Faculty of Resource Science and Technology

PRELIMINARY STUDY OF MORPHOLOGICAL AND GENETIC DIVERSITY
ON ASIAN GREEN MUSSEL (Perna viridis) IN SANTUBONG, SARAWAK

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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree qualification of any other university or institution of higher learning.

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<td>µl</td>
<td>Microliter</td>
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<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cethyl-trimethyl Ammonium Bromide</td>
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<td>COI</td>
<td>Cytochrome Oxidase I</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonuceic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diaminetetra-acetic acid</td>
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<td>EtBr</td>
<td>Ethidium Bromide</td>
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<td>EtOH</td>
<td>Ethanol</td>
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<td>MgCl₂</td>
<td>Magnesium Chloride</td>
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<td>Mitochondria DNA</td>
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<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>RAPD</td>
<td>Random Amplified Polymorphic DNA</td>
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<td>RAM</td>
<td>Random Amplified Microsatellite</td>
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<td>rpm</td>
<td>Round per minutes</td>
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<td>S</td>
<td>Seconds</td>
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<td>TBE</td>
<td>Tris-borate-EDTA</td>
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Preliminary Morphological and Genetic Diversity on Asian Green Mussel (*Perna viridis*) in Santubong, Sarawak based on Cytochrome Oxidase I (COI) Gene Analysis

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**ABSTRACT**

The Asian green mussels (*Perna viridis*) belonging to the Family Mytilidae plays important role due to its commercial value and feeding behaviour. Up to October 2013, there is no genetic information Cytochrome Oxidase I (COI) gene of *P. viridis* from Malaysia deposited in the GenBank. Thus, this study aims to sequence COI gene of *P. viridis* collected in Santubong, Sarawak and study the morphology and genetic diversity of *P. viridis* sampled from Santubong based on COI gene sequences analysis. A total of 12 Asian green mussels were subjected to assessment of morphological characteristics. Detail of the colour shell, shape and range shell morphology of all samples are similar to *P. viridis*. Approximately 550bp COI gene had been successfully sequenced. The genetic divergence values between *P. viridis* in Santubong, Malaysia and China ranged from 0% to 0.3%. While genetic divergence between *P. viridis* and other *Perna* sp. ranged from 60.2% to 60.5%. The topologies of all constructed phylogenetic tree based on COI gene showed that *P. viridis* is monophyletic since all *P. viridis* were grouped together forming one clade with 100% bootstrap value. This study involved only limited samples thus could be regarded as preliminary study only.

Keyword: *Perna viridis*, Cytochrome Oxidase I, genetic divergence, bootstrap value

**ABSTRAK**

Asian green mussel (*Perna viridis*) milik kepada Family Mytilidae memainkan peranan penting kerana mempunyai nilai komersial dan cara permakanan. Sehingga bulan Oktober 2013, tidak ada maklumat genetik Cytochrome Oxidase I (COI) gen *P. viridis* dari Malaysia. Oleh itu, kajian ini bertujuan untuk urutan gen COI dari *P. viridis* di Santubong dan mengkaji morfologi dan kepelbagaian genetik *P. viridis* sampel dari Santubong berdasarkan COI analisis jujukan gen. Sejumlah 12 individul Asian green mussel digunakan bertujuan untuk mengenal pasti ciri-ciri morfologinya. Ciri morfologi semua sampel menunjukkan sama seperti *P. viridis*. Kira-kira 500bp gen COI telah berjaya urutan. Pencaran genetik antara *P. viridis* dari Santubong, Malaysia dan China adalah di antara 0 % kepada 0.3%. Sementara, perbezaan genetik antara *P. viridis* dan species *Perna* lain adalah di antara 60.2 % kepada 60.5 %. Topologi semua pokok filogenetik dibina berdasarkan gen COI menunjukkan bahawa sampel *P. viridis* adalah monophyletic kerana semua *P. viridis* telah dikumpulkan bersama-sama membentuk satu klad dengan 100 % nilai bootstrap. Kajian ini hanya melibatkan beberapa sampel. Oleh itu ia hanya dijadikan permuakan kajian sahaja. Katakunci: *Perna viridis*, Cytochrome Oxidase I, perbezaan genetik, nilai Bootstrap
1.0 Introduction

*Perna viridis* is a Bivalvia belonging to the Family Mytilidae Linnaeus 1758. This mussel is under Order Mytiloida. Within the genus *Perna*, four species exist namely the *P. viridis* Linnaeus, 1758, *P. perna* Linnaeus, 1758, *P. indica* Gmelin, 1791 and *P. picta* Born, 1780. English common name for *P. viridis* are Asian green mussel and green-lipped mussel. In Malaysia local people called Asian green mussel by many names for example the Malay call them ‘siput sudu’, Indian called them ‘kallumalkai’ and ‘kadukka’.

*P. viridis* is an oviparous and dioecious whose life cycle exhibits a long larval dispersal period and a sedentary adulthood (Rosell, 1991). Sexual reproduction occurs when gametes are released into the water column where fertilization takes place. The pelagic larval stages last for about 3 weeks, providing many opportunities for larval dispersal and thereby promoting gene flow (Yamanaka & Fujio, 1984).

*P. viridis* is commercially cultivated in several countries and its aquaculture has been reviewed by Vakily (1989). According to Spencer (2002) the mussels in the tropics is mainly *P. viridis*, which is cultivated in India, Indonesia, the Philippines, Singapore, Thailand and Malaysia. *P. viridis* has been attracting lot of attention not only as a potential organism for commercial cultivation (Parulekar *et al*. 1982), but also as a serious pest organism in cooling water conduits of marine industries (Rajagopal *et al*. 1996, 2003). According to Monirith *et al*. (2003), *P. viridis* also used as a biomonitoring agent for heavy metal contamination in various Asian countries. In Malaysia, *P. viridis* has been proposed by Ismail *et al*. (2000) as a potential biomonitoring agent for heavy metal contamination in the Straits of Malacca; which is one of the busiest shipping lanes in the world.
Based on literature search, there is no genetic information Cytochrome Oxidase I (COI) gene of *P. viridis* from Malaysia. Thus, this study aims to (1) document detail morphology of *P. viridis* in Satubong (2) sequence COI gene from *P. viridis* in Santubong (3) study the genetic diversity of *P. viridis* sampled from Santubong based on COI gene sequences analysis.
Literature Review

2.1 Taxonomy of Asian Green Mussel

Asian green mussel belongs to Genus *Perna* of the Family Mytilidae. The Family Mytilidae is under Order Mytiloida. Taxonomy of Asian green mussel was stated by Linnaeus 1758 as below:

**Kingdom:** Animalia

**Phylum:** Mollusca

**Class:** Bivalvia

**Subclass:** Pteriomorphia

**Order:** Mytiloida

**Superfamily:** Mytiloidea

**Family:** Mytilidae

**Genus:** *Perna*

**Species:** *P. viridis* (Linnaeus, 1758)

*P. perna* (Linnaeus, 1758)

*P. picta* (Born, 1780)

*P. indica* (Gmelin, 1791)
2.2 Morphology of Asian Green Mussel

Rajagopal et al. (2006) had described the Asian green mussel in detailed. *P. viridis* is typically have two hinged shells closed by one or two adductor muscles. A strong ligament holds the two valves together at the hinge. The foot is generally laterally compressed with no operculum. *P. viridis* is characterized by narrow, elliptical, fan-shaped, thin valves which are of the same size; the absence of prominent hinge teeth; anterior adductor muscle reduced or absent; and often the presence of byssal threads for anchoring to hard substrates. The shell of *P. viridis* is bright green as a juvenile, fading to brown with green edges as it matures. The inner shell surface is bluish green and smooth, and the adductor muscle is kidney shaped. For juvenile, their shells can reach 8 cm to 10 cm in length and adults can reach 15 cm in shell length.

2.3 Distribution and Ecology

The native range of *P. viridis* stretches across the Indo-Pacific encompassing the Persian Gulf, India, Malaysia, Papua New Guinea and the South Pacific islands, and north to Japan (Sivalingam, 1977; Siddall, 1980; Vakily, 1989; Cheung, 1993). In Malaysia, they are widely distributed along the western coast of Peninsular Malaysia (Ismail et al., 2000) as well as in Sabah and Sarawak coasts but lack of proper documentation of this species are available. According to Hicks & Tunnell (1993), *P. viridis* have been vigorously invading new geographical regions. The introduction of *P. viridis* to new areas has been probably caused by international shipping, either as adults attached to ship hulls or as larvae in ballast water tanks (Hicks & Tunnell, 1993).
They generally inhabit marine intertidal, subtidal and estuarine environments with high salinity. The mussels form a thick carpet like growth on rocky surfaces and submerged structures like wharves, pilings, breakwaters and buoys (Huang et al., 1983 & Rao, 1990). These mussels attach to surfaces by byssus threads colonizing submerged rocks, wood, concrete, metal, old submerged logs, boats, PVC pipes, ropes, muddy sea bottoms and even seagrass beds and mangrove prop roots (Vakily, 1989; Agard et al., 1992; Rajagopal et al., 1997). One of the main reasons for the extraordinary invasive ability of the Asian green mussel is its tolerance to a wide range of environmental conditions. The mussel is quite hardy and individuals have been reported to do well in artificial seawater for more than 6 months. The green mussel is euryhaline and an optimal salinity range has been reported as 27-33 ppt (Nishida et al., 2003).

According to Bayne (1976), natural enemies of mussels fall into four categories: predators, competitors, parasites and shell borers. Algae, hydroids, free and tubiculous polychaetes, barnacles, amphipods and ascidians are important pests which colonise the outer surface of shell valves of *P. viridis* and compete for space. Cheung et al. (2004) studied the defensive responses of *P. viridis* on being challenged with two predators, the muricid gastropod, *Thais clavigera*, and the portunid crab, *Thalamita danae*. They observed that responses of the mussels were predator-specific. Mussels raised in the presence of crabs developed thicker shell at the umbo and lip margin, while those raised in the presence of gastropods had a thicker shell lip.

In *P. viridis*, the sexes are separated and fertilization is external. The spawning behaviour of *P. viridis* differs considerably (Vakily, 1989). According to Stephen and Shetty (1981), spawning is initiated by either sex, resulting in the release of streams of gametes into the
water and may also be induced by the presence of other spawning individuals in the area or also by a drop in salinity. Rajagopal et al. (1998) reported that there is an increase of settlement of *P. viridis* under high flow conditions. The high settlement intensity was attributed to enhanced propagule flux rate to the substratum, because of increased water flow. According to Neitzel et al. (1984), mussel larvae are capable of settling at high water velocities and it is reported that at velocities as high as 3.5 m s\(^{-1}\), mussels could settle and colonise new surfaces. The growth rate of green mussels is high compared to other species of mussel (Shafee, 1979).

As filter-feeders, they feed actively on particulate material in seawater with their frilled siphons. This mollusc is large, with shells typically reaching 8 to 10 cm in length and occasionally growing larger than 16 cm (Rajagopal et al., 2005).

### 2.4 Genetic Studies based on Cytochrome Oxidase I (COI) gene analysis

According to the Li et al. (2012) the complete mitochondrial (mt) genome of the *P. viridis* is 16,627 bp. *P. viridis* has a comparatively highest overall A+T content (68\%) among six available genomes of marine mussels to date. Comparison of the gene order demonstrated that the six marine mussels share no identical gene blocks although they belong to the same family, which indicates that this group should be a good model to study mtDNA evolution and mitochondria inheritance.

In the Genbank, there are 38 gene data belonging to *P. viridis* and they were deposited from many countries. COI gene was analysed by Divya et al. (2009) to resolve the taxonomic ambiguity among Indian marine mussel species. A total of 477 bp of aligned sequence of COI gene from two individuals each of green, brown and parrot mussel were used for the comparative study. It had been suggested that *P. indica*, which occurs only along the Indian
coast, is a synonym of the globally distributed *P. perna*. Along the south-west coast of India, where both *P. viridis* and *P. indica* co-exist, a third type existed referred to as parrot mussel. Result showed the sequence divergence between *P. indica* and parrot mussel was negligibly low (< 2%). *P. viridis* showed 20.87% of sequence divergence with *P. indica* as well as with the parrot mussel.

Gene flow is a process where genetic exchange occurs because fertilise individuals or gamates migrate within population (Campbell & Reece, 2002). A study conducted by Sotka (2004) based on COI gene and nuclear (elongation factor 1-alpha) loci showed significant differences in acorn barnacle (*Balanus glanudula*) collected across 475 km of California coastline between northern and southern populations.

Kojima (1995) also use Cytochrome Oxidase I gene to conduct phylogenetic studies on seven species of giant clam Calyptogena (Bivalve: Vesicomyidae). The results indicated that three species of Calyptogena (*C. kaikoi, C. phaseoliformis* and *C. fausta*) were phylogenetically different from the other four species (*C. soyoae, C. nautili, C. solidisima Calyptogena* sp.).

### 2.5 Genetic Studies of *Perna viridis* in Malaysia

Molecular genetic markers such as allozymes, Random Amplified Polymorphic DNA (RAPD) and Random Amplified Microsatellite (RAM) have been used to elucidate genetic information relating to local populations of *P. viridis*. Results based on allozymes support the use of *P. viridis* as a biomonitoring agent for heavy metal contamination in the Malacca straits (Yap et al. 2002). However, Yap et al. (2004) reported that there is a distinct genetic variation between *P. viridis* populations collected from contaminated and uncontaminated sites, in which a population from a contaminated site showed an excess of heterozygosity.
when compared to those of the populations from three uncontaminated sites. Moreover, a study by Chua et al. (2003) based on RAPD and RAM markers showed clustering of populations that differed from those derived from the use of allozyme marker data. Thus, a study was carried out to validate whether local populations of *P. viridis* collected from the coastal waters of peninsular Malaysia are genetically similar enough to be used as a biomonitoring agent for heavy metal contamination in the Straits of Malacca by using the more informative single locus DNA microsatellite markers compared to the findings by using allozymes (Yap et al. 2002). The analysis revealed low genetic variation within and among the 10 populations of *P. viridis* and this supports the use of local populations of *P. viridis* as a suitable biomonitoring agent for heavy metal contamination in the Straits of Malacca.

The feasibility of such studies is always limited by the lengthy and labour-intensive procedure of DNA isolation from the species. Realizing the need for a rapid and simple procedure in DNA isolation from marine species particularly in bivalve, a number of studies have reported on the development of a rapid procedure for DNA extraction (Banerjee et al., 1995; Estoup et al., 1996; Nelson et al., 1998; Taris et al., 2005; Aranishi & Okimoto, 2006). Chai et al. (2009) had developed a rapid, simple and inexpensive method for DNA isolation from the mantle of *P. viridis* for PCR amplification of RAM. The developed protocol was tested on frozen and ethanol-preserved mussels and the result shows both frozen and ethanol-preserved samples yielded comparable amounts of DNA. Previous studies have reported successful PCR amplification using standard Chelex-100 resin method (Chelex 100 combined with Proteinase K digestion) for DNA isolation from ark shells and scallops (Steiner & Muller, 1996), larvae and juveniles of oysters (Launey & Hedgecock, 2001) and
oyster parasites (Ko et al., 1999). Chai et al., (2009) however, DNA lysate obtained from Chelex 100-proteinase K digestion method yielded only a few weak bands in RAM analysis.

Yap et al. (2002) has carried out a research to determine the genetic distance among mussels using horizontal starch gel electrophoresis. The result shows that the population within small geographical distance are very closely related and that genetic similarity over a relatively large geographical distance (about 30 km) is probably the result of the dispersal distance of the pelagic mussel larvae.

2.6 The Importance of Asian Green Mussel

*P. viridis* is credited with considerable success as an invading species, conquering new geographical locations in the east and the west. In general, the life history traits that make a successful invader are: a short life span, rapid growth rate, rapid sexual maturity, high fecundity, greater ability to colonise a wide range of habitats, wide physiological tolerance, gregarious behaviour, suspension feeding and ability to repopulate following a population crash (Morton, 1997).

Bivalves are a key component of the fouling community that develops inside the cooling circuits of coastal power plants and green mussels were the most dominant species, in terms of biomass. *P. viridis* have proven to be a successful fouling species in a variety of maritime and industrial environments. Their widespread distribution and their ability to attach to different surfaces even at high flow rates and to make use of water flow to achieve fast growth rates and high population densities, make them highly suited to colonise cooling water systems (Rajagopal, 1997).
As filter-feeders, *P. viridis* feed actively on particulate material in seawater with their frilled siphons. Thus, *P. viridis* used as a biomonitoring agent for heavy metal contamination in various Asian countries (Monirith *et al.* 2003). According to Ismail *et al.* (2000), *P. viridis* used as a potential biomonitoring agent for heavy metal contamination in the Straits of Malacca; which is one of the busiest shipping lanes in the world. However, before this species can be used as a biomonitoring agent for heavy metal contamination in the Straits of Malacca, it needs to fulfil several recommended criteria. Among the criteria are that *P. viridis* collected from different geographical populations along the straits should have similar morphological characteristics for easy and correct species identification, and low-to-moderate degrees of genetic differentiation as they may genetically adapt to heavy metal stresses (Gyllensten & Ryman, 1985; Rainbow, 1995).

The main bivalve species for aquaculture operation in Malaysia is *P. viridis* (Ong & Rabihah, 1989). The culture activity started in the Johore Straits in the southern coast of Peninsular Malaysia due to availability of natural seeds. This activity then spread to the western coast of Peninsular Malaysia especially the state of Melaka where natural spats are available and Perak by obtaining the seeds for transplantation from Johore and Melaka. With the development of modern culture system through work done by Fisheries Research Institutes and other initiatives by the Malaysian government the mussel culture is now spreading to other parts of Peninsular Malaysia mainly by transplantation of young mussels collected on polypropylene ropes from sites with natural spat (Choo, 1979).
3.0 Materials and methods

3.1 Study Site

A total of 12 of green mussels was sampled in Santubong, Sarawak (Figure 3.1)

Figure 3.1: Location of sampling areas, Santubong (source: Google map)
3.1.1 Sample Collection and Preservation

Approximately 12 individuals of green mussel was collected in the field by manual collection. The specimen were stored in ice box filled with ice cubes during transportation. Upon arrival, the samples were stored at -20°C freezer in Aquatic Molecular Laboratory, Faculty of Resource Science and Technology for future analysis.

3.2 Morphological Identification

All 12 samples of *P. viridis* collected from Santubong were identified based on morphological characteristics following Rajagopal *et al.* (1998). Morphological characters were observed and measured which were mainly targeting on the shell as follows: colour shell, pallial line, posterior adductor muscle, posterior pedal retractor muscle, teeth, total shell length, shell width, and shell weight.
3.3 Laboratory Work

3.3.1 DNA Extraction

Total genomic DNA was extracted from adductor muscle tissue of *P. viridis* following modified cetyl-trimethyl ammonium bromide (CTAB) method as described by Doyle and Doyle (1987). A total of 0.5 mg of the sample tissue was minced using surgical blade. The minced sample was placed into 1.5 ml eppendorf tube, which later added with 700 µL of 2X CTAB buffer. Then, 5 µl of Proteinase K was added into the tube was followed by incubation in the water bath Protech, M-903 at 60°C for 60 minutes. A total of 700 µl of chloroform-isooamyl alcohol (CIA) was added into the tube. The tube vortexed using Gilson® GVLab for 1 – 2 minutes to mix the solution. Later, tube was centrifuged at 13 000 rpm for 15 minutes using himac CF15RX, High-Speed Micro Centrifuge. Three layers of mixture was observed in the tube after centrifugation process, but only upper layer of the aqueous phase was taken out slowly using micropipette and transferred into a new tube. After that, 500 µl of 100% ethanol (EtOH) was added into the tube and the tube was inverted upside down to mix the mixture. The tube was stored overnight in the freezer at -20 °C. The mixture was centrifuged at 13 000 rpm for 15 minutes. The absolute EtOH was poured out and 500 µl of cold 70% EtOH was added into the tube. Then, the sample was centrifuged again at 13 000 rpm for 15 minutes. After that, the mixture was poured out and visible DNA pellet was observed. After that, DNA pellet was dried at room temperature for 2 hours. The pelleted DNA was redissolved in 50 µl of distilled water. The DNA was run by electrophoresis on a 1% agarose gel in 50 ml of 1X TBE (Tris-borate-EDTA) buffer containing ethidium bromide (EtBr) at 90V for 60 minutes. The gel was observed under UV light and was photographed for further record. Isolated genomic DNA was preserved at -20°C and ready to be used for PCR amplification.
3.3.2 Polymerase Chain Reaction (PCR)

Approximately 500 bp of Cytochrome Oxidase subunit 1 (COI) gene was amplified using a pair of metazoan invertebrate COI primer, LCOI1490 (5’ GGT CAA CAA ATC ATA AAG ATA TTG G- 3’) and HCOI2198 (5’-TAA ACT TCA GGG TGA CCA AAA AAT CA - 3’) by Folmer et al., (1994). PCR was done using 25 μl of total reaction volume containing 1μl DNA (~15-20ng), 1 μl Taq DNA polymerase (Fermentes), 2.5 μl of 10x PCR buffer (Fermentes), 1.5μl of 25 mM magnesium chloride (MgCl₂), 2.5 μl of 0.2 mM of deoxynucleotide triphosphosphate (Fermentes). Amplification was carried out using thermocyclerBio-Rad MyCycler™ Thermal Cycler following Divya et al. (2009). PCR cycles initiated with pre denaturation for 5 minutes at 95 °C, followed by 29 cycles of 45 seconds denaturation at 94°C, 30 seconds annealing at 51°C and 45 seconds primer extension at 72°C, with a final extension of 5 minutes at 72°C (Figure 3.2). PCR products were visualized in 1% agarose gel containing ethidium bromide at 90V for about 45 minutes with a low range 100bp ladder marker.

Figure 3.2: Diagram of PCR temperature profile for COI gene following Divya et al. (2009)
3.3.3 Sequencing

The purified PCR products and the gel electrophoresis photograph was sent to a private laboratory (1st BASE, Selangor, Malaysia) for sequencing using the ABI PRISM BigDye Terminator v3.0 kit of both forward and reverse strands.

3.3.4 Data Analysis

Chromatogram was viewed using Chromas Lite. Chromas Lite (Version 1.81) program was used to display fluorescence-based DNA sequencing results. The text was extracted and evaluated through Basic Local Alignment Search Tool (BLAST) program. Multiple sequence alignment for forward reactions were done using ClustalX program (Version 1.81) and subsequently aligned by eye. The pairwise genetic distance between populations was calculated using the Tamura-Nei (Tamura and Nei, 1993), based on unequal base frequencies and unequal ratios of transitions to transversions (Ti:Tv) implemented in MEGA 4.0 (Molecular Evolutionary Genetic Analysis) (Kumar et al., 2004). The phylogenetic confidence was estimated by bootstrapping.