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Research

Genetic Variability of Wild Populations of Invasive Redclaw Crayfish (*Cherax quadricarinatus*) von Martens 1868 Across Peninsular Malaysia

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ABSTRACT

The redclaw crayfish had been listed as an invasive species in Malaysia following the various negative impacts displayed on both environment and economy. The species are largely culture in Malaysia for food, but unluckily escaped and expands to several waterbodies across the country. For effective control management of redclaw crayfish species, a total of 52 wild samples were collected from six locations in Peninsular Malaysia and analyzed using a 16S mitochondrial DNA to assess their genetic diversity and introduction history. Five haplotypes were detected associated with an overall low genetic diversity (Hd = 0.385, π = 0.00133). A single genetic structure was detected with a phylogenetic relationship showing two clusters related to the haplotypes from Australia and Papua New Guinea. The finding of this study provides the basic data that will aid the appropriate Malaysian authorities for both monitoring and management strategies of redclaw crayfish in Peninsular Malaysia.

Key words: Biological invasion, *Cherax quadricarinatus*, genetic structure, mitochondrial DNA

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INTRODUCTION

The Australian redclaw crayfish, *Cherax quadricarinatus* (von Martens 1868), is a freshwater invertebrate native to northern Australia and southern New Guinea invaded several countries including Malaysia (Patoka *et al*., 2016; Norshida *et al*., 2021). A culture shock of redclaw crayfish was first introduced to Malaysia in the early 2000s and has predominantly been cultured in the southern part of Peninsular Malaysia (Chang, 2001). The redclaw crayfish were listed among the invasive alien species introduced into Malaysia through aquaculture trading (Naqiuddin *et al*., 2016). Improper handling of redclaw crayfish farming has led them into the native waterways and freshwater habitats across Peninsular Malaysia (Nasir *et al*., 2020).

Undeniably, the introduction of redclaw crayfish into Malaysia has some detrimental consequences both environmentally and economically. These include biodiversity loss (Norshida *et al*., 2021), habitat destruction (Nasir *et al*., 2020), as well as disease transfer to other native freshwater decapod crustaceans (Sallehuddin *et al*., 2021). By burrowing activity and grazing on macrophytes, they might disrupt the balance of the receiving environment via competition for shelter and resources, spread various diseases, direct predation on native species and fish eggs, and alter habitat (Naqiuddin *et al*., 2016). In addition, *Cherax quadricarinatus* can threaten human health since they are known to be intermediate hosts for parasitic digean flatworms (Romero & Jimenez, 2002; Lane *et al*., 2009) and also act as disease vectors for *Vibrio cholerae*, *Vibrio mimicus*, enterococcids, and *Escherischia coli* (Edgerton *et al*., 2002). The three economic costs associated with redclaw crayfish are management and environmental harm associated costs, and direct impacts on other species (Taryono *et al*., 2021). In Malaysia, Naqiuddin *et al*. (2016) has claimed that fishermen have lost money as a result of crayfish because the creatures harm their catch and fishing nets.

Redclaw crayfish are tolerant to a wide range of habitual geographical conditions which makes them resilient to natural changes (Haubrock *et al*., 2021). Norshida *et al*. (2021) deduced that redclaw crayfish are the only invasive non-native crayfish that have established wild populations in Malaysia. Their wild invasion success might be owing to the multiple introduction sources and different maternal lineages. Yet, there was limited information concerning the genetic diversity and population structure of the wild populations of redclaw crayfish in Malaysia.

Nowadays, molecular genetic studies have indeed become an efficient field for determining the levels of differentiation among populations (Khaleel *et al*., 2019; Abdullahi *et al*., 2021; Dali *et al*., 2021; Max-Aguilar *et al*., 2021). The use of mitochondrial DNA has increasingly become significant in resolving molecular genetic diversities and structures as well as tracking the origin of different species (Ha *et al*., 2017; Ahmad-Syazni *et al*., 2017; Khaleel *et al*., 2020). This is highly important for verifying the genetic history of redclaw crayfish as well as understanding their ability to survive in different geographical locations. As such, the baseline data generated could be useful for monitoring and surveillance of redclaw crayfish in future conservation plans and strategies. In the current study, therefore, we evaluate the genetic diversity of wild redclaw crayfish populations to analyze the number of introductions that allegedly took place across Peninsular Malaysia.

MATERIALS AND METHODS

Sample collection

A total of fifty-two wild samples of redclaw crayfish were captured from six freshwater bodies across Peninsular Malaysia from September 2018 to September 2020 (Table 1). All samples were captured by hand picking and locally made trap (*bubu*). At least 1 cm of tissue of redclaw crayfish was cut and placed in a microcentrifuge tube containing 99% absolute ethanol for preservation as described by Nasir *et al*. (2020).

Table 1. Sampling locations and their habitats

Sampling site	Coordinate	Habitat	Abbrev.	N
Batu Pahat, Johor	1.776227, 103.144992	Stream	BPJ	10
Ayer Keroh Lake, Melaka	2.273944, 102.300262	Lake	TAM	10
Seremban Lake, Negeri Sembilan	2.721650, 101.943522	Lake	TSN	10
Sungai Jarak, Pulau Pinang	5.471782, 100.505892	River	SJP	04
Puchong Perdana Lake, Selangor	3.009649, 101.606787	Lake	PPS	09
Felda Tenang, Terengganu	5.561758, 102.496244	Stream	FTT	09
Total				52

DNA extraction, Polymerase Chain Reaction (PCR), and sequencing

The DNA of redclaw crayfish was extracted using Favorgen DNA Extraction Mini Kit by following the kit's protocol. A 16S ribosomal RNA of the mitochondrial gene was amplified using primers 1471 (5´-CCT GTT TAN CAA AAA CAT-3´) and 1472 (5´-AGA TAG AAA CCA ACC TGG-3´) retrieved from Crandall and Fitzpatrick Jr. (1996). PCR amplification was carried out in a 20 µL reaction volume containing 2 µL dH_2O, 13 µL Bioline PCR Mastermix, 0.5 µL of both primers and 4 µL DNA template conducted on a Veriti 96 Well Thermal Cycler (Applied Biosystem, California, USA). The PCR began with an initial denaturation step at 95 °C for 4.36 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 47 °C for 30 s and extension 72 °C for 45 s; followed by a final extension at 72 °C for 10 min. The result of PCR products was run in the agarose gel, and the amplified PCR products were finally sequenced at Apical Scientific Sdn. Bhd using Sanger sequencing method. All five haplotype sequences discovered in the current study were registered in National Centre of Biotechnology Information (NCBI) GenBank (accession number: OP389118 — OP389122).

Data analysis

The sequence chromatograms from the automated sequencer were checked with Chromas version 2.4 (Technelysium Pty. Ltd., Queensland, Australia). The sequences were aligned and edited using GENETYX v9.1.3 multiple sequence alignment program. Nucleotide composition and several variable sites were assessed using DnaSP v.6 (Rozas *et al*., 2017). The genetic diversity of each sampling site was measured as haplotypic diversity (Nei, 1987) and nucleotide diversity (Tajima, 1983). The level of genetic population differentiation was tested by analysis of molecular variance (AMOVA) as implemented in ARLEQUIN v3.5 (Excoffier & Lischer, 2010), using the genetic distance matrix to estimate the components of variance that are attributable to differences among the populations and within populations. Genetic differentiation between populations was then tested by pairwise comparison F_{ST} with Slatekin's and Reynold's distances with 1,000 permutations as implemented in ARLEQUIN v3.5 (Excoffier & Lischer, 2010). Two neutrality tests were examined: Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997) as implemented in ARLEQUIN v3.5 (Excoffier & Lischer, 2010).

The resulting matrix distance was used to construct trees with Neighbour-Joining (NJ) and Maximum Likelihood (ML) algorithms employed in MEGA v.7. Statistical support for nodes for both NJ and ML was tested using 1000 bootstrap replicates. We followed the suggested Hasegawa-Kishino-Yano (HKY+I) and Kimura 2 parameter models that have been used for ML and NJ respectively (MEGA v.7, Kumar *et al*., 2016). We began with a phylogenetic tree constructed by NJ using the five haplotype sequences of the current study (acc. no.: OP389118 - OP389122), other sequences retrieved from NCBI GenBank included three sequences of *Cherax quadricarinatus* from China, Australia, and Papua New Guinea (acc. no.: MG199588, KJ920764.1, and EU244890 respectively), *Cherax destructor* as ingroup (acc. no.: MK000279), and *Procambarus clarkii* as an outgroup (acc. no.: KJ645826). The ML tree was constructed with an additional 15 sequences of *Cherax quadricarinatus* from the native range (acc. no.: EU24487 - EU24493) and an outgroup *Macrobrachium rosenbergii* (acc. no.: MK113948) was included.

RESULTS

Sequence variation

A total number of 500 base pairs (bp) of 16S fragments were successfully amplified from 52 samples taken from six sampling sites. Nine variable sites were discovered, which yielded five haplotypes (Table 2).

Table 2. Sequence variation among the five haplotypes

Haplotype Number	Nucleotides Position								
	49	306	354	365	376	391	452	497	500
Haplotype 1 (Hap1)	G	A	A	G	G	G	G	A	T
Haplotype 2 (Hap2)	-	G	-	-	-	-	-	-	-
Haplotype 3 (Hap3)	-	G	-	A	-	-	-	-	-
Haplotype 4 (Hap4)	-	-	-	-	C	C	C	G	G
Haplotype 5 (Hap5)	C	-	T	-	-	-	-	-	-

Genetic diversity

Among five haplotypes discovered, Hap1 was a major haplotype (76.92%) that was found to be present in all six sampling sites (Figure 1). A Hap2 was shared by seven samples from Johor and two samples from Selangor. The distinct Hap3, bears only one sample from Negeri Sembilan, while Hap4 and Hap5 represent two different samples from Selangor (Table 3).

The overall haplotype diversity observed from the 52 samples used was 0.385 and the nucleotide diversity of 0.00133. In Table 4, the result revealed that Selangor (PPS) had the highest value of haplotype diversity Hd, (0.694) and nucleotide diversity π, (0.00423), followed by Johor (BPJ) (Hd: 0.467; π:0.000930) and Negeri Sembilan (TSN) (Hd:0.200; π:0.00080). Sampling sites from Melaka (TAM), Pulau Pinang (SJP), and Terengganu (FTT) have zero values for both haplotype and nucleotide diversity. No significant Tajima *D* and Fu's *Fs* were observed in all sampling locations.

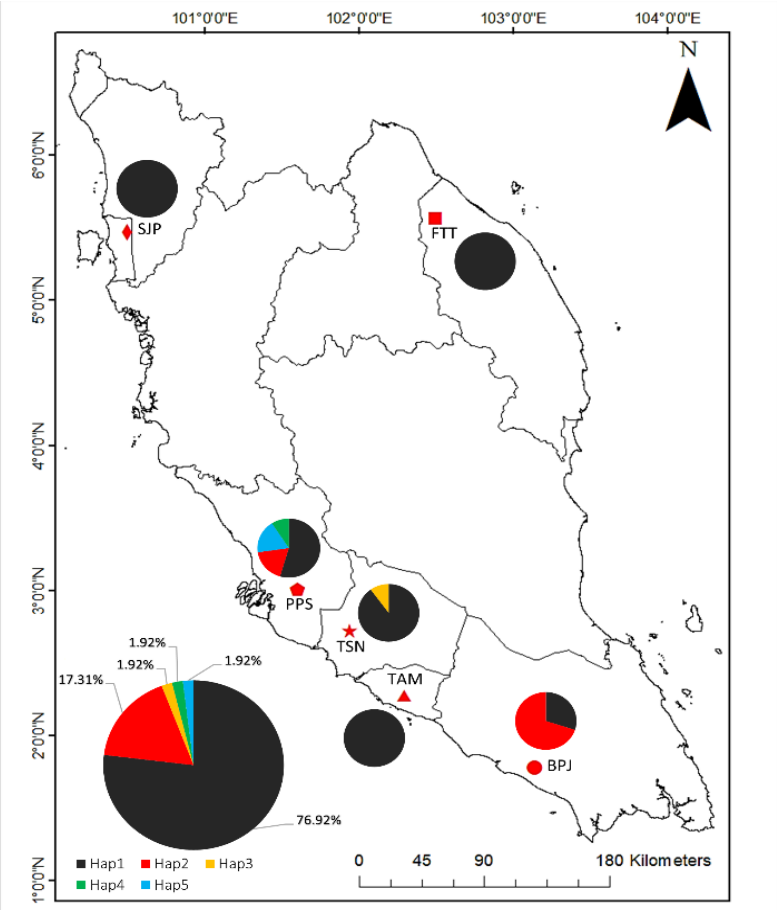

Fig. 1. Redclaw haplotypes distributions across the sampling locations in Peninsular Malaysia.

Table 3. Percentage of crayfish distributed among the haplotypes

Haplotype	BPJ	TAM	TSN	SJP	PPS	FTT	Samples % in the haplotype
Hap1	3	10	9	4	5	9	76.92
Hap2	7	-	-	-	2	-	17.31
Hap3	-	-	1	-	-	-	1.92
Hap4	-	-	-	-	1	-	1.92
Hap5	-	-	-	-	1	-	1.92
Total							100%

Table 4. Nucleotide sequence data of four sampling sites based on partial fragments of the 16S mitochondrial DNA region

SL	No. polymorphic Sites	No. of Haplotypes	Hd	π	Tajima D	Tajima D *P*-value	Fu's F_S	Fu's F_S *P*-value
BPJ	1	2	0.467	0.0009	0.820	0.869	-0.406	0.552
TAM	0	1	0.000	0.0000	0.000	1.000	0.000	NA
TSN	2	2	0.200	0.0008	0.402	0.399	0.020	0.55
SJP	0	1	0.000	0.0000	0.000	1.000	0.000	NA
PPS	8	4	0.694	0.0042	-0.689	0.223	0.850	0.235
FTT	0	1	0.000	0.0000	0.000	1.000	0.000	NA

* No Significant (P<0.05)

Population differentiation and phylogenetic tree

Analysis of molecular variance (AMOVA) results indicated the highest variation existed within populations (78.32 %) with a very weak fixation index value (F_{ST} = 0.21676; P<0.05) as shown in Table 5. The pairwise comparison F_{ST} results have further revealed weak population structure exited within the BPJ in the Johor sampling site (Table 6). The highest pairwise distance obtained from the current study was between Hap3 and Hap4 (0.014) while the lowest pairwise distance value obtained was between Hap1 and Hap2 (0.002) and between Hap2 and Hap3 (0.002). A phylogenetic analysis using NJ showed the presence of only a single cluster, consisting of all the haplotypes (Hap1 to Hap5) in the current study (Figure 2). Constructed Maximum Likelihood (ML) phylogenetic trees have also reaffirmed only one cluster existed (Figure 3).

Table 5. Analysis of molecular variance (AMOVA) data on the 16S mitochondrial gene from six sampling sites

Source of variation	df	Sum of squares	Variance components	Percentage of variation	F_{ST} Value
Among populations	5	4.521	0.07424	21.68	0.21676
Within populations	46	12.344	0.26836	78.32	
Total	51	16.865	0.34262		

$P < 0.05$

Table 6. Pairwise F_{ST} of the Population from six sampling sites

	BPJ	TAM	TSN	SJP	PPS	FTT
BPJ	—					
TAM	0.66667	—				
TSN	0.42982	0.00000	—			
SJP	0.55056	0.00000	0.12150	—		
PPS	0.15109	0.06404	0.01727	0.07294	—	
FTT	0.65251	0.00000	0.01124	0.00000	0.50000	—

DISCUSSIONS

The continuous growth and expansion of wild redclaw crayfish populations across the freshwater ecosystems in Peninsular Malaysia have become a serious concern. This prompted the current research to evaluate the genetic diversity of the wild redclaw crayfish populations and introduce events that might probably occur. It is essential to generate baseline information regarding the genetic resources of redclaw crayfish currently available in the wild for conservation and monitoring purposes. For two decades, wild redclaw crayfish have been moved around in Peninsular Malaysia, probably being exposed to the combined effects of genetic drift, founder events, and both direct/indirect selection over time. Subsequent translocations may likely modify and changes their genetic backgrounds which may lead to the formation of divergent redclaw crayfish species line (in the case of Puchong Perdana Lake in Selangor with high genetic diversity (Hd, 0.694; π, 0.0042)). As such, these wild specimens may probably be reintroduced into commercial hatcheries and production lines in the future. Therefore, the status regarding the degree of population differentiation and level of genetic diversity of

52 wild redclaw crayfish sampled from six sampling sites in Peninsular Malaysia has been assessed. The data was evaluated using molecular data derived from the 16S mitochondrial DNA of 52 sequences generated.

Five haplotypes (Hap1-Hap5) obtained in the current study have indicated the multiple maternal origins and introductions of redclaw crayfish in Peninsular Malaysia. Although, the findings may not reflect the mechanism and channel for which each haplotype has been introduced. But, a largely distributed Hap1 may be initially begun with the population from Johor 'first wild population recorded' (Chang *et al*., 2001; Naqiuddin *et al*., 2016), and certainly expand (transmitted via flooding or intentional release) and established down to the

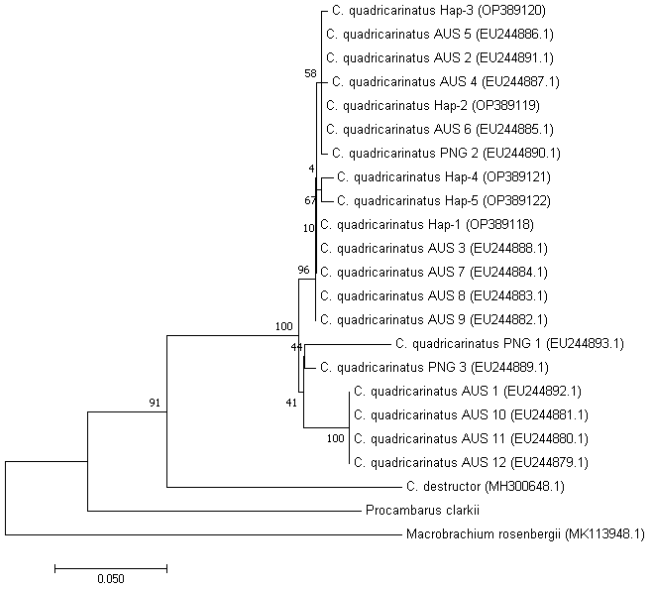

Fig. 2. Phylogenetic tree constructed using Neighbour-Joining Tree for five haplotypes (current study) together with two outgroups.

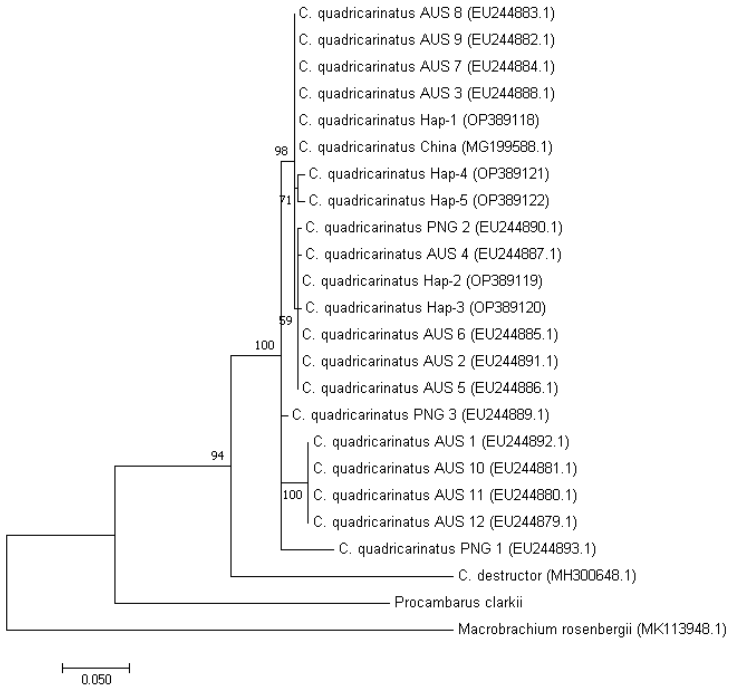

Fig. 3. Phylogenetic tree constructed using a Maximum Likelihood for five haplotypes of the current study, together with 15 *C. quadricarinatus* from native habitat range and three outgroups.

various freshwater bodies across the eastern and western Peninsular Malaysia (Norshida *et al*., 2021). For example, the capture of many young and matured redclaw crayfish from several freshwater habitats in Peninsular Malaysia indicated the ultimate reproductive success of the species (Nasir *et al*., 2020; Idrus *et al*., 2021; Sallehuddin *et al*., 2021). Except for Hap3, Puchong Perdana Lake in Selangor holds the entire haplotypes of redclaw crayfishes discovered in the present study. High haplotypic diversity in the lake, together with the discovery of a single cluster may probably suggest the likelihood of the lake to serve as an aquatic refuge for redclaw crayfishes in Peninsular Malaysia. During the sampling period, we observed that redclaws were most likely to be found in lake and stream compared to the river. This assumption was supported by the total percentages of redclaw specimens captured in the lakes (55.8%), streams (36.5%) and river (7.7%) habitats. Although only single sampling location represented the river in the current study, however, most previous studies reported the wild populations of redclaws from the streams and lakes (Idrus *et al*., 2021; Sallehuddin *et al*., 2021). Turbid nature (low transparency) of most rivers in Malaysia (Khaleel *et al*., 2021) could be a possible factor unsuitable for the redclaw to establish in the river habitats.

Despite the low genetic diversity observed in the redclaw crayfish examined, the invasion was successful as the species adapt well to the natural open-water bodies of Malaysia. In the previous study carried out within the native range of redclaws (Australia and Papua New Guinea), low genetic diversity has also been observed among the redclaw species examined (Baker *et al*., 2008). Their findings further revealed that wild populations of redclaws consist of two highly divergent lineages each from Australia and Papua New Guinea respectively. Generally, a high genetic diversity of invasive species usually increases the chances of invasion success (Cassey *et al*., 2018). Although, other invasive species like peacock bass (*Cichla* spp.) with low genetic diversity was reported to be successful for invading a newly habitat (Carvalho *et al*., 2014). The neutrality test revealed no population expansion (genetic equilibrium) in the entire sampling location. This suggest that the high genetic diversity in Selangor population might not be due to the mutation or gene evolution occurred in the habitat when considering the introduction time range of the redclaw crayfish species in the lake. Hence, it may be due to the different maternal origin and sources of redclaw crayfish species introduced. These assumptions were strongly supported by the finding of the previous study conducted in northern Australia and Papua New Guinea on wild redclaw stocks (Baker *et al*., 2008).

Both AMOVA and pairwise comparison tests indicated a weak population differentiation within the redclaw crayfish specimens used and therefore, we considered the outcome as a single population. This can be seen as both NJ and ML phylogenetic trees resulting only one clade consisting of all the five haplotypes in this study. The low pairwise distance value (0.002 – 0.014) further reinforce the grouping of all haplotypes into one clade due to high sequence similarity (Azhar & Azmir, 2019; Azmi *et al*., 2022). In addition, it is also believed that all the haplotypes from this study came from the native area of this crayfish as the pairwise distance between the haplotypes and *C. quadricarinatus* from Papua New Guinea and Australia was 0.000 to 0.009, respectively (Zhao *et al*., 2021; Azmi *et al*., 2022). Although, it has been widely believed that redclaws were introduce through aquarium trade in Malaysia (Naqiuddin *et al*., 2016). However, little is known about the genetic information regarding the commercial breeds currently available in the country. Thereby, it is quite difficult to discuss and compare the culture and wild redclaw crayfish species existed in Malaysia.

CONCLUSION

We discovered a total of five haplotypes from the 52 mitochondrial DNA (16s region) of wild redclaw crayfishes collected from six sampling locations in Peninsular Malaysia. It was indicated that multiple maternal origins and more than one propagule pressure event took place during the introduction of redclaw crayfish species in Peninsular Malaysia. The overall genetic diversity among the sampling locations was low, but redclaw crayfish from the Selangor sampling site displayed the highest genetic variations. Our study confirms the identification of one cluster which can be traced to the redclaw crayfish species originating from China, Australia and Papua New Guinea. We provided the baseline data concerning the genetic background of wild redclaw crayfish species present in the freshwater habitats and canals of Peninsular Malaysia for future monitoring and conservation purposes. We recommend further studies regarding nuclear markers and other mitochondrial regions couple with larger sample size to check on other factors like effective population size and inbreeding coefficient of both wild and culture stock of redclaw crayfish species in Malaysia.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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