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Utility of Single Stranded Conformation Polymorphism (SSCP) method to identify two related freshwater prawn species: *Macrobarchium rosenbergii* (De Man) and *Macrobrachium malcolmsonii* (Milne Edwards) – A preliminary investigation

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Abstract

Freshwater culture prawn industry is mainly based on *Macrobrachium rosenbergii* (De Man) in many countries. However, now there is a growing interest in many Asian countries to use *Macrobrachium malcolmsonii* (Milne Edwards) as a potential candidate in aquaculture programs due to its high growth rate. In Sri Lanka too, freshwater prawn culture is mainly based on *M. rosenbergii*. However, there is a possibility to use *M. macolmsonii* in future in aquaculture programs, as the presence of this species is reported from the eastern part of Sri Lanka. Although, the adults of two species show a few morphological differences, the appearance of young individuals of both species are identical. Therefore, in many occasions identification and separation of the above two species is a troublesome task. The delineation of species levels using reliable and cost effective methods is an important point in effective management programs in aquaculture.

This preliminary study was designed to determine the utility of Single Stranded Confirmation Polymorphism (SSCP) method to delineate two economically important freshwater prawn species, *M. rosenbergii* and *M. malcolmsonii* in Sri Lanka. Partially amplified mitochondrial 16S rRNA gene region was sequenced for both species and the number of nucleotide differences was estimated. The sequence length of the both DNA fragments were approximately 430-440 bp and the difference between two sequences was approximately 19bp. A cost effective molecular marker; SSCP technique was carried out using the same PCR product to determine the utility of this technique in delineation of above two species. However, the SSCP technique failed to construct the proper secondary structures and unable to differentiate DNA fragments derived from the two species. This may be due to the formation of more stable and similar secondary structures. The possibility of using more variable gene regions in the identification of *M. rosenbergii* and *M. malcolmsonii* species using SSCP method is suggested.

Keywords: freshwater prawn, *Macrobrachium rosenbergii*, *Macrobrachium malcolmsonii*, SSCP, mitochondrial 16S rRNA gene

Introduction

Freshwater prawn farming became an economically beneficial industry in many countries all over the world. In many Asian countries freshwater prawns are highly priced product which have a high demand in both domestic and export markets. Eighty six *Macrobrachium* species are recognized as economically important and among them at least 11 species have gained great commercial value in different countries (Jayachandran, 2001).

Macrobrachium rosenbergii is the largest palamonid prawn that cultured almost 43 countries across continents. However, in south Asian countries *M. malcolmsonii* is also gain an important place in the freshwater prawn industry. Nandeesha (2003) reported that there is potential in rearing *M. malcolmsonii* both in India and China commercially. Their high growth rate, ability to farm in poly-culture with compatible fish types such as carps helps to gain more attention on this species (Radheyshyam, 2009). In Sri Lanka freshwater prawn industry is mainly based on capture fishery and still the production could not fulfill the demand of the local market. *M. rosernbergii* is the only freshwater prawn species that attempted to culture so far which is available in many brackish water ecosystems in Sri Lanka. *M. malcolmsonii* which is named as monsoon river prawn is mainly recorded from the eastern part of Sri Lanka and many occasions *M. malcolmsonii* from *M. rosrnbergii* could not be distinguished due to their high morphological similarity.

In Sri Lanka *M. macrobrachium and M. malcolmsonii* species live sympatric in nature where both species can be found (personal communication with fisheries officers- NAQDA). Adults of the two species can be identified by experienced person using characters of rostrum and the 2nd periopod. However, the identification of larval stages is a troublesome task but, this is important as some culturing programs are designed to collect larvae from the wild and introduce into grow out ponds. In this matter species identification and delineation at larval stages is important using a cost effective marker.

Single stranded conformation polymorphism method is a simple and cost effective technique and allows us to discriminate between DNA fragments of the same size which are different in their nucleotide sequence (Ortì *et al.*, 1997; Sunnucks *et al.*, 2000). The technique is widely applied in the biomedical field, but very recently used in identification and delineation of species levels of different animal groups (Livi *et al.*, 2006, Khamnamtong *et al.*, 2005, Tranah *et al.*, 2003). As sequencing has become more affordable, it is still a limiting step when the assessment of a large number of samples is required. One of the main challenges when using SSCP is to achieve high resolution in order to discriminate sequences that differ for a single nucleotide. Therefore, experimental conditions need be optimized to improve SSCP sensitivity (Hayashi, 1991, 1992; Hongyo *et al.*, 1993; Highsmith *et al.*, 1999; Liù *et al.*, 1999; Nataraj *et al.*, 1999; Yip *et al.*, 1999).

This study aimed to analyse the usefulness of the SSCP method by employing the 16S rRNA gene as a genetic marker for species identification of two economically important freshwater prawn species, *M. rosenbergii* and *M. malcolmsonii* in Sri Lanka.

Materials and Methods

Freshwater prawn samples *M. rosenbergii* and *M. malcolmsonii* were collected from Walawe River estuary and Gal-Oya river system respectively (Figure 1). Fifteen individual were collected from each species with the aid of fishermen. Muscle samples were collected from each individual and preserved in 70% ethanol at the research laboratory of the Department of Zoology, University of Ruhuna and then were transported to the Genetech Laboratory, Colombo for genetic analysis. The rest of the specimens were deposited as reference materials.

Total DNA was extracted according to the Chelex® protocol (Walsh *et al.*, 1991). Partial mitochondrial 16S region was amplified using 1471-5'CCTGTTTANCAAAAACAT3'and 1472 5'AGATAGAAACCAACCTGG3' Primers which were initially designed for freshwater crayfish by Crandall *et al.* (1999). Samples were amplified in a total volume of 25 µl containing 5 µl of genomic DNA, 2.5 µl of 10X PCR buffer, 2.5 µl of 3.5 mM dNTP, 2.5 µl of 5 µM each primer, 2.5 µl of 15 mM Mg Cl_2 and 0.25 µl of 100 mM Taq polymerase and deionised water up to 25 µl. Amplifications were performed with an initial 3min pre-denaturation at 95°c, followed by 34

cycles of denaturation at 95°C for 1 min, 55°C at 1 min and 72°C at 1 min. Final step was performed at 72°C for 5 min. The products were electrophoresed on 1% agarose gel to confirm the PCR product.

Partial sequences for 16S mitochondrial gene region of *M. rosenbergii* and *M. malcolmsonii* were obtained using ABI Genetic Analyzer, Applied Biosystems, USA sequencing machine. Three individuals were sequenced from each species and consensus sequences were determined for each species. Nucleotide differences and percentage of identity between two species was estimated. All sequence analyses were carried out using Bio Edit (version 7.0.5) program.

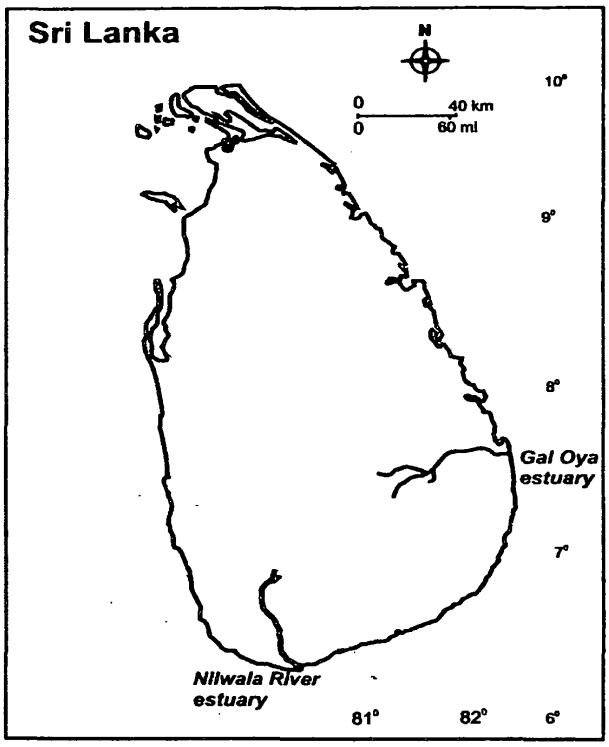

Figure 1: Map indicates two river systems where samples were collected for the current study

PCR products of 30 samples (15 samples from each species) were used to determine whether estimated nucleotide differences help to distinguish two species by SSCP analysis. Approximately 3 µl of each PCR reaction was diluted in denaturing solution, denatured at 95 °C for 5 min, chilled on ice and resolved on 6% polyacrylamide gel. The electrophoresis was carried in a vertical unit in 1x TBE buffer. Electrophoretic bands were visualized by silver staining method. SSCP gels were analysed by the naked eye. All 30 samples of the both species were loaded and run on the same gel and examined.

Results and Discussion

Total of 436 bp fragment of sequence was resulted for partially amplified 16S gene region (Figure 2). All three samples of each species produced identical sequences. A comparison (BLAST results) of derived two sequences is given in the Figure 3. Nucleotide composition of the sequence

of *M. rosernbergii* and *M. malcolmsonii* is given in the Table 1. The results indicated that both species are A+T rich in nucleotide composition. Genetic identity between two species was 93% and the number of nucleotide differences was nineteen. These results are congruent with the previous study conducted by Munasinghe (2010).

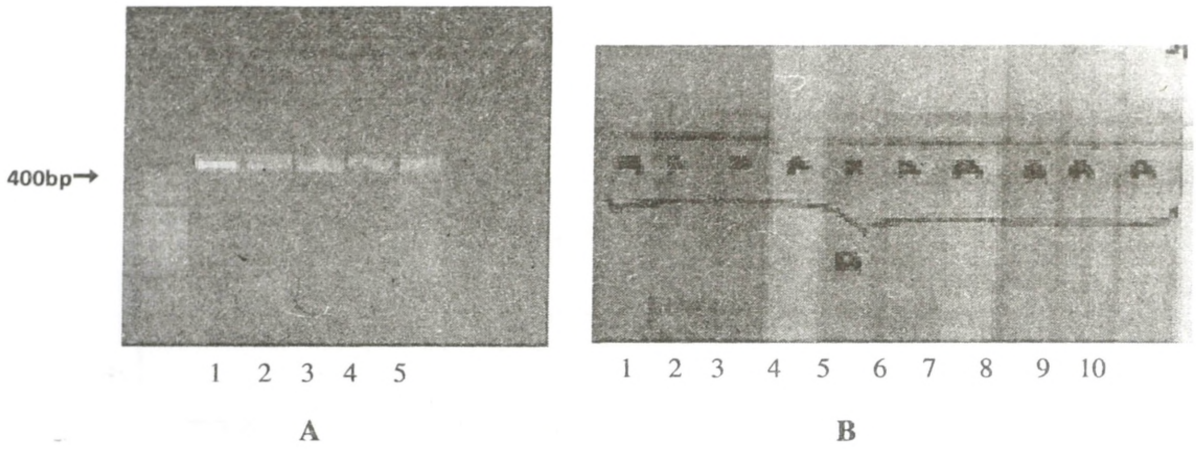

Figure 2: **(A).** A photograph of an agarose gel which shows the PCR product amplified from mitochondrial 16S rRNA gene region (430-440bp): 1-3 lanes - *M. rosenbergii*, 4-5 lanes- *M. malcolmsonii* **(B).** A photograph of a section of polyacrylamide gel with resulted banding pattern from SSCP method: 1-5 lanes - *M. rosenbrgii*, 6-10 lanes - *M. malcolmsonii*.

The results of the SSCP method are given in the Figure 2. According to observations this technique did not resolve PCR products derived from two *Macrobrachium* species. This indicates SSCP method that carried out by employing the 16S rRNA gene as a genetic marker is not supportive to delineate closely related two *Macrobrachium* species.

Table 1: Estimated nucleotide composition for partially amplified mitochondrial 16S gene region (436bp) for two *Macrobrachium* species

Species/nucleotide	A % (number)	T% (number)	C% (number)	G% (number)
M. rosenbergii	28.21% (123)	35.55% (155)	11.70% (51)	24.54% (107)
M. malcolmsonii	27.75% (121)	33.72% (147)	12.84% (56)	24.54% (111)
Nucleotide difference between two species	02	08	05	04

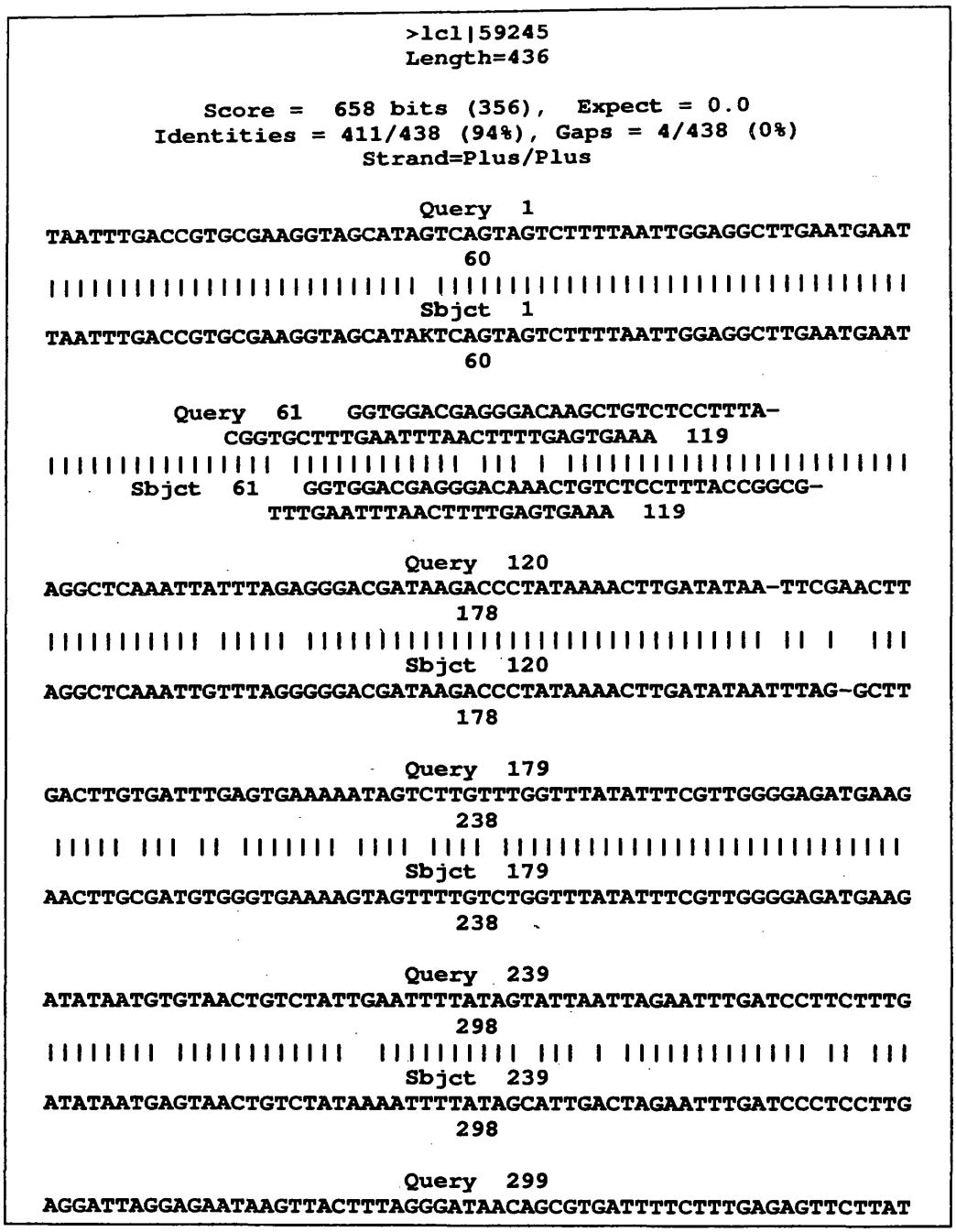

Figure 3: Comparison (BLAST results) of two nucleotide sequences derived from mitochondrial 16S rRNA gene. Upper sequence: *Macrobrachium malcolmsonii*, Lower sequence: *Macrobrachium rosenbergii*

In SSCP technique overall methods are rapid and simple if conditions are optimized. This technique first amplify target DNA fragment using specific primers and the product is denatured which result single-stranded DNA. This leads to form folded structure that is determined by intra molecular interactions. In SSCP analysis, a mutated sequence is detected as a change of mobility

in polyacrylamide gel electrophoresis caused by its altered folded structure. However, it was found that because of its high resolving power, polyacrylamide gel electrophoresis can distinguish most conformational changes caused by subtle sequence differences such as one base substitution in a several-hundred-base fragment (Hayashi, 1991). However, the detection of mutations depends on the conformational changes of the single-stranded molecule induced by the mutation, and therefore, sensitive to physical environment in the gel, e.g., temperature, concentration of ions, and solvents. Length of the fragment of the PCR product also plays a major role when forming secondary folded structures during the SSCP process. It has been found out that shorter fragments are more productive than larger fragments in SSCP analysis (Garcia–Vazquez *et al.*, 2006; Muhaghegh and Goswami, 2006).

According to the results of the current study, the SSCP method did not support to delineate two *Macrobrachium* species even the number of nucleotide difference between two species recorded as 19bp. The possible reason for this may be during the process of SSCP method both sequences form similar stable conformations which cannot be distinguished by polyacrylamide gel electrophoresis. Thorough examinations of two sequences indicated more conserved regions in between variable sequences may cause another reason for the formation of identical stable conformations. However, recently Khamnamton *et al.* (2005) successfully used SSCP analysis of 560 bp length fragment of 16S ribosomal DNA to identify five penaeid prawn species in Thailand waters. DNA fragments of the same gene region also have been used to identify fish larvae and eggs successfully (Garcia–Vazquez *et al.*, 2006).

Conclusion

Current study is significant as this is the first attempt to use SSCP method in species delineation process in Sri Lanka. This study partially amplified mitochondrial 16S gene region to distinguish two closely related *Macrobrachium* species: *M. rosenbergii* and *M. malcolmsonii.* DNA sequences of the relevant fragments indicated 19 nucleotide differences between two species. However, SSCP method did not support to differentiate the two *Macrobrachium* species. Formation of more stable, similar secondary structures could be the possibility of these consistent results. Therefore, it can be suggested to use shorter fragment of DNA sequence to perform SSCP method from more variable regions such as mitochondrial control gene region or Cytochrome Oxidase I (COI) region.

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