Genetic diversity of redclaw crayfish *Cherax quadricarinatus* von Martens 1868 using 16S mitochondrial DNA marker

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Abstract. There have been reported around 11 aquatic invasive alien species (IAS) found in Malaysian waters. *Cherax quadricarinatus*, or commonly known as Australian redclaw crayfish, is one of the invasive species. *C. quadricarinatus* has the potential of causing negative impacts ecologically and economically in the local environment as it has reported in several countries if they get established and not monitored properly. Habitat alteration, native species depletion and spreading of diseases are among the reported negative impacts of *C. quadricarinatus*. This study was conducted with the aim to assess the genetic diversity of *C. quadricarinatus* from 4 different populations. Uncovering the diversity and population structure of the redclaw crayfish will help in enhancing the understanding of adaptation and survival of *C. quadricarinatus*. Thus, the information can be used in monitoring and management of this invasive crayfish in future. DNA of *C. quadricarinatus* was successfully extracted from its tissue and amplified via polymerase chain reaction (PCR) using mitochondrial DNA (mtDNA) 16S gene then proceeded for sequencing and analysed using several genetic analysis software to understand the diversity, phylogeny and population structure of this invasive crayfish species. A total of 493 bp fragments of 32 samples from four sampling sites were obtained. Four haplotypes were observed which Hap-1 was the most common haplotype. The highest genetic variation is Selangor (Pi = 0.00248, Hd = 0.694). However, low levels of both haplotype and nucleotide diversity indicates the loss of genetic diversity. Analysis of molecular variance (AMOVA) results revealed that the percentage of genetic variation within the population was 69.58% while among populations was 30.42%, indicating significant genetic differentiation among population (P < 0.05). The maximum likelihood tree showed that all haplotypes were clustered and grouped together with United States, Czech Republic, China and Australia.

1. Introduction

Introduced or alien species can be defined as species that were placed outside its natural or origin range, either accidentally or on purpose, by various means [1]. The Australian redclaw crayfish, *Cherax quadricarinatus*, is also known as an invasive alien species (IAS) that has been established and reported in Malaysian waters. This IAS has the potential of causing negative effects both economically and ecologically in the local environment as it has been reported in several countries; especially if they get
established and not monitored properly. The introduction of the redclaw crayfish into Malaysian waters might be due to escaping from aquaculture and aquarium industries, similar to previous reports in Israel, Mexico, Puerto Rico and Singapore [2][3][4][5][6]. The establishment of alien species scenario shows that the study of genetic diversity is very crucial. According to [7], stated that in theory, novel genotypes could be created since admixtures and founder effects dilutes where it might create the colonization of new habitat.

Moreover, there is undoubtedly lack of reports of any native species displacement or other ecological impacts in Malaysia caused by *C. quadricarinatus* introduction. Since the redclaw crayfish is a crustacean, this species causes a lot of environmental and economic damages. For example, *C. quadricarinatus* destroys fishing nets and in turn causes loss to the fishermen. The worst part of it is the introduction of redclaw crayfish into the native environment has also high potential to spread disease to the freshwater ecosystem. This study aims to assess the status of genetic diversity of *C. quadricarinatus* population using molecular genetics since it has become a powerful tool to determine the levels of differentiation among populations. Besides, this study would also act as a baseline data to help develop proper management by related authorities in Malaysia. According to [8], genetic diversity is an excellent tool for assessing biological qualities of an organism. Current research stated that the genetic diversity was expressed based on haplotype and nucleotide diversity [9]. Genetic diversity shows the ability of the organisms to survive in any kind of environment in the long term. Based on [10], mitochondrial DNA or mtDNA has a distinct haplotype and acts as an indicator or DNA markers to identify a species since it is maternally inherited. The use of mtDNA gene as a genetic marker has become the major consideration of molecular technique due to highly conserved between closely related species.

2. Materials and methods

2.1. Sample collection

The Australian redclaw crayfish had been collected at four different locations in states across Peninsular Malaysia. They are the states of Terengganu, Pulau Pinang, Selangor and Johor. The amounts of total sample collections obtained were 32 samples of *C. quadricarinatus*. The samples were obtained based on its availability at the sampling sites (Figure 1; Table 1). The preserved *C. quadricarinatus* tissue was cut and placed in a microcentrifuge tube containing 99% ethanol.

| No | Sampling state                      | Abbreviations | Sample size |
|----|-------------------------------------|---------------|-------------|
| 1  | FELDA Tenang, Terengganu            | FTT           | 9           |
| 2  | Sg Jarak, Pulau Pinang              | SJP           | 4           |
| 3  | Puchong Perdana Lake, Selangor      | PPS           | 9           |
| 4  | Batu Pahat, Johor                   | BPJ           | 10          |
|    | Total                               |               | 32          |

Table 1. The list of sampling state, abbreviations and sample sizes.
2.2. DNA extraction, Polymerase Chain Reaction (PCR) and sequencing

The DNA of *C. quadricarinatus* was extracted by following the protocol of Favorgen DNA Extraction Mini Kit. 16S ribosomal RNA of mitochondrial gene 1471 (5´-CCTGTTTANCAAAAACAT-3´) and 1472 (5´-AGATAGAAACCAACCTGG-3´) [11] were used and amplified by PCR. The total reaction volume needed for PCR reaction is 20 µL which includes 2 µL of dH$_2$O, 13 µL of Bioline PCR Mastermix, 0.5 µL of forward and 0.5 µL of reverse primer (1471 and 1472) and 4 µL of DNA template on a thermal cycler Applied Biosystems™ Veriti™ 60 Well Thermal Cycler. The thermal cycling conditions were 95°C for 4.36 min; 35 cycles at 95°C for 30 s, 47°C for 30 s and 72°C for 45 s; followed by a final extension at 72°C for 10 minutes. The result of PCR products were analysed by 100 V and 500 mA for 40 minutes and the gel was visualized using Luminescent Image Analyzer LAS-1000plus v.2.0. The PCR products were then sent for sequencing to Apical Scientific Sdn. Bhd. using Sanger.

2.3. Data analysis

The sequences were aligned and edited using ClustalW multiple sequence alignment. The sequences were visualized using Chromas v2.6 and aligned and trimmed based on chromatogram. DnaSP v6 helps in finding the degree of variation for nucleotide diversity and identifying the haplotype diversity [12]. Genetic diversity in each population was measured as haplotype diversity (H) and nucleotide diversity [13]. Population structure and genetic variation were analyzed using ARLEQUIN v3.5 [14]. Analysis of Molecular Variance or AMOVA is used from the aligned sequences to analyse the population differentiation and genetic variation within sampling sites [15]. Hierarchical genetic structure of populations and pairwise Fst values among populations can be evaluated using AMOVA which was implemented in Arlequin 3.5.2 [14]. Two neutrality tests: Tajima’s D [16] and Fu’s Fs [17] were examined to study population demography either possible for population expansion or bottleneck. MEGA version 7 was used to determine appropriate models for evolutionary analyses and construct phylogenetic trees [18]. Phylogenetic tree was constructed using the Maximum Likelihood (ML) method based on the Hasegawa-Kishino-Yano model. Positions with gaps and missing data were removed. Also, the sequences of four genus of Cherax as ingroup and four outgroups were retrieved from the National Centre of Biotechnology Information (NCBI) and included in the tree.
3. Results and discussion

3.1. Sequence variation

A total of 493 bp fragments of thirty-two samples from four sampling sites were successfully sequenced and aligned. Table 2 shows that all variable sites were parsimony informative sites. There were 4 variable sites and 493 invariable (monomorphic) sites out of 493 sites. For Johor, Pulau Pinang and Terengganu, there were no presence of variable sites while Johor and Selangor have single mutation sites. Additionally, the Selangor population contains more variable sites compared to the other sampling sites.

Table 2. Sequence variation of four haplotypes in 16S mtDNA of 32 samples and nucleotide position.

| Haplotype number | Nucleotide position |
|------------------|---------------------|
|                  | 299 | 347 | 369 | 384 |
| Hap-1            | A   | A   | G   | G   |
| Hap-2            | G   | .   | .   | .   |
| Hap-3            | .   | .   | C   | C   |
| Hap-4            | .   | T   | .   | C   |

3.2. Genetic diversity and population structure

The excellent tool to evaluate biological qualities of an organism is genetic diversity [8]. In addition, current research by [10] stated that the genetic diversity was expressed based on haplotype and nucleotide diversity. Haplotype diversity is the possibility that two sampled alleles are different from each other while nucleotide diversity can be defined as the average number of nucleotide differences per site in selected haplotypes. Table 3 shows that four haplotypes were observed from the samples. Haplotypes indicate that the sequence set of DNA by an individual is inherited from a single parent which means that different haplotypes would give different numbers.

Table 3. Number of Australian redclaw crayfish from four sampling sites according to haplotype distribution.

| Haplotype | Johor | Pulau Pinang | Selangor | Terengganu | Sample % in haplotype |
|-----------|-------|--------------|----------|------------|-----------------------|
| Hap-1     | 3     | 4            | 5        | 9          | 65.63%                |
| Hap-2     | 7     | 4            | 2        | -          | 28.13%                |
| Hap-3     | -     | 3            | 1        | -          | 3.12%                 |
| Hap-4     | -     | 3            | 1        | -          | 3.12%                 |

Table 4. Nucleotide sequence data of four sampling sites based on partial fragments of the mtDNA 16S region, haplotype and nucleotide diversity, and neutrality test.

| Sampling site | Sample size | No. of polymorphic sites | No. of haplotypes | Haplotype diversity | Nucleotide diversity Fu’s Fs P-value | Tajima’s D | Tajima’s D P-value |
|---------------|-------------|--------------------------|-------------------|---------------------|--------------------------------------|------------|-------------------|
| Johor         | 10          | 1                        | 2                 | 0.467               | 0.00095                              | 0.55200    | 0.81980 1.00000    |
| Pulau Pinang  | 4           | 0                        | 1                 | 0.000               | 0.00000                              | N.A        | 0.00000 1.00000    |
| Selangor      | 9           | 4                        | 4                 | 0.694               | 0.00248                              | 0.23500    | -0.68914 0.22300   |
| Terengganu    | 9           | 0                        | 1                 | 0.000               | 0.00000                              | N.A        | 0.00000 1.00000    |

Among the total samples of *Cherax quadricarinatus*, all sampling sites have haplotype 1 (Hap-1) which contain 21 samples and cover 65.63% of total samples. This shows that Hap-1 is the most common and widespread haplotype among the other sampling sites. Furthermore, two haplotypes were shared in more than one state. Haplotype 2 (Hap-2) contains 2 samples from Johor and Selangor while both haplotype 3 (Hap-3) and haplotype 4 (Hap-4) contain 1 sample from Selangor, respectively. This means that Hap-
3 and Hap-4 are the distinct haplotype, which means it can only be found in a particular sampling site and is therefore, unique.

Table 4 shows that Selangor has the highest haplotype and nucleotide diversity value which are (0.694) and (0.00248), followed by Johor with (0.467) and (0.00095), respectively. However, both Pulau Pinang and Terengganu have zero value for haplotype and nucleotide diversity since the detected variable sites in the sequences are lacking. Genetic diversity and population size are related with each other. This was further explained by [9] that a small number of samples and sampling sites might cause low nucleotide diversity and haplotype diversity. This study shows four haplotypes that obtained from 16S mtDNA helps in proving the phylogenetic history of Australian redclaw crayfish in Malaysia. The analysis of all samples shows low levels of average genetic diversity where the haplotype diversity value is 0.5040 and nucleotide diversity value is 0.00135. Low levels of both haplotype and nucleotide diversity indicates the loss of genetic diversity, and it may cause an occurrence of population bottleneck phenomenon [19]. Also, demographic bottlenecks happen as the population of a species encounters a severe or temporary reduction in size. The evolutionary biologist has studied extensively on the genetic effects of reduced population size, as the distribution of genetic variation within and between population may be influenced by bottlenecks [20][21].

Two statistical tests had been used to investigate demographic events. According to [16], stated that Tajima’s D test is based on the allele frequency of segregating nucleotide sites where a positive value shows a bias towards intermediate frequency alleles. However, a bias towards rare alleles shows a negative value, the end result of recent population expansion. Similar to Tajima’s D test, Fu's FS test [17] with negative values shows recent population growth but it is based on the distribution of alleles or haplotypes. Population in Selangor may undergo recent population growth since it has distinct haplotypes and negative values on Tajima’s D test.

AMOVA procedure was starting to carry out the analysis for DNA haplotypes and to any marker system. Also, it is a powerful tool that aids in stating the hypothesis of population structure due to isolation [9]. However, the AMOVA result that shown in Table 5 below revealed that the percentage of variation among the population is 30.42% and within the population is 69.58% with significant value (P < 0.05) respectively.

**Table 5.** Data of 16S mtDNA nucleotide of four sampling sites using analysis of molecular variance (AMOVA).

| Source of variation     | Df | Sum of squares | Variance components | Percentage of variation |
|-------------------------|----|---------------|---------------------|------------------------|
| Among populations       | 3  | 3.292         | 0.10911             | 30.42                  |
| Within populations      | 28 | 6.989         | 0.24960             | 69.58                  |
| Total                   | 31 | 10.281        | 0.35871             |                        |

Fixation index $F_{ST}$: 0.30416

**Table 6.** Pairwise difference $F_{ST}$ value of population differentiation.

|                  | Johor | Pulau Pinang | Selangor | Terengganu |
|------------------|-------|--------------|----------|------------|
| Johor            | -     | -            | -        | -          |
| Pulau Pinang     | 0.55056 | -            | -        | -          |
| Selangor         | 0.20771 | 0.04348     | -        | -          |
| Terengganu       | 0.65251 | 0.00000     | 0.08333  | -          |

*Significant level (P< 0.05) of $F_{ST}$ value
Table 7. $F_{ST}$ P value

|       | Johor       | Pulau Pinang | Selangor    | Terengganu |
|-------|-------------|--------------|-------------|------------|
| Johor | -           | -            | -           | -          |
| Pulau Pinang | 0.07324 + 0.0083 | -   | -           | -          |
| Selangor | 0.05078 + 0.0069 | 0.55957 + 0.0139 | -   | -          |
| Terengganu | 0.00195 + 0.0014 | 0.99902 + 0.0002 | 0.22461 + 0.0124 | -          |

The fixation index ($F_{ST}$) is 0.30416 ($P < 0.05$), which shows that there is a significant difference in genetic differentiation of the populations. According to [22], the degree of differentiation among populations are shown by $F_{ST}$. This further explained that the greater $F_{ST}$ value between 0 to 1, the greater the degree of differentiation of the population, the higher the degree of differentiation. Since the $F_{ST}$ value is 0.30416 indicating that the population has moderate degrees of differentiation. The percentage of variation within populations (69.58%) is higher than among populations (30.42%). Based on AMOVA analysis, this indicate that the species has population structure and no gene flow. However, the P value from AMOVA result shows the opposite. This means that the Australian redclaw crayfish population dispersal is restricted as they are separated by lakes or ponds.

It has been reported that Australian redclaw crayfish has the ability to tolerate from fast flowing rivers to slower rivers and lakes [23] and it has adapted all the harsh conditions in its native range, which made this species to become one of the species that has wide tolerance in the environment. Table 5 shows a pairwise difference between four sampling sites and Table 6 shows the $F_{ST}$ P value. Since the fixation index ($F_{ST}$) is 0.30416 ($P < 0.05$) which is significant, there must be at least one population that has population structure and no gene flow. From Table 5, population between Pulau Pinang and Johor shows that there might be a population structure. This means that the populations are unique but it is not too high. Some of the population have structured while some of them are not, may be caused by the geographical barrier that make the individual cannot move to the other places. Thus, no gene flow was observed. This inconsistent result might derive from the unbalanced number of samples since some of sampling site contain 10 samples, some of it contain 9 samples and Pulau Pinang state contain only 4 samples.

3.3. Phylogenetic tree

Phylogenetic tree was constructed using the Maximum Likelihood (ML) method based on the Hasegawa-Kishino-Yano model shows that the phylogenetic relationships among four haplotypes of Australian redclaw crayfish where four sampling sites are clustered into one clade. All of the haplotypes were strongly supported by a bootstrap value of 100%. The presence of single clade may originate from the same single ancestor in maternal lineage. From the figure, the phylogenetic tree consists of two main clades where the first clade consists of outgroups of crayfish named Procambarus clarkii, Astacus astacus, Cambarellus patzcuarensis and Orconectes rusticus while the second clade consists of Cherax sp. The second clade was divided into two subclades where the first subclade consists of Cherax parvus, Cherax punctatus and Cherax destructor. The second subclade consists of all haplotypes in the same clade as the other Australian redclaw crayfish in other countries and Cherax nucifraga. Also, these haplotypes derived from the same 16S mtDNA with the other countries to examine the phylogenetic relationship. As for the sequence that had been studied by Czech Republic, this freshwater crayfish had invaded Indonesia at the transitional zone between Australia and Asia named Wallace Line. From the result of previous study, it can be concluded that the population had been identified to have occurred in the southern part of the Papua Province while having the highest potential for dispersing throughout Indonesia [24].
4. Conclusion

In conclusion, 32 samples of *Cherax quadricarinatus* were successfully extracted and amplified by PCR using 16S mtDNA marker. Australian redclaw crayfish were collected from four different states which are Johor, Pulau Pinang, Selangor and Terengganu. This species has high haplotype and nucleotide diversity in Selangor. However, low genetic diversity between sampling sites was observed. AMOVA results in this study shows that there is a significant population structure for Johor-Pulau Pinang populations. As a recommendation for further studies, it is suggested to use a possible minimum of 10 sample size or larger per sampling site to reveal more genetic variation in particular populations since this study shows low genetic diversity of Australian redclaw crayfish caused by inaccurate results. The related authority needs to construct or develop proper management in order to eradicate or reduce the number of invasive species. Also, it is recommended to educate fishermen and hobbyists in the future about the harmful effects of invasive species to aquatic environments.

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