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POLYMERASE CHAIN REACTION (PCR) OPTIMIZATION FOR 16S rRNA GENE FRAGMENT OF CICADAS (HOMOPTERA: CICADOIDEA) FROM PENINSULAR MALAYSIA

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ABSTRACT

A molecular study to obtain the best amplification conditions for 16S rRNA gene fragment of cicadas (Homoptera: Cicadoidea) from Peninsular Malaysia was conducted and presented herewith. DNA was extracted from a total of six cicada specimens, representing species of four genera, *Platypleura*, *Purana* (Cicadidae), *Lemuriana* and *Abroma* (Tibicinidae). Successful amplification was from all three samples of the two Tibicinid genera, *Lemuriana* spp. and *Abroma maculicollis* (Guerin). For the successful amplification, the reactions of the PCR should consist of 5 µl 10X PCR buffer, 1 µl 10mM dNTPs, 4 µl 5.0 mM $MgCl_2$, 0.5 µl 20 pmol/primer, 0.5 µl 2.5U *Taq polymerase* and 36.5 µl ddH_2O. The final total volume was 50 µl. The PCR process should follow 30 cyles of denaturation (30 seconds; 94°C), annealing (120 seconds; 49°C), extention (90 seconds; 72°C) and final

extension (9 minutes; 72^{o}C). The estimated PCR product size for this gene fragment was around 600 basepairs.

ABSTRAK

Satu kajian peringkat molekul untuk mendapatkan keadaan amplifikasi terbaik bagi fragmen gen 16S rRNA riang-riang (Homoptera: Cicadoidea) dari Semenanjung Malaysia telah dijalankan dan dipersembahkan di sini. DNA telah diekstrak daripada sejumlah enam spesimen, mewakili spesies daripada empat genus, *Platypleura, Purana* (Cicadidae), *Lemuriana* dan *Abroma* (Tibicinidae). Amplikasi yang berjaya adalah daripada semua tiga sample daripada dua genus Tibicinid, *Lemuriana* spp. and *Abroma maculicollis* (Guerin). Untuk mendapatkan amplifikasi terbaik, campuran tindakbalas PCR haruslah mengandungi larutan penimbal PCR 5 µl 10X, 1 µl 10mM dNTP, 4 µl 5.0 mM $MgCl_2$, 0.5 µl 20 pmol/pencetus, 0.5 µl 2.5U *Taq polymerase* dan 36.5 µl ddH_2O. Isipadu akhir adalah 50 µl. Proses PCR haruslah mengikuti 30 kitaran yang melibatkan proses penyahaslian (30 saat; 94^{o}C), penyepuhan (120 saat; 49^{o}C), pemanjangan (90 saat; 72^{o}C) dan pemanjangan akhir (9 minit; 72^{o}C). Anggaran saiz produk PCR bagi fragmen gen ini adalah sekitar 600 pasangan bes.

INTRODUCTION

Mitochondrial DNA (mtDNA) provides useful genetic markers for phylogenetic study. Markers from this genome have been used in a great deal to address problems related to phylogenetic reconstruction, population genetic structure, dating cladogenic events, and historical biogeography (Avise 1994; Evans et al. 1999; Hillis et al. 1996). MtDNA analysis has, over the past two decades, been a popular and well suited source of data for systematic studies of potentially closely related species. The molecule's high rate of evolution, small size, easy recovery, maternal inheritance and lack of recombination, all facilitate it to be manipulated in the laboratory to provide the rich

phylogenetic informative data (Melnick et al. 1992; Melnick & Hoelzer 1993).

To date, little work has been done on the molecular systematics of the Asian- Pacific cicadas. Asian cicada systematics have been based on morphological data (e.g., Duffels & Zaidi 1999; Schouten & Duffels 2002; Azman & Zaidi 2002). Of the little molecular work, most has been on cicadas from New Zealand, Australia and New Caledonia (Buckley et al. 2002). Since there has been no publication of molecular systematic work on Malaysian cicadas, this article represents the first attempt of providing molecular approach to be used, in the near future, to possibly resolve the phylogeny of some of the Malaysian cicadas.

Preliminarily, PCR optimization to get the right PCR conditions for successful mtDNA amplification was conducted for selected specimens of cicada species of 4 genera from Peninsular Malaysia, and the results are presented herewith. In this study, the mitochondrial 16S rRNA gene was chosen mainly because this locus has been previously shown to be informative for insect systematic studies, particularly Hymenoptera (Belshaw & Quickle 1997) and cicadas (Buckley et al. 2002).

MATERIAL AND METHODS

Samples

A total of 6 cicada specimens, representing species of 4 genera, *Platypleura, Purana* (Cicadidae), *Lemuriana* and *Abroma* (Tibicinidae), from Peninsular Malaysia, was used. The specimens were collected during two scientific expeditions. The first expedition was in Tasik Chini, Pahang, in May 2004, organized by Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The second expedition was in Gunung Mandi Angin, Terengganu, in August 2004, organized by Peninsular Malaysia Forestry Department. The cicada specimens were obtained through the usual light-trapping methods (e.g., Azman & Zaidi (2004). The samples used in this study were all alcohol-preserved spesimens and they were as listed in the Table 1.

The Genomic DNA Extraction and Amplification

Total genomic DNA was extracted from tissues, using Phenol-Chloroform extraction (Hillis et al. 1996). The tissues with liquid nitrogen were ground, using a mortar and pestle, and crested to powder form before transferring them to appendorf tubes. An amount of 8-10 volume of the lyses buffer was then added together with 3 µl of RNAase (10 mg/µl) and incubated for 1½ hours in the water bath at 37^{o}C. Proteinase K (20 mg/µl) of 6 µl was added after the first incubation and continued with the second incubation at 50^{o}C for 2-4 hours in an incubator shaker. One volume of each phenol and chloroform was then added and thoroughly mixed. The mixture was then centrifuged at 6000 rpm for 15 minutes. The aqueous part was transferred to a new appendorf tube, and this phenol: chloroform process was repeated for 3-4 times. Once the process was completed, an amount of 1 ml of absolute ethanol and 20 µl 3M sodium acetate were added, and the precipitation were carried-out at -20^{o}C, overnight. The pellet obtained on the second day was dissolved in 30 µl of ddH_2O and the total DNA was kept at -20^{o}C for further steps.

The 16S rRNA amplification of 50 µl in GeneAmp 2400 thermal cycler was carried out. Two primers, 16S-F (5'-CACCGTTTTATCAAAAACAT-3') and 16S-R (5'-CGTCGATTTGAACTCAAATC-3'), were used to amplify segment of 16S rRNA gene. The PCR products were then loaded onto 1 % agarose gel electrophoresis.

RESULTS AND DISCUSSION

DNA extraction products were visualized, at 1 % agarose gel in 1 X TAE at 90V (Figs. 1 and 2). DNA sizes for all samples were estimated about 20 KB base pairs. Most of the bands were unclear and smeared. Nevertheless, successful amplification of the 16S rRNA fragment was obtained from all the three specimens of the species of the two Tibicinid genera, *Lemuriana* spp. and *Abroma maculicollis* (Guerine). The amplification was not successful for the Cicadid species (*Purana guttalaris* (Walker) (SC070); *Platypleura* sp. 1 (TC090); *Platypleura* sp. 3 (TC111)), even though the parameters, such as the concentration of the DNA

template, $MgCl_2$, and annealing temperatures, were variedly employed (Table 2). The decrease of $MgCl_2$ concentration appeared to be ineffective, since the expected results were nil for the Cicadid species. Likewise, negative results were obtained even though the concentration of the DNA template has been diluted to five times. Thus, it is believed that the annealing temperature may vary between cicadas of the two families. In view of this, further studies should be conducted, probably with various annealing temperatures and $MgCl_2$ concentrations, to obtain the expected positive results for Cicadidae species.

Fig. 3 shows the successful amplification for the Tibicinid species. The successful amplification was based on the PCR reactions that consist of 5 µl 10X PCR buffer, 1 µl 10mM dNTPs, 4 µl 5.0 mM $MgCl_2$, 0.5 µl 20 pmol/primer, 0.5 µl 2.5U *Taq polymerase* and 36.5 µl ddH_2O. The final total volume for the PCR reaction was 50 µl. The PCR process was conducted in the GeneAmp 2400 thermal cycler with 30 cyles of denaturation (30 seconds; 94°C), annealing (120 seconds; 49°C), extension (90 seconds; 72°C) and final extension (9 minutes; 72°C). Based on the visualization on agarose gel, the PCR product size for 16S rRNA gene fragment was estimated to be around 600 basepairs.

In any PCR optimization, the right mixture of PCR reaction components is important in getting positive result with a good and clear band. This is because polymerase chain reaction has a degree of sensitivity and specificity to species used. This is found to be true when we compared the PCR conditions of this study with those of other insects, such as fruitflies and wasps (Table 3). The temperature for denaturation for Tibicinid cicada of this study was the same as that of fruit flies, *Bactrocera*, (Izhan-Shahrin (2003) but different from that of the wasps (Hymenoptera:Ichneumonidae) (Hoh 2003). However, for annealing and extention process, the required temperature was the same for the Tibicinid cicadas and Ichneumonidae but different from the fruit flies. In any PCR, it is very difficult to provide a specific condition as the best or optimum for amplification for varied samples. This is because the interaction between PCR components is very complexs especially between primers and DNA samples (Hoong 2001). Incidentally, this study managed to

show the optimum PCR conditions required to amplify 16S rRNA for the Tibicinid cicadas. However, further studies need to optimize the PCR conditions suited to show their specificity and sensitivity to the Malaysian Cicadid cicadas. When these could be obtained, then the following procedure of DNA sequencing could be carried out to provide the currently much needed molecular systematic data to clarify phylogenetic relationships among Malaysian cicadas, species of various genera of the two families, Cicadidae and Tibicinidae.

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Table 1 Details of the samples

Taxa Cicadidae	**Code No.**	**Sex**	**Locality**
Purana guttilaris (Walker)	SC 070	M	Sg Cemerung, Mandi Angin, Terengganu
Platypleura sp. 1	TC 090	M	Tasik Chini, Pahang
Platypleura sp. 3	TC 111	F	Tasik Chini, Pahang
Tibicinidae *Lemuriana* sp.	SC 120	F	Sg, Cemerung, Mandi Angin, Terengganu
Lemuriana sp.	SC 123	M	Sg, Cemerung, Mandi Angin, Terengganu
Abroma maculicollis (Guerin)	SC 130	M	Sg, Cemerung, Mandi Angin, Terengganu

Table 2 DNA template dilution and the amplification product of the samples

No.	Taxa	Code No.	Concent	DNA Dilution -ration(10^{o}x)	Amplification result
1	**Cicadidae** *Purana guttalaris* (Walker)	SC 070	1/3x	10^{-1}x- 10^{-5}x	NIL
2	*Platypleura* sp. 1	TC 090	3x	10^{-1}x- 10^{-5}x	NIL
3	*Platypleura* sp. 3	TC 111	3x	10^{-1}x- 10^{-5}x	NIL
4	**Tibicinidae** *Lemuriana* sp.	SC 120	3x	10^{-1}x	Yes
5	*Lemuriana* sp.	SC 123	3x	10^{-1}x	Yes
6	*Abroma maculicollis* (Guerine)	SC 130	1.5x	10^{-1}x	Yes

The concentration of the total DNA was based on the comparison with the first band of ë-Hind III (50 ng/µl).

Table 3 PCR optimum conditions of 16S rRNA amplification of the studied Tibicinid cicadas in comparison with fruit flies and wasps

	Tibicinid cicadas (This study)	**Fruit flies Izhan-Shahrin (2003)**	**Wasps Hoh (2003)**
ddH_2O	36.5 µl	36.5 µl	32.5 µl
10X PCR buffer	5 µl	5 µl	5 µl
10mM dNTPs	1 µl	1 µl	1 µl
Each primer	0.5 µl (20pmol)	0.5 µl (20pmol)	0.5 µl (20pmol)
$MgCl_2$	4 µl (5.0mM)	4 µl (5.0mM)	8.0 µl (3.0mM)
2.5U *Taq polymerase*	0.5 µl	0.5 µl	0.5 µl
DNA template	2 µl	2 µl	2 µl
Denaturation	30 seconds 94°C	30 seconds 94°C	50 seconds 94°C
Annealing	120 seconds 49°C	15 seconds 51°C	120 seconds 49°C
Extention	90 seconds 72°C	4 minutes 72°C	90 seconds 72°C
Final extension	9 minutes 72°C		9 minutes 72°C
Cycle numbers	30	37	35

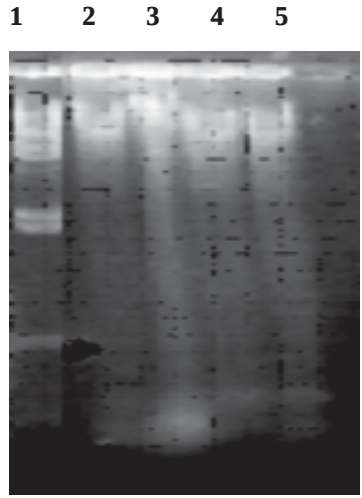

Fig. 1 DNA extraction product of *Lemuriana* and *Platypleura***.** Lane 1, λ-Hind III; Lane 2, *Lemuriana* sp. (SC120); Lane 3, *Lemuriana* sp. (SC123); Lane 4, *Platypleura* sp. 1 (TC090); Lane 5, *Platypleura* sp. 3 (TC111).

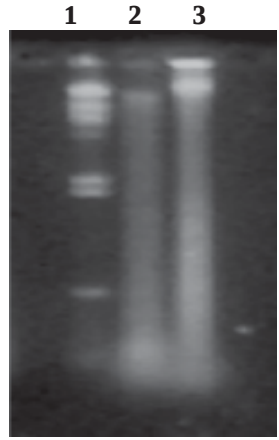

Fig. 2 DNA extraction product of *Purana* and *Abroma***.** Lane 1. λ-Hind III; Lane 2. *Purana gittalaris* (SC070); Lane 3. *Abroma maculicollis*. (SC130)

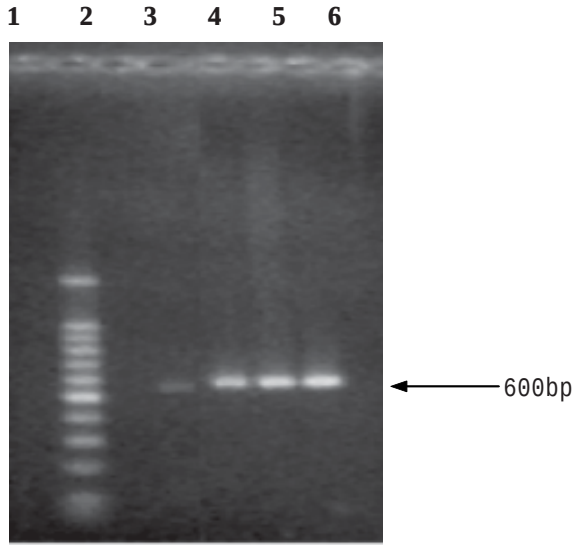

Fig. 3 PCR product of 16S rRNA. Lane 1, Marker 100bp; Lane 2, Negative control; Lane 3, Positive control (Braconid wasp from Mr. Mazlan); Lane 4, *Lemuriana* sp. (SC120); Lane 5, *Lemuriana* sp. (SC123); Lane 6, *Abroma maculicollis* (SC130)