Chapter 8

# Genetic Diversity and Conservation of an Endemic Taiwanese Species, *Platyeriocheir formosa*

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Additional information is available at the end of the chapter

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## 1. Introduction

Mitten crabs (Brachyura, Varunidae, Varuninae) are native to east Asia and currently classified into eight species belonging to four genera: *Eriocheir* De Haan, 1835, with *E. japonica* (De Haan, 1835) and *E. ogasawaraensis* Komai et al., 2006; *Paraeriocheir* gen. nov., with *P. hepuensis* (Dai, 1991) and *P. sinensis* (H. Milne Edwards, 1853); *Platyeriocheir* Ng et al., 1999, with *P. formosa* (Chan et al., 1995) and *P. guangdonga* sp. nov.; and *Neoeriocheir* T. Sakai, 1983, with *N. leptognatha* (Rathbun, 1913); plus an eighth, currently nameless species with the status of a *species inquirenda* [1].

Catadromous mitten crabs have the unusual life history of spawning in the sea and growing up in rivers. Fertilized eggs hatch into zoea, which leave the female and begin life in the sea as plankton. After passing through five ecdysis cycles over a period of several weeks, they metamorphose into megalopa (post-larval stage) that live in estuaries and migrate upstream to freshwaters where a second metamorphosis, into juvenile crabs, occurs [2]. The larval stage drifts passively with coastal currents, providing high potential for gene flow within coastal waters. Larvae mainly drift in proximity to the coastline rather than the open sea, so long distance dispersal across open seas is restricted [3]. Juvenile crabs move into rivers and dwell in their middle or upstream reaches where they grow until adulthood. They generally inhabit clear rushing waters as well as hiding in rock crevices by day and coming out at night to feed, their main food being periphyton growing on rocks and aquatic vegetation. They spend most of their lifetime (1–3 years) in freshwater and migrate downstream to coastal waters when mature to mate and spawn [3].

Of this group, only two genera and species, *Paraeriocheir hepuensis* and *Platyeriocheir formosa*, are native to Taiwan. The distribution of *P. hepuensis* in Taiwan extends from Dasi, Yilan

County to Wu Stream, Taichung City. *P. formosa*, a Taiwan endemic, is mainly distributed in rivers of eastern Taiwan (Figure 1). Both species are of economic importance; nevertheless, neither have been artificially cultured from egg to maturity, and as a result, people catch large numbers of them from the wild for sale. Therefore, these wild populations must be able to tolerate intense exploitation. In addition, they also suffer other adverse impacts, such as habitat destruction, natural population declines, climatic oscillations, and so on. Too, many rivers were severely impacted when typhoon Morakot hit the eastern and southern portions of Taiwan in 2009 by mudflows that covered riverbeds, resulting in a significant decrease in the *P. formosa* population size. In addition, wild crab catches are insufficient to meet consumer demand. Farmers directly import juvenile crabs of *P. sinensis* from China for culture until they are grown to adults for sale. However, when *P. sinensis* escape from farms and invade Taiwan rivers, hybrids with native species can occur to degrade the genetic structure of native species. At present, populations of these two native species are dwindling in population size due to habitat destruction by natural disasters and unrestricted over-harvesting. Moreover, *P. sinensis* has also been introduced into Taiwan for short-term aquaculture, disregarding the consequences of potential threats to native species. It is clear that conservation of *P. formosa* should be taken more seriously.

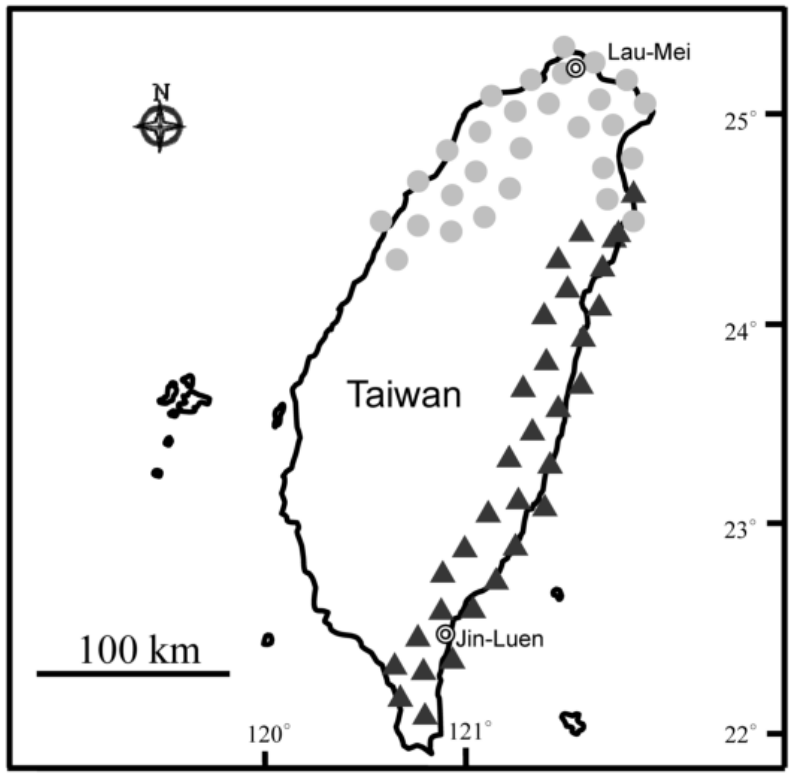


**Figure 1.** Map showing the distribution of two native mitten crabs in Taiwan and sampling sites. Black triangles indicated *Platyeriocheir formosa* and ashy circles indicated *Paraeriocheir hepuensis.*

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*P. formosa*, *P. hepuensis*, and *P. sinensis* have similar morphological characters. Consequently, species identification was clarified using a molecular marker, mitochondrial DNA cytochrome oxidase subunit I (COI), which is often used in taxonomy, biodiversity assessments, phylogenetics, and phylogeographic studies [4-8]. Here we discriminate these three species using mt COI sequences. Because microsatellites have large mutation rates of $10^{-5}$~$10^{-2}$ per generation [9-10], they are widely used as markers for studying genetics, population structure, kinship, and mating system [11-14]. In a previous study, the intraspecific genetic diversity of *P. formosa* was analyzed using COI sequences, with results showing insignificant genetic differences among samples from different streams [15]. To conserve *P. formosa* population diversity and ensure the sustainable use of this natural resource, its genetic diversity needed to be determined using polymorphic microsatellite loci.

This study attempted to distinguish *P. hepuensis*, *P. sinensis*, and *P. formosa,* and explored interspecies and intraspecific genetic diversity, using mt COI gene sequences. In addition, the genetic diversity of an endemic Taiwanese species, *P. formosa,* was examined by microsatellite loci. Effective management and conservative strategies also are proposed herein.

## 2. Experimental section

### 2.1. Sample collection

A total of 40 *Platyeriocheir formosa* specimens were collected in November 2010 from Jin-Luen, Taitung (120°55′ E, 22°32′ N), southeastern Taiwan. All *Paraeriocheir hepuensis* (n = 20) were collected from Lau-Mei Stream in New Taipei City and 2 *P. sinensis* individuals were collected from an aquaculture farm in Pingtung County.

### 2.2. Genomic DNA isolation

Muscle tissues from all specimens were preserved in 95% ethanol until DNA extraction. Genomic DNA was isolated and purified from the muscle tissue of all individuals. Five hundred milligrams of tissue with 1 mL lysis buffer was digested with 55 μL proteinase K solution. Small amounts of DNA were extracted for polymerase chain reactions (PCR) using a Puregene core kit A (Qiagen, Valencia, CA, USA).

### 2.3. COI subcloning and analysis

The complete COI gene was amplified using the specific forward primer 5′-CTCTAACR-GATTCCCCATCTTCTC-3′ and reverse primer 5′-ATCCTACACATCTGTCTGCC-3′ designed by the authors. A PCR consisted of approximately 50 ng genomic DNA, 50 pmol each of the forward and reverse primers, 25 mM dNTP, 0.05 ~ 0.1 mM $MgCl_2$, 10× buffer, and 5 U *Taq* polymerase (Takara Shuzo, Shiga, Japan), and brought up to 100 μL with Milli-Q water (Millipore, Billerica, MA, USA). The PCR program included one cycle of 4 min at 95 °C, 38 cycles of 1 min at 94 °C, 50 s at 50 °C, and 1 min at 72 °C, followed by a single further extension

of 10 min at 72 °C. We evaluated 8 μL of each product on 0.8% agarose gel to check PCR success and confirm product sizes. The remaining PCR products were run on 0.8% agarose gels and purified using a DNA Clean/Extraction kit (GeneMark, Taichung, Taiwan). Purified DNA was subcloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* JM109. Plasmid DNA was isolated using a mini plasmid kit (Geneaid, Taichung, Taiwan). Clones from all individuals were sequenced on an Applied Biosystems (ABI, Foster City, CA, USA) automated DNA sequencer 377 (ver. 3.3) using a Bigdye sequencing kit (Perkin-Elmer, Wellesley, MA, USA).

In total, 42 COI sequences were subcloned. All sequences were aligned using Clustal W [16] and then checked with the naked eye. Intraspecific and interspecific genetic distances and numbers of different nucleotides were calculated using MEGA software [17]. The interspecific variable site numbers and intraspecific nucleotide diversities were computed by DnaSP v5 [18]. The phylogenetic trees for COI sequences were constructed using neighbor-joining [19] and maximum parsimony methods [20]. Cluster confidence was assessed using a bootstrap analysis with 1000 replications [21]. The minimum spanning tree (MST) was computed from the matrix of pairwise distances between all pairs of haplotypes in each sample using a modification of the algorithm [22]. We evaluated whether sequences had evolved under strict neutrality. Fu's *Fs* [23] and Tajima's neutrality tests [24] were performed in Arlequin 3.1 [25]. The significance of the statistics was tested by generating random samples under the hypothesis of selective neutrality and population equilibrium, using a coalescent simulation algorithm [26]. Tajima's test is based on an infinite-site model without recombinations. A significant *D* value can be due to factors other than selective effects, like population expansion, a bottleneck, or heterogeneity of mutation rates [27]. The possible occurrence of historical demographic expansions was examined using the mismatch distribution [28] implemented in Arlequin [25]. The distribution is unimodal in samples following a population demographic expansion [29].

### 2.4. Genotyping and data analysis

All 18 microsatellite loci [30] were amplified in this study. A PCR was performed in a volume of 25 μL that included ~10 ng genomic DNA, 10 pmol reverse primer, 10 pmol forward primer, 25 mM dNTP, 0.05–0.1 mM $MgCl_2$, 10× buffer, and 0.5 U *Taq* polymerase (Takara Shuzo, Tokyo, Japan) with Milli-Q water. The PCR products were subjected to a 1.5% agarose gel and allele sizes were checked by comparison with a DNA ladder and the length of the original sequence. Forward primers were labeled with FAM, TAMRA, or HEX fluorescence markers. PCR amplifications were carried out in a Px2 Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) with the following temperature profile: 1 cycle of 95 °C for 4 min, followed by 38 cycles of 94 °C for 30 s, and annealing at 50–60 °C for 30 s and 72 °C for 30 s. Each 5 μL of PCR product from three loci labeled with different fluorescence tags was mixed and precipitated with 95% alcohol. Semi-automated genotyping was performed using a capillary ABI 3730XL DNA Analyzer (ABI). Genotypes were scored with GeneMapper 4.0 (ABI).

The total number of alleles (*na*) and effective allele numbers were estimated for each locus using Popgene [31]. Observed ($H_O$) and expected ($H_E$) heterozygosities were independently calculated for each locus. Deviations from Hardy-Weinberg expectations (HWEs) were

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examined by an exact test using GENEPOP [32]. Linkage disequilibrium among all pairs of loci was determined using Burrow's composite measure [33] and $\chi 2$ values.

## 3. Results and discussion

### 3.1. Interspecific diversity

Molecular systematics and historical population dynamics were also analyzed using mt COI gene sequences. The full-length of all 42 COI sequences in *Platyeriocheir formosa* (n = 20), *Paraeriocheir hepuensis* (n = 20), and *P. sinensis* (n = 2) are consistent with 1534 bp in length. The average percentage of nucleotide components consist of 64% A + T for *P. sinensis*, 63% for *P. hepuensis*, and 62.6% for *P. formosa,* with slight differences among these three species.

The numbers of interspecific nucleotide differences ranged 196 - 221 with an average of 205.08 ± 4.14 between *P. formosa* and *P. hepuensis*, 199 - 214 with an average of 204.82 ± 3.30 between *P. formosa* and *P. sinensis*, and 60 - 79 with an average of 66.55 ± 3.87 between *P. hepuensis* and *P. sinensis*. Some interspecific nucleotide variable sites are shown in Figure 2.

The interspecific genetic distances ranged 0.142 - 0.163 with an average 0.150 ± 0.006 for *P. formosa* vs. *P. hepuensis*, 0.144 - 0.157 with an average 0.148 ± 0.002 for *P. formosa* vs. *P. sinensis*, and 0.041 - 0.054 with an average 0.045 ± 0.003 for *P. hepuensis* vs. *P. sinensis*. All 42 sequences in the study and one outgroup sequence from *Xenograpsus testudinatus* (NCBI accession number NC013480) were used to construct a phylogenetic tree by neighbor-joining (NJ) and maximum-parsimony (MP) methods. Phylogenetic trees presented significant clustering among the three species indicated *P. formosa*, *P. hepuensis*, and *P. sinensis* are an individually monophyletic group and share a common ancestor in two genealogical trees (Figure 3a, b). *P. hepuensis* and *P. sinensis* have closer relationships than *P. formosa,* indicating that the former two share a common recent ancestor. In addition, we also concluded that the COI gene is an effective genetic marker for distinguishing these mitten crabs having similar morphological characteristics.

The complete COI gene can be translated into a 511 amino acid sequence. The number of different amino acids ranged 0 - 10 within *P. formosa* containing two identical sequences and 0 - 16 within *P. hepuensis* containing four identical sequences. Intraspecific genetic distances ranged 0 - 0.020 (mean, 0.009 ± 0.004) within *P. formosa* and 0 - 0.033 (mean, 0.009 ± 0.007) within *P. hepuensis*. Interspecific genetic distance ranged 0.020 - 0.050 (mean = 0.026 ± 0.007) between *P. formosa* and *P. hepuensis*, 0.012 - 0.027 (mean = 0.019 ± 0.004) between *P. formosa* and *P. sinensis*, and 0.012 - 0.037 (mean = 0.017 ± 0.005) between *P. hepuensis* and *P. sinensis*. The NJ tree constructed from amino acid sequences reveals that all three species belong to one monophyletic group (Figure 4). The amino acid sequences of *P. hepuensis* and *P. sinensis* expressed higher similarity, suggesting that they have a closer relationship than either has with *P. formosa*.

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[            1 1111111111 2222222222 2222222233 3333333333 3333334444 4444444444 ]
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PF02 .......... ...G....T. .......... .....C.... .......... ........A. ..........
PF05 ..G....... ...G...... .........C ..C..C.... .......... .......... A.........
PF06 .......... ...G...... .......... .....C.... .......... .......... ..........
PF07 .......... ...G...... .......... .....C.... .......... .......... ..........
PH01 A..A.T.CAA TACGCG...T G.CTG...C. GCAATGAGCA ATATT..ATC .ATAACCT.C AAATTTC.AC
PH02 A..A.T.CAA TA.GC....T G.CTG..... GCAATGA.CA ATATT..ATC .ATAACCT.C AAATTTC.AC
PH03 A..A.T.CAA TACGC....T G.CTG..CC. GCAATGA.CA ATATT..ATC .ATAACCT.C AAATTTC.AC
PH04 A..A.T.CAA TACGC....T G.CTG...C. GCAATGA.CA ATATT..ATC .ATAACCT.C AAATTTC.AC
PH05 A..A.T.CAA TACGC....T G.CTG...C. GCAATGA.CA ATATT..ATC .ATAACCT.C AAATTTC.AC
PS01 AT.ACTGCAA .ACG..AT.T .GCT.CC.C. .CAATAA.CA CTATTGA.TC CATAACCT.. AAATTTCCA.
PS02 AT.ACTGCAA .ACG..AT.T .GCT.CC.C. .CGATAA.CA CTATT...TC CATAACCT.. AAATTTCCA.

[    4444444444 4455555555 5555566666 6666666666 6666666777 7777777777 7777777788 ]
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PF02 ........A. ........T. .......G.. .......... .......... .......... ..........
PF05 ........A. ........T. .......G.. .......... .......... .......... ..........
PF06 ........A. ........T. .......... .......... .......... .......... ..........
PF07 ........A. ........T. .......G.. .......... .......... .......... ..........
PH01 ..TCTATTAT CGTTTGTATA ATCAT.C.G. TCGGT.TA.A CATACGCCC. CC..TCT..T ATAT..TTGG
PH02 ..TCTATTAT C.TTTGTATA ATCAT.C.G. TCGGT.TA.A .ATACGC.C. CC..TCT..T ATAT..TTGG
PH03 ..TCTATTAT C.TTTGTATA ATCATTC.G. TCGGT.TA.A CATACGC.C. CC..TCT..T ATAT..TTGG
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PS01 CATCTATTAT C..TAGTATA CTCATAC.G. TC.GTC.AGA CATACGC.CT CCCGTC..CT ATATGGTT..
PS02 CATCTATTAT C..TAGTATA C.CATAC.G. TC.GTC.AGA CATACGC.CT CC.GTC..CT ATATG.TT..

[            1 1111111111 2222222222 2222222233 3333333333 3333334444 4444444444 ]
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PF02 .......... ...G....T. .......... .....C.... .......... ........A. ..........
PF05 ..G....... ...G...... .........C ..C..C.... .......... .......... A.........
PF06 .......... ...G...... .......... .....C.... .......... .......... ..........
PF07 .......... ...G...... .......... .....C.... .......... .......... ..........
PH01 A..A.T.CAA TACGCG...T G.CTG...C. GCAATGAGCA ATATT..ATC .ATAACCT.C AAATTTC.AC
PH02 A..A.T.CAA TA.GC....T G.CTG..... GCAATGA.CA ATATT..ATC .ATAACCT.C AAATTTC.AC
PH03 A..A.T.CAA TACGC....T G.CTG..CC. GCAATGA.CA ATATT..ATC .ATAACCT.C AAATTTC.AC
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PH05 A..A.T.CAA TACGC....T G.CTG...C. GCAATGA.CA ATATT..ATC .ATAACCT.C AAATTTC.AC
PS01 AT.ACTGCAA .ACG..AT.T .GCT.CC.C. .CAATAA.CA CTATTGA.TC CATAACCT.. AAATTTCCA.
PS02 AT.ACTGCAA .ACG..AT.T .GCT.CC.C. .CGATAA.CA CTATT...TC CATAACCT.. AAATTTCCA.

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PF01 TGCTCGCGGC TACCCACTCG TCTCCCTATT CTAACTATAT TTCTTATTTC TTTACTCATC CGCGAACCAA
PF02 ........A. ........T. .......G.. .......... .......... .......... ..........
PF05 ........A. ........T. .......G.. .......... .......... .......... ..........
PF06 ........A. ........T. .......... .......... .......... .......... ..........
PF07 ........A. ........T. .......G.. .......... .......... .......... ..........
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PH03 ..TCTATTAT C.TTTGTATA ATCATTC.G. TCGGT.TA.A CATACGC.C. CC..TCT..T ATAT..TTGG
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PS01 CATCTATTAT C..TAGTATA CTCATAC.G. TC.GTC.AGA CATACGC.CT CCCGTC..CT ATATGGTT..
PS02 CATCTATTAT C..TAGTATA C.CATAC.G. TC.GTC.AGA CATACGC.CT CC.GTC..CT ATATG.TT..
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**Figure 2.** Partial interspecific variable sites within 12 COI sequences from *Platyeriocheir formosa, Paraeriocheir hepuensis* and *Paraeriocheir sinensis.*

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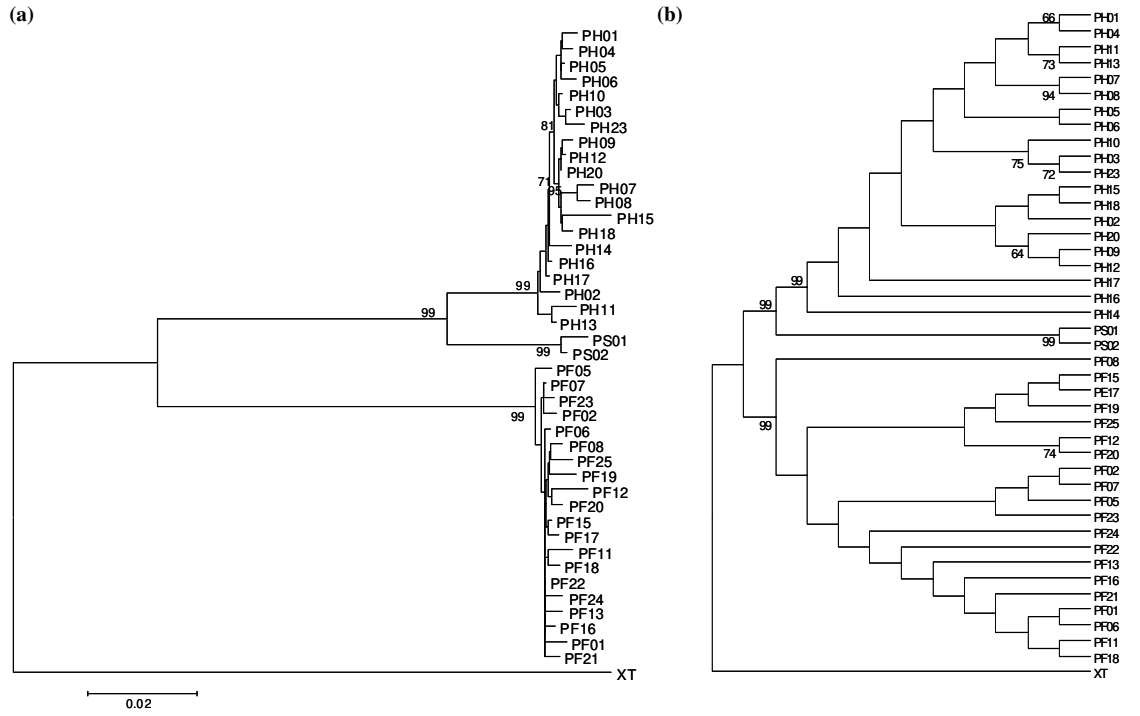


**Figure 3.** (a)Neighbor-joining tree and (b)Minimum parsimony tree constructed by 43 COI gene sequences from *Platyeriocheir formosa* (PF), *Paraeriocheir hepuensis* (PH) and *Paraeriocheir sinensis* (PS) and the outgroup *Xenograpsus testudinatus* (XT). Bootstrap values >60% (out of 1000 replicates) are shown at the nodes.

### 3.2. Intraspecific diversity and historical population dynamics

In total, 86 and 84 variable sites were respectively observed within intraspecific sequences of *P. formosa* and *P. hepuensis*. All 20 COI sequences from *P. formosa* contain seven highly variable sites (Figure 5a). The intraspecific number of nucleotide differences ranged 2 - 20. Haplotype diversity ($H_d$), the mean number of nucleotide differences (k), and mean nucleotide diversity ($\pi$) are 1, 9.73 ± 3.63, and 0.006 ± 0.003, respectively. A total of 86 substitutions containing 75 transitions and 11 transversions occur within these 20 sequences. There are 23 highly variable sites observed within 20 COI sequences of *P. hepuensis* (Figure 5b). Intraspecies nucleotide differences ranged 1 - 27. Haplotype diversity ($H_d$), the mean number of nucleotide differences (k), and mean nucleotide diversity ($\pi$) are 1, 11.36 ± 4.83, and 0.007 ± 0.004, respectively. The 85 substitutions include 68 transitions and 17 transversions. Intraspecific genetic distances of *P. formosa* ranged from 0.001 - 0.013, with an average of 0.006 ± 0.002. In contrast to *P. formosa*, *P. hepuensis* had similar intraspecific genetic distances that ranged from 0.001 to 0.018, with an average of 0.007 ± 0.003. Extremely high levels of $H_d$ and low to moderate levels of $\pi$ were discovered in these two species.

A similar genetic pattern is observed in many marine species [34]. The most likely explanation is that the accumulation of mutations over time in a rapidly growing population leads to an increase in the number of haplotypes; even so, population sizes suffer seriously when there is

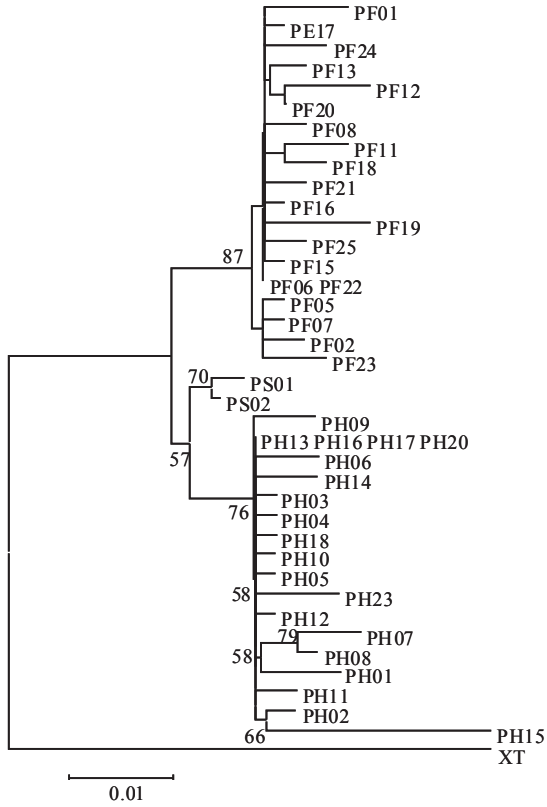


**Figure 4.** The neighbour-joining tree of COI amino acid sequences from *Platyeriocheir formosa* (PF), *Paraeriocheir hepuensis* (PH) and *Paraeriocheir sinensis* (PS) and the outgroup *Xenograpsus testudinatus* (XT). Numbers above the branches indicate the bootstrap values.

low genetic diversity. Except for wild mitten crabs, which must counteract overharvesting, probable natural causes include climate oscillations that result in temperature and water quality changes, mudflows covering riverbeds that can block migratory pathways, and any other environmental factor that affects adult reproduction and larval survival in estuaries. A second explanation for low levels of genetic diversity in these species could be due to their high dispersal potential during the planktonic egg and larval stages, resulting in strong gene flow among populations. Intraspecific genetic diversity in *P. formosa* analyzed by COI sequences indicate insignificant genetic differences among different populations [15]. It is interesting to note that *P. formosa* spawns in the sea and planktonic stage dispersal trends northward due to seasonal currents along the eastern Taiwan coast. This easily explains why *P. formosa* has low genetic variability among samples from different streams.

Four groups of marine fishes were defined based on the haplotype diversity ($h_d$) and nucleotide diversity ($\pi$) of various mtDNA coding regions [35]. The most widespread group possesses a high number of haplotypes ($H_d > 0.5$) and moderate to low levels of sequence divergences ($0.4\% < \pi < 0.8\%$). *P. formosa* and *P. hepuensis* were found in this study to have high haplotype diversity ($H_d = 1$) and moderate nucleotide diversities (0.6% & 0.7%), which fit the most common pattern

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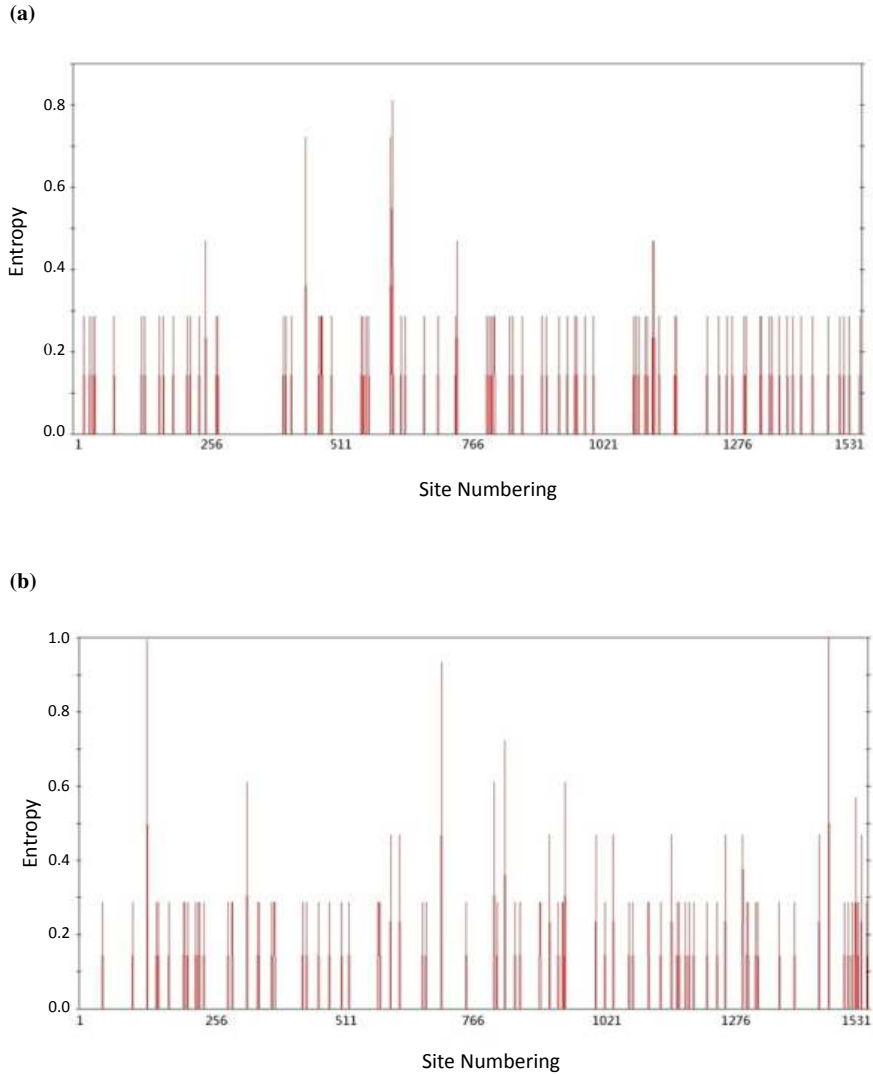


**Figure 5.** Seven and 23 highly variable sites observed within COI sequences. (a) *Platyeriocheir formosa* (b) *Paraeriocheir hepuensis*.

observed in marine fishes. High haplotype diversity within regional populations can be maintained through historically rapid population increases, resulting in the accumulation of mutations in populations [36]. Nevertheless, the shallow mtDNA branch structure of the NJ tree in these two mitten crabs might have resulted from catastrophic reductions in population size, which would produce low values of $\pi$.

The *D* values of Tajima's *D* neutral tests were analyzed to test this. *P. formosa* and *P. hepuensis* were -2.451 ($p$ <0.001) and -2.129 ($p$ <0.003), respectively. Negative Fu's *F*s values of -12.646 ($p$ = 0) in *P. formosa* and -11.333 ($p$ = 0) in *P. hepuensis* suggested that these two species experienced a recent population expansion event. The mismatched distribution analysis presented average intraspecific nucleotide differences among COI sequences of 9.726 ± 7.731 in *P.*

*formosa* and 11.358 ± 6.344 in *P. hepuensis,* which were unimodally distributed (Figure 6) and indicated that the population experienced a historical expansion event. One and two central haplotypes were found in the minimum spanning tree (MST) of *P. formosa* and *P. hepuensis,* and most of the haplotypes were located at the tips (Figure 7), implying that adaptive radiation occurred.

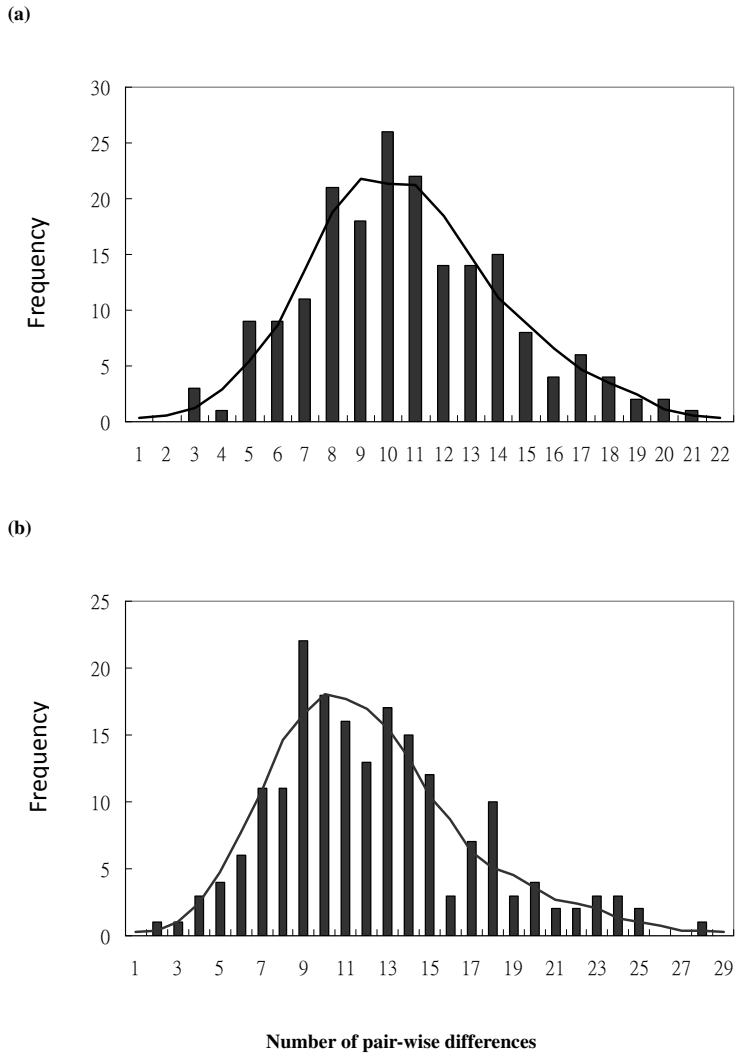


**Figure 6.** Mismatch distributions obtained from mtDNA COI data. The bars of the histogram represent the observed pairwise differences. The curve is the expected distribution under the sudden expansion model. (a) *Platyeriocheir formosa* (b) *Paraeriocheir hepuensis*.

Allele numbers and the effective allele numbers of all 18 microsatellite loci in *P. formosa* ranged from 3 - 14 and 2.25 - 10.26, respectively. Allele sizes within these loci ranged from 68 to 239 bp in length. The allele sizes of three loci (Pfo-15, -31, and -34) were all shorter than 100 bp (Table 1). Heterozygous individuals have been found at all loci except for Pfo-15. When

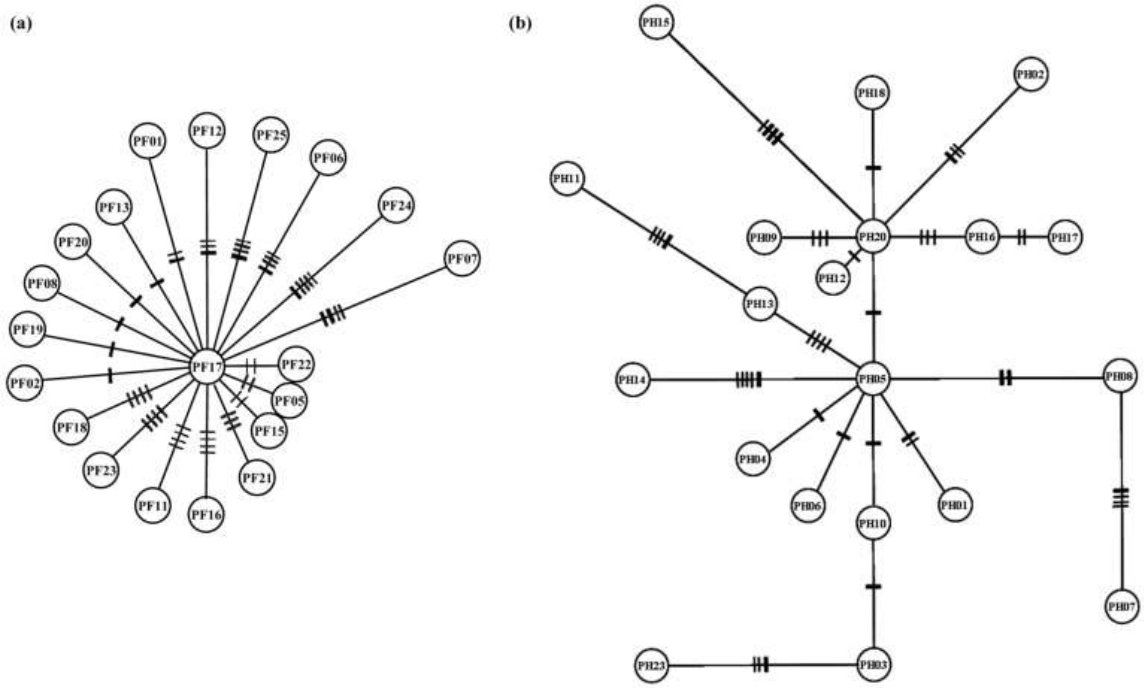


**Figure 7.** Minimum spanning tree constructed from COI data. (a) *Platyeriocheir formosa* (b) *Paraeriocheir hepuensis*.

excluding the homozygous Pfo-15 locus, the observed and expected heterozygosities ($H_O$ & $H_E$) ranged from 0.20 - 0.95 (mean = 0.55) and 0.57 - 0.93 (mean = 0.818), respectively. Thirteen of these loci departed from the Hardy-Weinberg (HW) equilibrium, suggesting that *P. formosa* suffers from an intense inbreeding effect, bottleneck, or other possibility. Burrow's composite measure for linkage disequilibrium (LD) among the 18 loci was estimated for the entire dataset. The total variance of interlocus allele disequilibrium ($D_{IT}^2 = 0.018$) was slight. It is unlike the marine swimming crab, *Portunus trituberculatus,* which has a significantly higher mean heterozygosity ($H_O > 0.8$), while a similar $H_O$ (0.55) was found in the catadromous *P. sinensis* [37]. The mean allele number per locus (*na*) in the catadromous *P. formosa* was estimated to be 9.61, which was higher than that of *P. sinensis* on average (*na* = 4.94), but lower than that of the marine *P. trituberculatus* (*na* = 22) [37-38]. These results agree with the observation that catadromous *P. formosa* have lower genetic diversity than marine *P. trituberculatus* species.

| Microsatellite locus | Major repeats | $T_a$ (°C) | Allelic size range (bp) | *na* | *ne* | $H_O/H_E$ | NCBI accession no. |
|---|---|---|---|---|---|---|---|
| PFO-4 | $(CA)_{29}$ | 56 | 145-183 | 13 | 6.45 | 0.75/0.87 | JQ582816 |
| PFO-5 | $(TC)_6$ | 54 | 141-149 | 3 | 2.25 | 0.55/0.57 | JQ582817 |
| PFO-7 | $(GT)_{22}$ | 52 | 217-239 | 10 | 6.06 | 0.65/0.86 | JQ582818 |
| PFO-9 | $(CA)_{31}$ | 54 | 156-194 | 14 | 9.41 | 0.40*/0.92 | JQ582819 |
| PFO-10 | $(CA)_{10}$ | 50 | 94-128 | 8 | 7.27 | 0.65/0.88 | JQ582820 |
| PFO-12 | $(CA)_{32}$ | 56 | 186-216 | 14 | 7.84 | 0.45*/0.89 | JQ582821 |

| Microsatellite locus | Major repeats | $T_a$ (°C) | Allelic size range (bp) | *na* | *ne* | $H_O/H_E$ | NCBI accession no. |
|---|---|---|---|---|---|---|---|
| PFO-15 | $(GT)_{16}$ | 60 | 68-76 | 5 | 2.99 | 0.00*/0.68 | JQ582822 |
| PFO-18 | $(CA)_{33}$ | 60 | 134-170 | 13 | 6.84 | 0.35*/0.88 | JQ582823 |
| PFO-19 | $(CA)_{32}$ | 50 | 141-175 | 13 | 9.20 | 0.25*/0.91 | JQ582824 |
| PFO-31 | $(CA)_{17}$ | 52 | 79-93 | 6 | 2.74 | 0.35*/0.65 | JQ582825 |
| PFO-34 | $(CA)_{20}$ | 50 | 71-77 | 3 | 2.38 | 0.70*/0.59 | JQ582826 |
| PFO-36 | $(CA)_{35}$ | 54 | 101-125 | 10 | 6.11 | 0.50*/0.86 | JQ582827 |
| PFO-37 | $(CA)_{31}$ | 50 | 166-200 | 13 | 8.79 | 0.90/0.91 | JQ582828 |
| PFO-51 | $(CA)_{18}$ | 58 | 85-101 | 8 | 2.94 | 0.20*/0.68 | JQ582829 |
| PFO-52 | $(CA)_{12}$ | 50 | 97-109 | 6 | 4.10 | 0.95*/0.78 | JQ582830 |
| PFO-54 | $(GT)_{18}$ | 50 | 141-173 | 10 | 5.52 | 0.70*/0.84 | JQ582831 |
| PFO-60 | $(GT)_{17}$ | 50 | 118-146 | 11 | 7.62 | 0.60*/0.89 | JQ582832 |
| PFO-79 | $(GT)_{26}$ | 52 | 113-143 | 13 | 10.26 | 0.50*/0.93 | JQ582833 |

**Table 1.** Characterization of the core region and levels of genetic variation at 18 microsatellite loci from *Platyeriocheir* formosa. $T_a$, PCR annealing temperature; *na*, observed number of alleles detected at each locus; *ne*, effective number of alleles; $H_O$, observed heterozygosity within a sample; $H_E$, expected heterozygosity within a sample. *significant Hardy-Weinberg deviation ($p$ <0.05).

*P. formosa* and *P. hepuensis* have a peculiar migratory history. Juvenile crabs migrate from the sea into rivers where they grow until adulthood. Mature adults move from their habitats in middle and upstream river reaches down to coastal waters for reproduction. Consequently, the most important conservation considerations are high water quality, freely flowing channels, reduced harvesting by humans, and preventing the invasion of the exotic *P. sinensis*. Focus must start on the catadromous journey of juvenile crabs from estuaries, and good water quality is the key to their survival and sustainable populations. Secondly, uninterrupted river flows are necessary, as mitten crabs must migrate throughout rivers in order to complete their full life cycle regardless of whether they are upstream-swimming juveniles or downstream-swimming adults. When river bottoms are buried under mudflows, previously established aquatic organisms are lost. However, temporarily created waterways will allow aquatic organisms to survive and complete their life cycles. Furthermore, overfishing results in decreasing crab resources and increases in their selling prices which results in additional overharvesting. However, capture can be banned for temporary periods of time to allow populations to recover. Finally, *P. sinensis* must be prevented from invading Taiwan's rivers. Because there are insufficient harvests of *P. formosa* and *P. hepuensis* to meet human demand, aquaculturists import large numbers of juvenile *P. sinensis* crabs and raise them to adulthood for sale. However, there are some problems with the culturing process. For one thing, *P. sinensis* effortlessly escapes from aquaculture even though ponds are equipped with anti-slipping nets. What is more, market prices may collapse if supply exceeds demand, and the industry might give up raising crabs and dump them. It is, therefore, certainly possible that these non-native crabs might colonize all of Taiwan's streams, causing an ecological catastrophe to native Taiwanese crab populations. Preventing the invasion of non-native mitten crabs should be of universal concern to Taiwan crab management and conservation.

The genetic structure and population dynamics of Taiwan's *P. formosa* population diversity must be continually monitored to ensure the sustainable use of this valuable natural resource.

## 4. Conclusions

The interspecific and intraspecific genetic diversity of two native Taiwanese mitten crabs, *P. formosa* and *P. hepuensis,* were determined in this study using the mtDNA COI gene and microsatellite loci. These two species possess similar genetic patterns with extremely high haplotype diversity and low to moderate nucleotide diversity. These results suggest that their population sizes historically underwent expansions but are currently undergoing serious decreases. Consequently, a conservation policy is proposed here that includes maintaining free-flowing stream channels and good water quality, preventing overharvesting by limiting harvesting to specific seasons, preventing the establishment of non-native mitten crabs, and conducting research on improved methods for the aquaculture of native mitten crabs.

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## References

[1] Sakai K. A review of the genus *Eriocheir* De Haan, 1835 and related genera, with the description of a new genus and a new species (Brachyura, Grapsoidea, Varunidae). Crustaceana 2013;86:1103-38

[2] Shy JY, Yu HP. Complete larval development of the mitten crab *Eriocheir rectus* Stimpson, 1858 (Decapoda, Brachyura, Grapsidae) reared in the laboratory. Crustaceana 1992;63: 277-90

[3] Xu J, Chan TY, Tsang LM, Chu KH. Phylogeography of the mitten crab *Eriocheir sensu stricto* in East Asia: Pleistocene isolation, population expansion and secondary contact. Molecular Phylogenetics and Evolution 2009;52: 45-56

[4] Thaler AD, Plouviez S, Saleu W, Alei F, Jacobson A, Boyle EA, Schultz TF, Carlsson J, Dover CLV. Comparative population structure of two deep-sea hydrothermal-vent-associated decapods (*Chorocaris* sp. 2 and *Munidopsis lauensis*) from Southwestern Pacific Back-Arc Basins. PLoS One 2014; 9(7) e101345. doi: 10.1371/journal.pone.0101345

[5] Zhan A, Bailey SA, Heath DD, Macisaac HJ. Performance comparison of genetic markers for high-throughput sequencing-based biodiversity assessment in complex communities. Molecular Ecology Resources 2014;14(5) 1049-59.

[6] Korn M, Rabet N, Ghate HV, Marrone F, Hundsdoerfer AK. Molecular phylogeny of the Notostraca. Molecular Phylogenetics and Evolution 2013;69(3) 1159-71. doi: 10.1016/j.ympev.2013.08.006

[7] Cornils A, Blanco-Bercial L. Phylogeny of the Paracalanidae Giesbrecht, 1888 (Crustacea: Copepoda: Calanoida). Molecular Phylogenetics and Evolution 2013;69(3) 861-72. doi: 10.1016/j.ympev.2013.06.018

[8] Fernández MV, Heras S, Viñas J, Maltagliati F, Roldán MI. Multilocus comparative phylogeography of two aristeid shrimps of high commercial interest (*Aristeus antennatus* and *Aristaeomorpha foliacea*) reveals different responses to past environmental changes. PLoS One 2013;8(3) e59033. doi: 10.1371/journal.pone.0059033

[9] Weber JL, Wong C. Mutation of human short tandem repeats. Human Molecular Genetics 1993;2: 1123-8

[10] Ellegren H. Mutation-rates at porcine microsatellite loci. Mammalian Genome 1995;6: 376-77

[11] Cristescu MEA, Colbourne JK, Radivojac J, Lynch M. A microsatellite-based genetic linkage map of the waterflea, *Daphnia pulex*: on the prospect of crustacean genomics. Genomics 2006;88: 415-30

[12] Kang JH, Park JY, Kim EM, Ko HS. Population genetic analysis and origin discrimination of snow crab (*Chionoecetes opilio*) using microsatellite markers. Molecular Biology Reports 2013;40(10) 5563-71

[13] Bentzen P, Olsen JB, McLean JE, Seamons TR, Quinn TP. Kinship analysis of Pacific salmon: insights into mating, homing, and timing of reproduction. Journal of Heredity 2001;92: 127-36

[14] Jensen PC, Bentzen P. A molecular dissection of the mating system of the Dungeness crab, *Metacarcinus magister* (Brachyura: Cancridae). Journal of Crustacean Biology 2012;32(3) 443-56

[15] Shiao CY. The genetic diversity of *Eriocheir formosa* (Crustacea, Decapoda, Grapsidae). Master Thesis. Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan: 2007.

[16] Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research 1994;22(22) 4673-80. doi: 10.1093/nar/22.22.4673

[17] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution 2013;30(12) 2725-2729. doi:10.1093/molbev/mst197

[18] Librado P, Rozas J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 2009;25: 1451-2

[19] Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 1987;4: 406-25

[20] Fitch WM. On the problem of discovering the most parsimonious tree. The American naturalist 1977;111: 223-57

[21] Hillis DM, Bull JJ. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Systematic Biology 1993;42(2) 182-92. doi: 10.1093/sysbio/42.2.182

[22] Rohlf FJ. Algorithm 76. Hierarchical clustering using the minimum spanning tree. Computer Journal 1973;16: 93-5

[23] Fu YX. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 1997; 147: 915-25

[24] Tajima F. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 1989;123: 585-95

[25] Excoffier L, Laval LG, Schneider S. Arlequin vers. 3.0: an integrated software package for population genetics data analysis. Evolutionary Bioinformatics 2005;1: 47-50

[26] Hudson RR. Genealogies and the coalescent process. In: Furuyama D and Antonovics J (eds.). Oxford Surveys in Evolutionary Biology. New York: Oxford University; 1990

[27] Tajima F. The amount of DNA polymorphism maintained in a finite population when the neural mutation rate varies among sites. Genetics 1996;143: 1457-65

[28] Schneider S, Excoffier L. Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: application to human mitochondrial DNA. Genetics 1999;152: 1079-89

[29] Harpending H. Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. Human Biology 1994;66: 591-600

[30] Cheng HL, Lee YH, Hsiung DS, Tseng MC. Screening of new microsatellite DNA markers from the genome of *Platyeriocheir formosa*. International Journal of Molecular Sciences 2012;13: 5598-606

[31] Yang RC, Yeh FC. Multilocus structure in *Pinus contoria* Dougl. Theoretical and Applied Genetics 1993; 87: 568-76

[32] Raymond M, Rousset F. GENEPOP (version 1.2) population genetic software for exact tests and ecumenicism. Journal of Heredity 1995;86: 248-9

[33] Cockerham CC, Weir BS. Quadratic analyses of reciprocal crosses. Biometrics 1977; 33: 187-203

[34] Bowen BW, Meylan AB, Ross JP, Limpus CJ, Balazs GH, Avise JC. Global population structure and natural history of the green turtle (*Chelonia mydas*) in terms of matriarchal phylogeny. Evolution 1992; 46: 865-8l

[35] Grant WS, Bowen BW. Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. Journal of Heredity 1998;89: 415-26

[36] Rogers AR, Harpending H. Population growth makes waves in the distribution of pairwise genetic differences. Molecular Biology and Evolution 1992;9: 552-69

[37] Gao XG, Li HJ, Li YF, Sui LJ, Zhu B, Liang Y, Liu WD, He CB. Sixteen polymorphic simple sequence repeat markers from expressed sequence tags of the Chinese mitten crab *Eriocheir sinensis*. International Journal of Molecular Sciences 2010; 11: 3035-38

[38] Xu Q, Liu R. Development and characterization of microsatellite markers for genetic analysis of the swimming crab, *Portunus trituberculatus*. Biochemical Genetics 2011;49: 202-12