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Optimization of Polymerase Chain Reaction (PCR) of Mitochondrial Cytochrome c Oxidase I (COI) Gene in Two Bornean Fanged Frogs

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ABSTRACT

Limnonectes kuhlii and *Limnonectes leporinus* are two of the Bornean fanged frogs (without advertisement call) which are widely distributed, thus thought to exhibit different evolutionary lineages and the existence of genetically cryptic species. Yet, the two species are still under study especially at the molecular level. Hence, cytochrome c oxidase I (COI) of mitochondrial gene was used to investigate suitable parameters for DNA amplification using the Polymerase Chain Reaction (PCR) method. Three PCR programmes (varied in the temperatures and period of each PCR step) were employed to identify the most efficient parameters in amplifying PCR products for both species. From the three programmes, Programme B (Initial denaturation: 96°C for 5 min; denaturation: 95°C for 45 sec; annealing: 48-53°C for 1 min 30 sec; extension: 72°C for 1 min 30 sec; final extension: 72°C for 10 min, 30 cycles) showed the highest percentage (53%) of optimal PCR products. The other two programmes showed non-specific products or "primer-dimers". The results also suggest that the annealing temperature of 52°C, 0.025-0.05 units/µl of 1.5mM *Taq* polymerase, 0.04 mM of dNTPs mix and optimal concentrations of magnesium in 50 µl of reaction mixture were sufficient enough to amplify high quality PCR products for both species. However, using Programme B, the re-amplification of the PCR products yielded "primer-dimer". In addition, a 'Hot-Start' PCR method was also applied and mostly yielded in an optimal PCR amplification. Nevertheless, further research on the second amplification of the two species should be conducted to determine the causes of the primer-dimer production.

Keywords: **Polymerase Chain Reaction (PCR) conditions, optimization, annealing temperature, Hot-Start PCR**

INTRODUCTION

Bornean fanged frogs, categorized under the sub-genus *Limnonectes* in the family of Ranidae (Dubois, 1992; Emerson and Ward, 1998; Frost *et al*., 2006) are divided into four species, namely *Limnonectes leporinus*, *L. ingeri*, *L. kuhlii*, and *L. ibanorum* (Inger, 1996; Emerson and Inger 1992; Dubois, 1992; Frost *et al*., 2006). Most of the *Limnonectes* species (except for *L. kuhlii*) are grouped in the *grunniens* (Emerson and Ward, 1998), and consist all the putative species which are difficult to identify due to the high similarity in their external morphology. Hence, there is confusion in taxonomically categorizing the species and its systematic relationship using the conventional methods (Emerson and Ward, 1998). With the revolution of the molecular techniques, the studies on the phylogenetic and taxonomy of the Bornean fanged frogs can be applied as an alternative method (Avise, 1994; Duellman and Trueb, 1994).

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One of the steps which are essential in any molecular technique is the polymerase chain reaction (PCR) amplification (Mullis, 1990; Mullis and Faloona, 1987). Polymerase chain reaction (PCR) is a technique which is used to amplify the number of copies of a specific region of DNA to produce enough DNA to be adequately tested (Mullis and Faloona, 1987). Basically, it works on two designated and oriented primers; anneal complementary to the 'targeted' regions of the denatured DNA templates that are to be amplified and continued with primer extension (Mullis, 1990; Mullis and Faloona, 1987). The cycle of denaturation, annealing and extension is repeated, resulting in exponential copies of the targeted regions, approximately 2^n where n is the number of performed amplification cycles (Mullis, 1990; Mullis and Faloona, 1987). A PCR mixture reaction contains DNA template, primers, *Taq* polymerase, deoxynucleotide triphosphates mix (dATP, dCTP, dGTP, and dTTP), 10X reaction buffer that contains Tris-HCL (pH 7.5-9.0), KCl and additive(s) to increase the PCR reaction, and finally $MgCl_2$ (Erlich *et al*., 1991). Previous studies (Saiki, 1989; Erlich *et al*., 1991) showed that the components of PCR, as well as the PCR protocols such as temperature and duration of each step, could influence the yield of the PCR amplification.

The main objective of this study is to optimize the PCR amplification of *Limnonectes leporinus* and *Limnonectes kuhlii* by modifying the concentrations of certain components in the reaction mixtures, the annealing temperatures and duration of each phase of the COI primers.

MATERIALS AND METHODS

Samples Collection

Samples of *Limnonectes leporinus* and *Limnonectes kuhlii* were collected from Santubong (1°44'00.00"N 110°20'00.00"E), Matang Ranges including Matang Wildlife Center and Kubah National Park (1°36'44.61"N 110°11'38.47"E) and Bau District (1°21'05.45"N 110°14'16.81"E), which are in the Kuching Division. Muscle tissues were extracted from the hind legs of the captured frogs and these were then preserved in vials containing 20% DMSO, and 0.25M EDTA before they were kept in -20°C freezer.

PCR Analysis

Total genomic DNA was extracted following the protocols of Pure-Gene™ Tissue DNA Kit (BioSynTech, Subang Jaya). The extracted DNA was subsequently amplified using the Whatman Biometra® (050-551 T-*personal* 48). Two universal mitochondrial DNA primers, namely COI-f (forward) and COI-e (reverse) with the sequences of '5-CCTGCCGGAGGAGGTGAYCC-3'and '5-CCAGTAAATAACGGGAATCAGTG-3' (Palumbi *et al*., 1991), were used in the study. The optimization of the PCR amplification was done by varying the concentrations of Mg^{2+} and *Taq* DNA polymerase (Fermentas) (*see* Table 1), following Arnheim (1992). Three PCR programmes were designated for the optimization procedures (Table 2), following Emerson and Ward (1998) and Ramlah (1998). The amplification products were electrophorese to check for successful amplification products. Successful PCR products were excised from the gel (without purification) and re-amplified for the second PCR amplification (weight, per comm.).

TABLE 1
PCR reaction mix used for DNA amplification

PCR component	Volume in a 50 µl reaction mix, µl	Final concentration in a 50 µl reaction mix, mM except *Taq* in units/µl
10X PCR reaction buffer[a]	5	1X (10 mM of Tris-HCl; 50 mM KCl)
$MgCl_2$	2.0-4.0	1.0-2.0 mM
Deoxynucleotide triphosphates mix (dATP, dCTP, dGTP and dTTP)	1.0	0.04 mM of each dATP, dTTP, dCTP and dGTP
Taq polymerase	0.25-0.5	0.025-0.05 units /µl
Primer CO1-e	2.5	$7.56X10^{-3}$ mM
Primer CO1-f	2.5	$3.28X10^{-2}$ mM
DNA template	1-4	Variable[b]

[a]1 ml of 10X PCR Reaction Buffer contains 100mM Tris-HCl, 500 mM KCl, and 0.8% Nonidet P40.
[b]Depending on the concentration of the total genomic DNA extraction.
Note: Sterile distilled water was added for each PCR reaction mix to maximize the volume of 50 µl.

TABLE 2
Three PCR programmes with different sets of parameters, the number of reaction performed and cycling temperatures, following Emerson and Ward (1998) and Ramlah (1998)

PCR programme	Cycling temperature	
A	Denaturation: 94˚C for 30 sec Annealing: 43˚C for 30 sec Extension: 72˚C for 45 sec	5 cycles
	Denaturation: 94˚C for 30 sec Annealing: 45˚C for 30 sec Extension: 72˚C for 45 sec	25 cycles
	Storage: 4˚C for forever	
B	Initial denaturation: 96˚C for 5 min Denaturation: 95˚C for 45 sec Annealing: 48-53˚C for 1 min 30 sec Extension: 72˚C for 1 min 30 sec Final extension: 72˚C for 10 min	30 cycles
	Storage: 4˚C for forever	
C	Initial denaturation: 95˚C for 5 min Denaturation: 94˚C for 45 sec Annealing: 52˚C for 1 min 30 sec Extension: 72˚C for 1 min 30 sec Final extension: 72˚C for 10 min	30 cycles
	Storage: 4˚C for forever	

Note: Programmes A and C were tested on 2, and 52 PCR reactions, respectively. In Programme B, an amount of the PCR reactions in the bracket was prepared based on the respective annealing temperatures: 48˚C (2); 49˚C (2); 50˚C (3); 51˚C (17); 51.5˚C (33); 52˚C (78); 52.5˚C (5); and 53˚C (5).

The visualization step was repeated and the successful PCR product was then purified to remove any excess of reaction components. For the Hot-Start PCR, *Taq* polymerase was added into an uncompleted reaction mixture incubated in a thermal cycler at a temperature between 80°C- 82°C before entering the annealing stage of the first cycle.

The PCR results were calculated as the percentage of the total positive reaction products (positive results that consist either single or multiple bands and primer dimer) per total reaction samples. In addition, positive results were further divided into three categories of single band, multiple bands and primer dimer. Meanwhile, the percentage was calculated as the total products (single band/ multiple band or primer dimer) per total positive results. Only single band PCR products were considered to be successful.

RESULTS AND DISCUSSION

Cycling Parameter and Mg^{2+} Concentration

The results showed that the annealing temperatures between 48°C and 52°C (*Figs 1* and *2a,b*) were sufficient to produce PCR products (*see* Table 3). However, the 500bp PCR products produced the brightest band at 52°C (*Fig. 3*). Programme B was more preferable than Programme C due to the highest occurrence of primer–dimer products (69.1% out of 30.9 % of positive results) in the latter programme (*see* Table 4). The magnesium concentrations of 1.5 mM and 1.75 mM yielded good PCR products, either using the Programme B, or C with an annealing temperatures ranging from 51°C to 52°C (*Fig. 3*). The result is consistent with the cycling parameter and Mg concentration used by Ramlah (1998: 2009) in producing good PCR products.

TABLE 3

Overall results of PCR products in three various programmes; A, B and C

No. of PCR reaction	Program (Annealing temperatures, °C)	Positive result (%)	Negative result (%)
2	A (43-45˚C)	-	100
132[a]	B (48-53˚C)	53.3	44.7
52	C (52.0˚C)	33.9	66.6

[a]refer to the note listed under Table 2

TABLE 4

Detail results of PCR products using Programmes B and C (with annealing temperature of 51-52°C)

No. of reaction samples	PCR programme	Parameter (°C)	Obtained results in %				
			Positive results	Single band		Multiple bands	Primer-dimer
				Good	Poor		
15	B	51.0	60.0	-	20.0	40.0	40.0
30		51.5	73.3	-	20.0	53.3	26.7
72		52.0	59.7	26.4	29.2	4.1	40.3
55	C	52.0	30.9	23.6	7.3	-	69.1

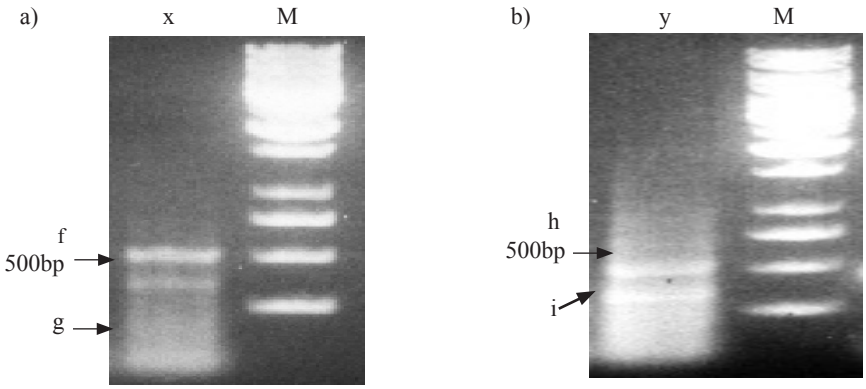
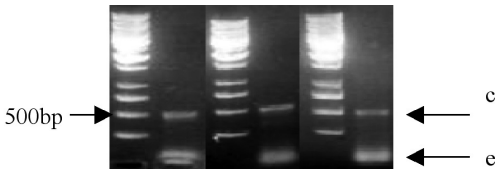
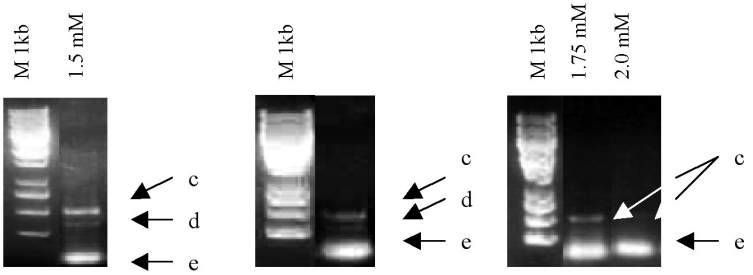


Fig.1: PCR products (bands f and h) and truncated products (bands g and i) of L. leporinus *of Matang obtained at annealing temperatures of 49˚C (lane x) and 48˚C (lane y). The PCR products in lane y were the least purified as compared to lane x. M= 1kb Ladder (Fermentas)*



(a) Annealing temperature



(b) [Mg^{2+}]

Fig. 2: (a) Reaction mixtures of Limnonectes leporinus *from Bau contained 0.025 units/µl Taq polymerase, 10 mM Tris HCl, 50 mM KCl, 0.04 mM dNTPs, 1.5 mM Mg^{2+}, and 3µl template. Amplification used Programme B with annealing temperature ranging from 51˚C to 52˚C. (b) Reaction mixtures were prepared as in (a) except with a variation in Mg^{2+} concentrations from 1.5 mM (o), 1.5mM with HS (p), 1.75mM (r) to 2.0 mM (s). Amplification was at 52˚C (H. S. = Hot-Start PCR). Bands c were first amplification products and Bands d were non-specific products. Bands e were primer-dimers. M= 1kb Ladder (Fermentas)*

Enzyme Concentration and Hot-Start PCR

An amount of 0.025 units/µl of *Taq* successfully amplified eight PCR products of *L. kuhlii* from the Santubong area (*Fig. 3*). However, poor products were mostly produced when it was tried on *L. leporinus* (*Fig. 4*). The optimization of *Taq* polymerase concentrations, ranging from 0.035 units/µl to 0.05 units/µl, successfully produced PCR products (*Fig. 4*) at the 0.025 units/µl. This is consistent with the result obtained by Ramlah (2009) when the same concentration of *Taq polymerase* used was found to produce good PCR products of the *Hylarana* frog. Additionally, the Hot-Start PCR produced far better results than the conventional PCR method (see *Fig. 4*).

PCR Cycle Profile and Hot-start Method

The success of the PCR amplification in generating clean products is determined by the PCR cycling system. Temperature and duration time for each step in a PCR profile must be perfectly suitable to initiate the amplification processes. Some previous studies showed that the success of amplification was determined by the annealing temperature which ranged between 50°C to 72°C (Ramlah, 1998; Ramlah, 2009; Roux, 1995; Innis and Gelfand, 1990). Hoelzel and Green (1992) reported that annealing temperature is influenced by the sequence and length of the primers used and the targeted DNA. In this study, the amplified products were detected at the annealing temperature above 48°C (*Figs. 2* and *3*) although they were contaminated with background smear and non–specific products. The emergence of the unwanted or false products was due to mis-incorporation between the primer(s) and template, or mis-extension of incorrect nucleotides at the 3' end of the primer(s) (Innis and Gelfand, 1990; Kidd and Ruano, 1995).

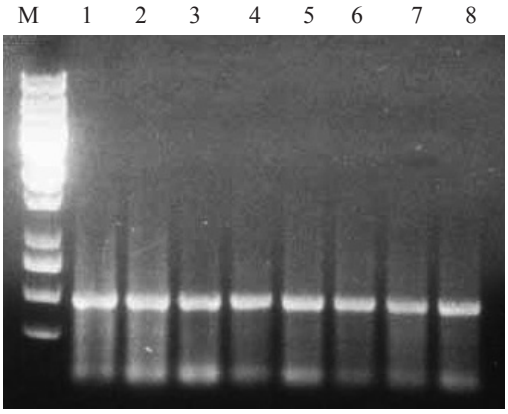


Fig. 3: PCR products of Limnonectes kuhlii *of Santubong from the first successful amplifications using programme B (52˚C). The reaction mixtures were 10 mM Tris-HCl; 50 mM KCl, 1.5 mM Mg^{2+}, 0.025 units/µl Taq and 3.5 µl of template. M= 1kb Ladder (Fermentas)*

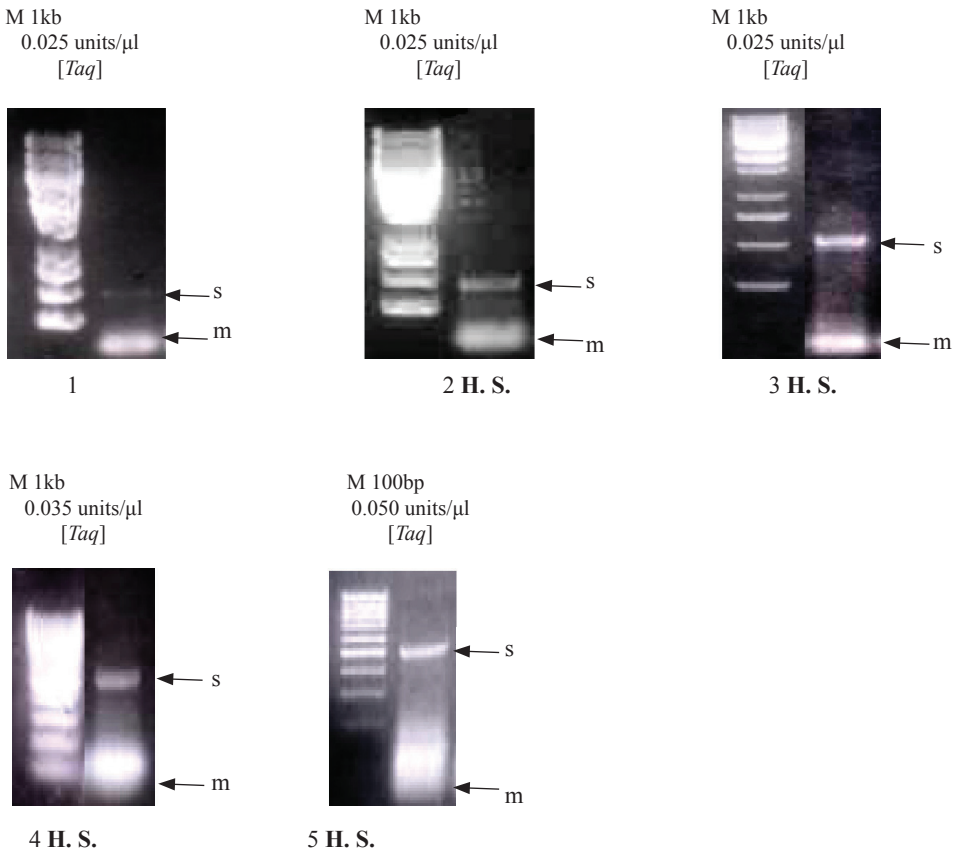


Fig. 4: The reaction mixtures of Limnonectes leporinus *samples taken from Bau were prepared as in Fig. 3, with a range of enzyme variations from 0.025-0.050 units/µl and concentrations of template (s= 500 bp PCR products; m= primer-dimer; Lanes 1, 2 and 4= First amplification products; Lanes 3 and 5= Second amplification products; H.S.= Hot-Start PCR). The template volumes of 3.5 µl, 3.0 µl, 1.0 µl, 4.0 µl, and 2.0 µl were pipetted respectively in the reaction mixtures of lanes 1, 2, 3, 4, and 5. M= Gene Ladder (Fermentas)*

When, the temperature was increased to 52°C, no production of spurious products was observed (*Fig. 3a*). In addition, the duration of annealing time within a range of 30 sec to 2 min could avoid the disturbances of the secondary structure in the template and the concentration of primer(s) although other study showed that shorter time was sufficient for hybridization (Hoelzel and Green, 1992). Hence, the duration of 1 min and 30 sec was sufficient to permit all the primers to bind to the target regions.

The denaturation step can influence the PCR amplification as incomplete separation of the targeted template and/or amplified products in initial denaturation, and further cycles will cause a formation of "primer-dimer", while an amplified double-stranded fragment was short in length, i.e. roughly 50bp. Therefore, both Programmes B and C had 5 min initial denaturation before the first cycle was begun. Preference of Programme B than C may be due to the longer strands, and/or the target region of the COI gene of those two species which are rich in G+C bases (Kidd and Ruano,

1995). The temperature and period of denaturation in Programme B was not too high and too long, respectively, for a *Taq* polymerase to degrade quickly as it has a half-life activity of about 5-6 min (Gelfand and White, 1990). The extension step, at 72°C for 45 sec, was seemingly sufficient to polymerize complementary all annealed temperatures.

The number of cycles may have an impact on the efficiency of the PCR amplification. Thirty cycles in the three programmes were proven to yield enough amplification products; however, some of them were very low in yield or contaminated, which were probably due to fewer cycles or excess in the initial DNA template in the sample reactions (Kidd and Ruano, 1995). For the re-amplified PCR products, substantial primer-dimers could be seen, which might be due to the emergence of a "plateau effect" after 20-25 cycles in the secondary amplification (Innis and Gelfand, 1990). Many factors, such as competition for reactants between primer-dimers and targeted products, re-annealing of targeted product at concentrations above 10^8 M, and insufficient denaturation of DNA at higher DNA concentration may contribute to the effect. Hence, it is essential to maintain the copied template at a lower concentration. The application of the Hot-Start PCR method (Roux, 1995; Brownie *et al.*, 1997) can halt the non-specific hybridization of primer and template by mixing the reaction mixture together at a temperature above the annealing temperature. The substitute method has been proven to be efficient in producing outstanding results in most sample reactions in this study.

Optimization of the Buffer Components

Generally, the concentration of $MgCl_2$, *Taq* polymerase and template DNA can influence the specificity, and efficiency of the PCR amplification (Saiki, 1989). According to Kidd and Ruano (1995), too much magnesium will initiate spurious binding of primer(s) to incorrect template region that will lead to an accumulation of undesired products, while too little will disturb the extension reaction, and lead to amplified products reduction. Gelfand (1989) and Chamberlain and Chamberlain (1994) reported that the exact requirement of the ionic concentration was dependent on the concentration of dNTPs. As for the *Taq* concentration, a range within 0.025-0.050 units/µl can seemingly be used; however, it depends on the initial template or/and copied template concentration in the PCR reaction buffer. Too much template could inhibit the amplification although a maximum amount of 0.050 units/µl of the enzyme had been used. In other cases, an amount of 0.035 units/µl of *Taq* had been tried, but the outcomes were non-specific products (*Fig. 4*). In this experiment, the amount of 0.050 units/µl produced desired products in some sample reactions. It is assumed that the DNA template is not the only main factor for the amplification of non-specifics products, as it may also be due to the primer itself (Innis and Gelfand, 1990), or the concentration of magnesium (Viguera *et al*., 2001).

Additionally, storage buffer (20% DMSO) used for preserving frogs' muscle tissues might not be fully washed out from the DNA extraction and carried into the PCR reaction mixture, and this thus disturbed the activity of *Taq* to polymerize annealed primer(s). More than 10% of DMSO could lessen *Taq* activity by 50%, and thereby decreased the yield (Hoelzel and Green, 1992). Moreover, the addition of an additive, Nonidet P40 in PCR reaction buffer could reverse the DMSO effect. Previous experiments had proved that 5% of NP40 could increase the *Taq* activity (Hoelzel and Green, 1992).

CONCLUSIONS

PCR parameters and cycling temperatures of initial denaturation: 96°C for 5 min; denaturation: 95°C for 45 sec; annealing: 48-53°C for 1 min 30 sec; extension: 72°C for 1 min 30 sec; final extension: 72°C for 10 min, 30 cycles) were adequate to produce optimal PCR products. The results also suggest that the annealing temperatures of 52°C, 0.025-0.05 units/µl of 1.5mM *Taq* polymerase, 0.04 mM of dNTPs mix and optimal concentrations of magnesium in 50 µl of reaction mixture were sufficient to amplify high quality of PCR products for both species. Moreover, the Hot-Start PCR was found to be effective in yielding intended PCR products.

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