of the fragments for variety I (A: 36.56%, G: 9.79%, C: 9.79%, T: 43.86%) and variety II (A: 36.03%, G: 9.96%, C: 8.79%, T: 45.22%) were A, T-rich, as is usual for the region in many invertebrate species. Thirty-five fixed nucleotide substitutions were found between the two varieties. The haplotype diversity (h) for variety I was high for all populations (99.9%), with values from 99.3% (ECS) to 1 (SCS) (Table 1). Nucleotide

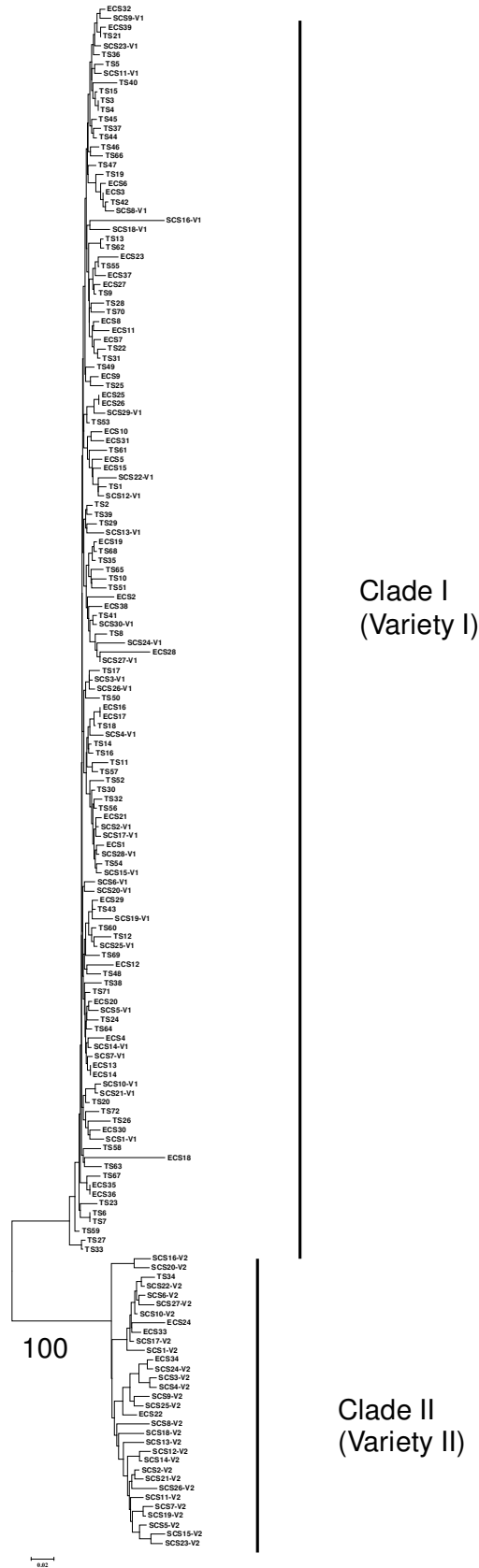


Figure 2. Neighbor-joining tree for kuruma shrimp (*P. japonicus*) species complex off China.

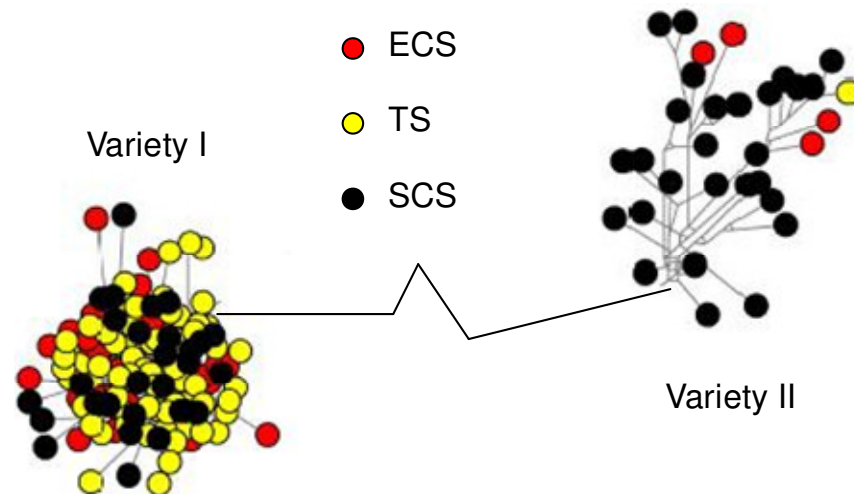


Figure 3. Alleles network of kuruma shrimp (*P. japonicus*) species complex off China. Small symbols indicate 1 individual, while large ones indicate more than 1 individual.

Table 2. F_{ST} value in kuruma shrimp (*Penaeus japonicus*) species complex off China.

	ECS	TS	SCS-VI
TS	0.0036ns		
SCS-VI	-0.0019ns	-0.0022ns	
SCS-VII	0.7457**	0.7619**	0.7459**

** $P < 0.01$, ns = not significant ($P > 0.05$). The abbreviations for the sampling locations are defined in Table 1.

diversity (π) for variety I was low for all populations (0.0321), with values from 0.0285 (TS) to 0.0361 (ECS). The h and π for variety II (SCS_VII) were 1 and 0.0446, respectively (Table 1).

The conventional population statistic F_{ST} revealed no significant genetic structure for variety I populations (Table 2). The F_{ST} values between variety I and II populations showed significant genetic differences. AMOVA of three variety I populations yielded a Φ_{ST} value of 0.0021, indicating no significant heterogeneity between any pair-wise combination of these 3 populations (Table 3).

Significant Tajima's D values were obtained in ECS, TS and SCS_VI, but not in SCS_VII. The Fu's F_s tests were significant for any examined population (Table 4). The model of population expansion could not be rejected when all variety I populations were combined for Tajima's D and Fu's F_s statistical tests. The mismatch distribution including both varieties I and II was bimodal (Figure 4), with one mode corresponding to the number of differences within the varieties and the other to differences between the two varieties. The separate analysis of varieties I and II yielded in both cases a unimodal distribution, not significantly different (as measured by the sum of squared deviation; $p > 0.05$) from that predicted by the growth expansion model.

The τ values of varieties I and II were 13.054 (95% confidence interval (CI), 10.719-14.941) and 22.264 (95% CI, 16.297-25.301), respectively. Because of shrimp's short life span, a generation time of 2 years was used (Tzeng and Yeh, 1998). McMillan-Jackson and Bert (2003) roughly estimated a mutation rate of 19%/MY for the mtDNA control region of brown shrimp (*Farfantepenaeus aztecus*) and white shrimp (*Litopenaeus setiferus*). Using this rate, the estimated time of expansion for variety I was 75,667 (95% CI, 62,132-86,605) year ago. For variety II, was 129,052 (95% CI, 94,464-146,655) year ago.

DISCUSSION

The control region sequences revealed high level of haplotypic diversity (for variety I: 0.999 and variety II: 1) and the low level of nucleotide diversity (for variety I: 0.0321 and variety II: 0.0446) (Table 1). The h for variety I was higher than one found in East Asia ($h = 0.69$) (Tsoi et al., 2007). The π for variety I was lower than 0.094 found in Tsoi et al. (2007), but higher than 0.0251 found in Tzeng et al. (2004). The values of h and π for variety II were higher and lower than those found in variety II populations examined by Tsoi et al. (2007), respectively. It has been proposed that marine organisms can be classified into four categories based on different combinations of small and large values for h and π of mtDNA sequences to interpret different scenarios of population history (Grant and Bowen, 1998). They indicated that population with high h and low π probably underwent population expansion after a period of low effective population size. In this study, high haplotype diversity (for variety I: 0.999 and variety II: 1) and the lower nucleotide diversity (for variety I: 0.0321 and variety II: 0.0446) suggests that the variety I and II of kuruma shrimp of China had undergone population expansion.

Table 3. Analysis of molecular variation for variety I Kuruma shrimp populations of China.

Source of variation	Degree of freedom	Sums of squares	Variance component	Percentage of variation	F_{ST}	P
Among population	2	16.429	0.01552	0.20	0.00205	0.2594
Within populations	133	1006.582	7.56829	99.80		
Total	135	023.012	7.58381	100		

Table 4. Code of sampling site, sample size (n), Tajima's D , and Fu's F_s in kuruma shrimp (*Penaeus japonicus*) species complex off China.

Code	Sampling site	n	Tajima's D	Fu's F_s
ECS	East China Sea	35	-2.007*	-12.084**
TS	Taiwan Strait	71	-2.127*	-75.367**
SCS-VI	South China Sea	30	-2.09*	-17.959**
SCS-VII	South China Sea	27	-1.162ns	-12.627**
Total Variety I		136	-2.382**	-182.867**

ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$.

The neutrality of mtDNA control region mutations for variety I was rejected on the basis of Tajima's D ($D = -2.382$, $P < 0.01$) and Fu's F_s ($F_s = -182.867$, $P < 0.01$). Although, the Tajima's D value ($D = -1.162$) for variety II was not significant, the Fu's F_s was a significant negative value (Fu's $F_s = -12.627$). These two statistics are sensitive to factors such as bottlenecks or population expansion which tend to drive the values of Tajima's D and Fu's F_s towards more negative values (Tajima, 1996; Martel et al., 2004) and the latter one is the most sensitive to recent population growth (Fu, 1997). Indeed, significant negative values of these two statistics in this study also indicated that, variety I and II of kuruma shrimp of China had experienced population expansion.

The unimodal mismatch frequency distribution pattern based on the mtDNA sequence accorded well the predicted distribution under a model of population expansion (Figure 4) (Rogers and Harpending, 1992). This unimodal pattern has also been observed for other shrimp species in East Asia, such as *Parapenaeopsis hardwickii* (Tzeng, 2007), *Feneropenaeus chinensis* (Li et al., 2009). Although, the neighbor-joining tree of all populations was divided into two deep clades and shallow, and no significant genealogical branches were found within clades (Figure 2). The shallow phylogeny is consistent with a population expansion (Slatkin and Hudson, 1991).

Past geological and climatic events have undoubtedly played a major role in population expansion of kuruma shrimp species complex of China. Sea level was 130 to 150 m lower than the present level in the ECS and 100 to 120 m lower in the SCS during Pleistocene glaciations (2.4 Ma–10000 years). Consequently, the coastal areas along the SCS, together with the ECS and TS were exposed (Wang and Sun, 1994). The disappearance of habitat had restricted marine species to the relatively

limited areas and caused the mixing among populations and also, reduced the genetic variation between populations (Benzie and Williams, 1997). Two estimates of the time since the expansion for both varieties I and II were approximately 75,667 and 129,052 years ago, in agreement with the extension of the distribution for variety I and II of kuruma shrimp populations following the rise in the sea level of the studied areas since the late Pleistocene (1,600,000 to 10,000 years ago).

In this present study, four variety II individuals were also found in the ECS that were not reported before. We mainly agree that, the rise in sea level allowed greater dispersal of the two varieties (with variety I from the north and II from the south) (Tsoi et al., 2007). Presumably variety II is well adapted to the new, more tropical environment and extends throughout the entire East Asia during the interglacial period. The variety I population from the Japan Sea were genetically different from the ones in ECS, TS and SCS (Tzeng et al., 2004). Moreover, the ECS was only reduced into an elongated trough next to the Pacific during the last glacial maximum (Wang and Sun, 1994). It is therefore presume that, the distribution of variety I gradually extended northwestwards from the west of Pacific (south of Japan) to the top of ECS and southwards to the north of SCS corresponding to the rise of the sea level of the ECS, TS and SCS.

An analysis of the demographic history of this shrimp from the two distinct clades (Clade I and II) seems to indicate that the Clade I (variety I) display a steeper wave, which is typical of a smaller initial population prior to the expansion or bottleneck (Figure 4) (Rogers and Harpending, 1992). This picture suggests that, the variety I could have a more recent past than the variety II, whose pair-wise distribution mode is more clearly displaced to the right of the distribution pattern (Figure 4).

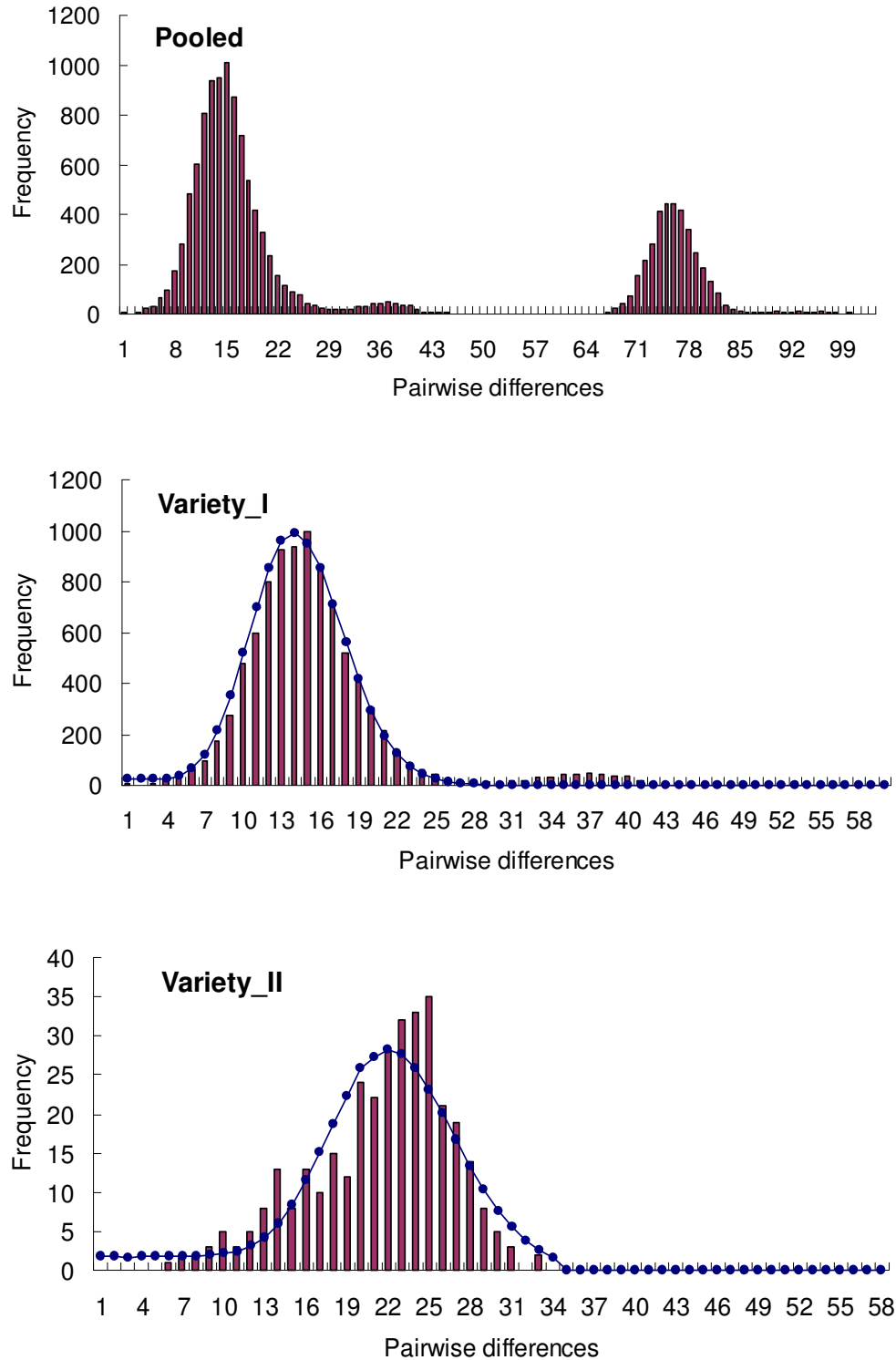


Figure 4. The observed pair-wise differences (bars) and the expected mismatch distributions under sudden expansion model (solid line) of haplotypes in kuruma shrimp (*P. japonicus*) species complex of China.

AMOVA did not detect significant differences at all hierarchical levels (Table 3), and all the conventional population F_{ST} statistics were not significant (Table 2),

indicating that, no significant population structure exists throughout the main distribution range of variety I of kuruma shrimp, this is consistent with findings of Tsoi et al.

(2007). However, the present result is different from the one obtained by Tzeng et al. (2004). This distinctness might result from small sample size used in previous study.

Conclusion

Results of this study indicate that, the variety II of *P. japonicus* had expanded into the East China Sea. The variety I of *P. japonicus* of China appears genetically homogeneous. The understanding of their genetic structure and demographic history are important in formulating knowledge-based fishery management and aquaculture development programs for this marine biological resource.

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