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*Methodology*

# Optimization of PCR conditions to amplify Cyt *b*, COI and 12S rRNA gene fragments of Malayan gaur (*Bos gaurus hubbacki*) mtDNA

**M.K.A. Rosli[1], A.S. Zamzuriada[1], S.M.F. Syed-Shabthar[1], M.C. Mahani[1], O. Abas-Mazni[2] and B.M. Md-Zain[1]**

[1]School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia
[2]Agro-Biotechnology Institute, Malaysia, Serdang, Selangor, Malaysia

Corresponding author: B.M. Md-Zain
E-mail: abgbadd@ukm.my / abgbadd1966@yahoo.com

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**ABSTRACT.** PCR has been extensively used for amplification of DNA sequences. We conducted a study to obtain the best amplification conditions for cytochrome *b* (Cyt *b*), cytochrome c oxidase I (COI) and 12S rRNA (12S) gene fragments of Malayan gaur mtDNA. DNA from seven Malayan gaur samples were extracted for PCR amplification. Various trials and combinations were tested to determine the best conditions of PCR mixture and profile to obtain the best PCR products for sequencing purposes. Four selected target factors for enhancing PCR, annealing temperature, concentration of primer pairs, amount of *Taq* polymerase, and PCR cycle duration, were optimized by keeping the amount of DNA template (50 ng/µL) and concentration of PCR buffer (1X), $MgCl_2$ (2.5 mM) and dNTP mixture (200 µM each) constant. All genes were successfully amplified, giving the correct fragment lengths, as assigned for both forward and reverse primers.

 

The optimal conditions were determined to be: 0.1 µM primers for Cyt *b* and COI, 0.3 µM primers for 12S, 1 U *Taq* polymerase for all genes, 30 s of both denaturation and annealing cycles for Cyt *b*, 1 min of both stages for 12S and COI and annealing temperature of 58.4°C for Cyt *b*, 56.1°C for 12S and 51.3°C for COI. PCR products obtained under these conditions produced excellent DNA sequences.

**Key words:** PCR; Malayan gaur; *Bos gaurus*; Cyt *b*; 12S rRNA; COI

## INTRODUCTION

In recent years, geneticists have discovered the usefulness of molecular data and its deployment in several studies such as phylogenetics, population studies and molecular systematics (Md-Zain et al., 2008, 2010a,b; Lim et al., 2010; Zainudin et al., 2010). A very popular basic technique in molecular research is the polymerase chain reaction (PCR). PCR is a rapid and powerful technique for the *in vitro* amplification of DNA. Basically, PCR enables researchers to amplify or clone, in a test tube, assayable quantities of almost any desired piece of DNA (Avise, 2004). The use of PCR technique has massively expanded among molecular scientists because of its ability to amplify target regions of template DNA in a much shorter time compared to other amplification methods, by the repetition of typically 30-50 replication cycles that double the target DNA molecules at each cycle (Lalam, 2006). Exploration and understanding of principles of the PCR itself can help ensure a good PCR product with less time and cost. Because of complex interactions among the components of PCR and the wide variety of its application, it is very unlikely that one set of amplification conditions would be optimal for all situations (Innis et al., 1990). Several changes can be made to the PCR composition in order to optimize the reaction (Gibson et al., 1996). Some parameters that should be of concern in order to facilitate the optimization process are chemical concentrations ($MgCl_2$, dNTP mix, PCR buffer, and *Taq* polymerase), primers used and PCR conditions that include the highest annealing temperature, optimal cycle number and amplification duration (Ernie-Muneerah et al., 2005; Tabone et al., 2009). A typical PCR consists of three stages: 1) a denaturation stage (1-2 min); 2) a primer annealing (hybridization) stage (1-2 min), and 3) an extension stage (1-2 min) for several cycles (Cha and Thilly, 1993).

A study, based on PCR method, was conducted on seven Malayan gaur genomic DNA samples to optimize the right PCR conditions for successful mitochondrial DNA (mtDNA) amplification. Locally known as seladang, the Malayan gaur (*Bos gaurus hubbacki*) is the only remaining wild cattle that can be found in Peninsular Malaysia. Gaurs live in the wild and face an increasing threat to extinction. There are an estimated 13,000 to 30,000 wild gaurs globally with the population of mature individuals between 5200 to 18,000 (Nguyen et al., 2007). Wild populations of gaur have declined significantly in Malaysia (Conry, 1989). Globally, *Bos gaurus* is currently classified as vulnerable by the International Union for the Conservation of Nature Red List. The Malayan gaur has been declared as a "Totally Protected" animal under the Wildlife Protection Act 76/72, Schedule I (wild animals) by the Malaysian government. Since 1982, *in situ* conservation efforts for the Malayan gaur by the Department of Wildlife and National Parks (DWNP) include stages to prevent its extinction in response to the growing concern that survival in the wild may be threatened by severe habitat reduction (Sahir, 2001).

 

Three genes of interest that lie in mtDNA were selected: cytochrome *b* (Cyt *b*), cytochrome c oxidase I (COI) and 12S rRNA (12S). Cyt *b* has been extensively used in molecular studies especially in systematics (Ang et al., 2011; Rosli et al., 2011). Located in the functional area of mtDNA with high definition of phylogenetic information, the variation ratio of the Cyt *b* gene sequence is higher than that of other functional areas (Bataille et al., 1999). It can also be detected easily using molecular approaches. The 12S gene is a small subunit of ribosomal DNA in mtDNA. It is considered as one conserved gene between taxa that is helpful for phylogenetic analysis (Matthee and Davis, 2001; Vun et al., 2011). According to Simon et al. (1990), this gene has an evolutionary rate that is almost similar to the evolutionary rate of mtDNA itself. On the other hand, the COI gene appears to possess a greater range of phylogenetic signal than any other mtDNA regions (Hebert et al., 2003). Compared to other protein-coding genes, there is a high rate of base substitution on its third-position nucleotides, leading to a rate of molecular evolution that is about three times greater than that of 12S or 16S rRNA (Knowlton and Weight, 1998). In general, each of the three genes used in this study had its own characteristic to be assayed by PCR. There are many conducted studies (Gunson et al., 2003; Lardeux et al., 2008) that can help improve PCR optimization.

In our study, optimization assays were conducted by using a set of constant parameters to simplify optimization stages. The results of the optimized PCR condition of these three genes are presented herewith.

## MATERIAL AND METHODS

### Samples and DNA extraction

A total of seven Malayan gaur samples from blood and tissues were used in this study. All seven samples were taken from a captive population in Wildlife Conservation Centre, DWNP, Jenderak, Pahang. All samples, as listed in Table 1, were collected by Agro-Biotechnology Institute (ABI), Malaysia, and DWNP. Total genomic DNA was extracted using the QIAGEN DNeasy Blood & Tissue Kit and the DNA were kept at -20°C for storage.

**Table 1.** Details of the Malayan gaur samples used in this study.

| No. | Name | Type of sample | Source |
|---|---|---|---|
| 1 | Awani | Blood | ABI |
| 2 | Waja | Blood | ABI |
| 3 | Seroja | Blood | ABI |
| 4 | Nino | Blood | ABI |
| 5 | Sarum | Tissue | DWNP |
| 6 | BGH 8 | Tissue | DWNP |
| 7 | BGH 17 | Tissue | DWNP |

*ABI = Agro-Biotechnology Institute, Malaysia; DWNP = Department of Wildlife and National Parks.

### Oligonucleotide primers and PCR

Annealing temperature for each universal primer pairs (Cyt *b*, 12S and COI) was first estimated by using the equation of Wallace rule ($T_m$ = 64.9 + 41 * (G+C-16.4) / (A+T+C+G))

(Wallace et al., 1979). Primers and estimated annealing temperature ($T_m$) details are listed in Table 2. PCR chemicals used in this study were obtained from Vivantis (Malaysia). DNA amplifications by PCR were initially carried out using PCR chemical concentrations (Table 3) suggested by the Vivantis manufacturer in a MultiGene Gradient PCR Machine (Labnet International Inc., Edison, NJ, USA) before further optimization stages in a Master Cycler (Eppendorf) and PTC-100 Thermal Cycler (MJ Research Inc., Waltham, MA, USA). Initial PCR was performed in a 25-µL mixture containing approximately 50 ng/µL DNA template; 1X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.1 at 20°C, and 0.1% Triton X-100); 400 nM concentration of each of the PCR primers; a 2.5 mM concentration of $MgCl_2$; a 200 µM concentration (each) of dATP, dCTP, dGTP, and dTTP) and 2 U *Taq* polymerase. Initial PCR consisted of a preheating at 95°C for 4 min; 30 cycles of 95°C for 30 s, gradient ranged annealing temperature for 30 s and 72°C for 1 min, and incubation at 72°C for 7 min. Negative controls, which contained all elements of the reaction mixture except DNA, were assigned. Successful bands were detected on 1.5% agarose gel in 1X TAE buffer.

**Table 2.** Details of forward and reverse primers used for PCR amplification.

| Primers | Sequences 5'-3' | Length (bases) | Amplicon size | G + C (%) | Estimated $T_m$ | Reference |
|---|---|---|---|---|---|---|
| 12SL1091 (12S For) | CTG GGA TTA GAT ACC CCA CTA T | 22 | 450 bp | 45 | 53°C | Kocher et al., 1989 |
| H1478 (12S Rev) | GAG GGT GAC GGG CGG TGT GT | 20 | 450 bp | 70 | 60°C | Kocher et al., 1989 |
| R-L14724 (Cyt *b* For) | CGA AGC TTG ATA TGA AAA ACC ATC GTT G | 28 | 1140 bp | 39 | 57°C | Kocher et al., 1989 |
| UH15155 (Cyt *b* Rev) | GGA ATT CAT CTC TCC CGG TTT ACA AGA C | 28 | 1140 bp | 46 | 60°C | Irwin et al., 1991 |
| LCO1490 (COI For) | GGT CAA CAA ATC ATA AAG ATA TTG G | 25 | 658 bp | 32 | 51°C | Herbert et al., 2003 |
| HCO2198 (COI Rev) | TAA ACT TCA GGG TGA CCA AAA AAT CA | 26 | 658 bp | 35 | 53°C | Herbert et al., 2003 |

$T_m$ = annealing temperature; For = forward; Rev = reverse.

**Table 3.** Initial and final concentrations of the PCR amplification mixture component used in this study.

| Reagents | Initial concentration | Final concentration | Volume (µL) |
|---|---|---|---|
| $ddH_2O$ | - | - | 17.65 |
| PCR buffer | 10X | 1X | 2.5 |
| dNTP mix | 10,000 µM | 200 µM | 0.5 |
| $MgCl_2$ | 50 mM | 2.5 mM | 1.25 |
| Primer (forward) | 10 µM | 0.4 µM | 1.0 |
| Primer (reverse) | 10 µM | 0.4 µM | 1.0 |
| *Taq* polymerase | 500 U | 2 U | 0.1 |
| DNA template | 50 ng/µL | 50 ng/µL | 1.0 |
| Total | - | - | 25.0 |

## PCR optimization assays

For optimization, the concentrations of PCR buffer, $MgCl_2$, dNTP mixture, and amount of genomic DNA were kept constant. The first parameter assayed for the optimization stage was the annealing temperature of each primer pair. PCR was performed using the suggested concentration together with 50 ng/µL genomic DNA (Awani). The best anneal-

ing temperature was then used for PCR amplification of other optimization experiments. The primer concentrations used in PCRs were then optimized by 0.1 µM decrement and increment from the previous initial primer concentration used (0.4 µM). The best primer concentration for each gene was assayed by observing the sharpness of the desired DNA band and the absence of primer dimers on the agarose gel. Optimization assays were conducted to determine the amount of *Taq* polymerase used in each PCR and ranged from 1 U *Taq* polymerase with gradual increments of 2 U from 2 to 10 U. The last parameter optimized was the profile of PCR cycles for each gene, focusing only to the cycle duration of denaturation and annealing stages. The best parameters obtained for each primer pair were then used as the main cocktail for PCRs of the respective gene.

## DNA sequencing

PCR products of successful amplifications were sent to a sequencing service company (First Base Sdn. Bhd., Malaysia) for DNA sequencing after purification using the Vivantis Purification Kit. The quality of amplified single-stranded DNAs was quantified by chromatogram of ABI file format using the BioEdit software. Open access internet software, ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used to quantify the quality of double-stranded DNA by getting the pairwise alignment scores.

# RESULTS AND DISCUSSION

## Annealing temperature

The success of PCR relies heavily on the specificity with which a primer anneals only to its target (and not nontarget) sequence so it is important to optimize the molecular interaction (McPherson and Moller, 2006). The most important part of PCR is the optimum $T_m$. The estimated $T_m$ calculated by primer sequences often varies from the actual $T_m$. An approach to detect the highest and correct annealing temperature of primer pairs can be conducted by ranging the temperature 5°C below and above the estimated $T_m$ (Rolfs et al., 1992). The requirement of an optimal PCR $T_m$ is to amplify a specific locus without any nonspecific by-products. Average estimated $T_m$ for Cyt *b*, 12S and COI are 58.4°, 56.1° and 51.3°C, respectively (Figure 1).

$T_m$ experiments were conducted in a gradient PCR machine with temperature that ranged from 53° to 63°C for Cyt *b*, 50° to 60°C for 12S and 45° to 55°C for COI. For Cyt *b*, the optimal $T_m$ is 58.4°C, almost similar to the estimated $T_m$. However, each $T_m$ tested for this gene produced products in which smearing and multiple bands were detected between 53° to 57.3°C and gradually fainter bands were obtained for $T_m$ between 59.4° to 63°C. For 12S gene, the optimal $T_m$ is 56°C, which is similar to the estimated $T_m$. Increasing and decreasing the $T_m$ formed multiple bands and smearing products. For COI, 51.3°C is the optimal $T_m$, which is approximately 1°C less than the estimated 52°C. At 52° to 55°C, faint single bands were detected while multiple bands were observed between 47.1° to 50.2°C. From these patterns of $T_m$ for each gene, we can conclude that the $T_m$ estimation equation by Wallace rule is highly applicable to all universal primers used in this study. However, as the $T_m$ is affected variously by the individual buffer components and even the primer and template concentrations, any calculated $T_m$ value should be regarded as an approximation (Roux, 1995). The highest $T_m$ that

can produce the best visible product on agarose gel was chosen for each gene to obtain the highest specificity of the annealing of the primer to its perfect matched template (McPherson and Moller, 2006). Evans (2009) reported that temperatures at or above the $T_m$ may ensure better specificity at the expense of sensitivity.

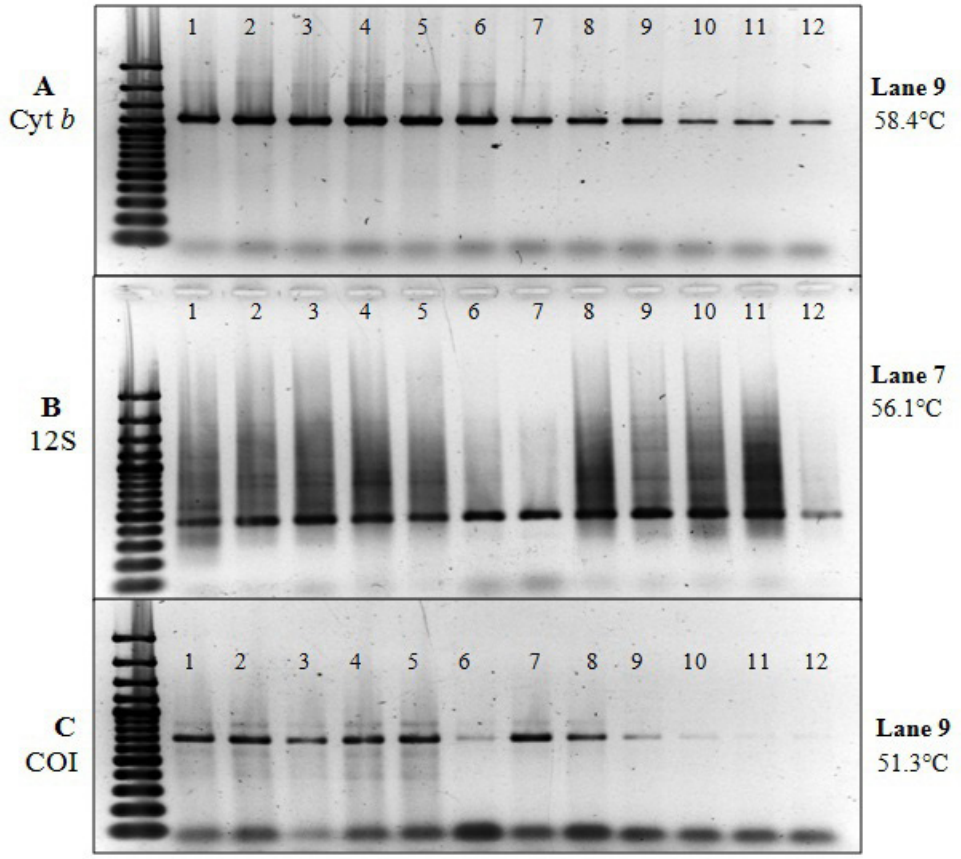


**Figure 1.** DNA bands of gradient PCR experiments captured on 1.5% agarose gel for Cyt *b*, 12S and COI genes. The best $T_m$ detected was 58.4°C for Cyt *b*, 56.1°C for 12S and 51.3°C for COI. Each lane on agarose gel indicates $T_m$ for Cyt *b*, 12S and COI genes, respectively: *lane 1* = 50°, 50°, 47.1°C; *lane 2* = 50.6°, 50.6°, 47.2°C; *lane 3* = 51.1°, 51.1°, 47.6°C; *lane 4* = 52.4°, 52.4°, 48.3°C; *lane 5* = 53.9°, 53.6°, 48.7°C; *lane 6* = 55.3°, 55.3°, 49.2°C; *lane 7* = 56°, 56.1°, 49.7°C; *lane 8* = 57.3°, 57.6°, 51°C; *lane 9* = 58.4°, 58.4°, 51.3°C; *lane 10* = 59.4°, 59.4°, 52°C; *lane 11* = 59.7°, 59.7°, 53°C; *lane 12* = 60°, 60°, 55°C.

## Primer analysis and final concentration

The DNA template and oligonucleotide primers must be considered in greater detail (Linz et al., 1990). Optimization stages for primers are essential because the efficacy and sensitivity of PCR depend largely on the efficiency of primers (He et al., 1994). All 3 primers used in this study were designed as universal primers for mammals and have been used in several

previous studies (Lavoue and Sullivan, 2004; Cai et al., 2007). Primers were analyzed by calculating the estimated $T_m$ as discussed before, G and C contents (%) and the relationship of both. GC% is an important characteristic of DNA and provides information about the strength of annealing (Abd-Eslam, 2003). The forward and reverse GC% were 39 and 46% for Cyt *b*, 45 and 70% for 12S and 32 and 35% for COI, respectively. According to Dieffenbach et al. (1995), primers should have GC content between 45 and 60%. In this study, the COI gene primer pair was observed to have the lowest GC% and gave the best result with the lowest $T_m$. However, the primer pair with the highest GC% does not necessarily require the highest $T_m$. This was proven by the $T_m$ for 12S, which was lower than the $T_m$ for Cyt *b* primers, despite having higher GC% than the Cyt *b* gene. A significant difference between Cyt *b* and 12S primers analyzed is the length of primer pairs. The length of the primers may also contribute to the increase or decrease of $T_m$.

Optimization stages were carried out to investigate the right amount of primer that would provide sufficient starter to amplify the DNA template. The concentration assayed was from 0.1 to 0.7 µM and the best possible DNA band with the least or no primer dimers indicated the ideal primer concentration (Figure 2). For Cyt *b* and COI genes, 0.1 µM primers was sufficient to produce solid bands with less dimers. The gradient of primer concentration for both genes also amplified the desired DNA but with higher number of dimers. On the other hand, a different pattern was observed for 12S. The best amount of primers for this region is 0.3 µM. Decreasing and increasing the concentration of 12S primers (PCR mixtures with wrong primer concentration) caused unspecific annealing and smearing. According to Gunson et al. (2003), adding the wrong concentration either too much or too little of primers to a PCR is likely to reduce the sensitivity, which may lead to false-negative results, and having a low concentration of primers may favor test specificity (Evans, 2009).

## Effects of *Taq* polymerase quantity on PCR products

DNA polymerase plays a central role in PCR as it can commit *in vivo* replication in the genome during each cell cycle to preserve the genetic information (Wang et al., 2004). The discovery and association of thermostable polymerase *Taq* from "Thermus aquaticus" in PCR have revolutionized modern *in vitro* molecular biology (Saiki et al., 1988). Assays to detect the right amount of *Taq* polymerase and its effects on PCR product were conducted for each gene with the final *Taq* unit set as 1, 2, 4, 6, 8, and 10 U. For the Cyt *b* gene, 1 U was observed to be sufficient for amplifying the whole region (1140 bp) with no unspecific annealing for several excess amounts of *Taq* trials, from 2 to 10 U. For 12S and COI genes, the amount of *Taq* needed to amplify both partial regions was also 1 U (Figure 3). For positive results with *Taq* amount of more than 1 U, COI could take up to 2 U while 12S could take up to 4 U. However, excess amounts of *Taq* starting from 6 to 10 U for 12S and from 4 to 10 U for COI were observed to create unspecific PCR products. The achieved result led to two possible conclusions: 1) For certain cases with correct annealing temperature condition, a good primer pair can anneal successfully with a positive result even in the presence of excess *Taq* polymerase and 2) the presence of excess *Taq* polymerase can generate non-specific products and may reduce the yield of the desired product (Saiki, 1989). The presence of excess polymerase will give extra force to the PCR resulting primers to bind at non-specific targets. In this study, we found that even though 2 U *Taq* was suggested for amplification of sequences less than 2 kb by the

Vivantis manufacturer, the usage of 1 U in the final 25 µL PCR mixture is sufficient to amplify a DNA region that is less than 1.5 kb. However, according to Adlimoghadam et al. (2008), if inhibitors are present in the reaction mix, higher amounts of *Taq* polymerase (2-3 U) may be necessary to obtain a better yield of amplification products.

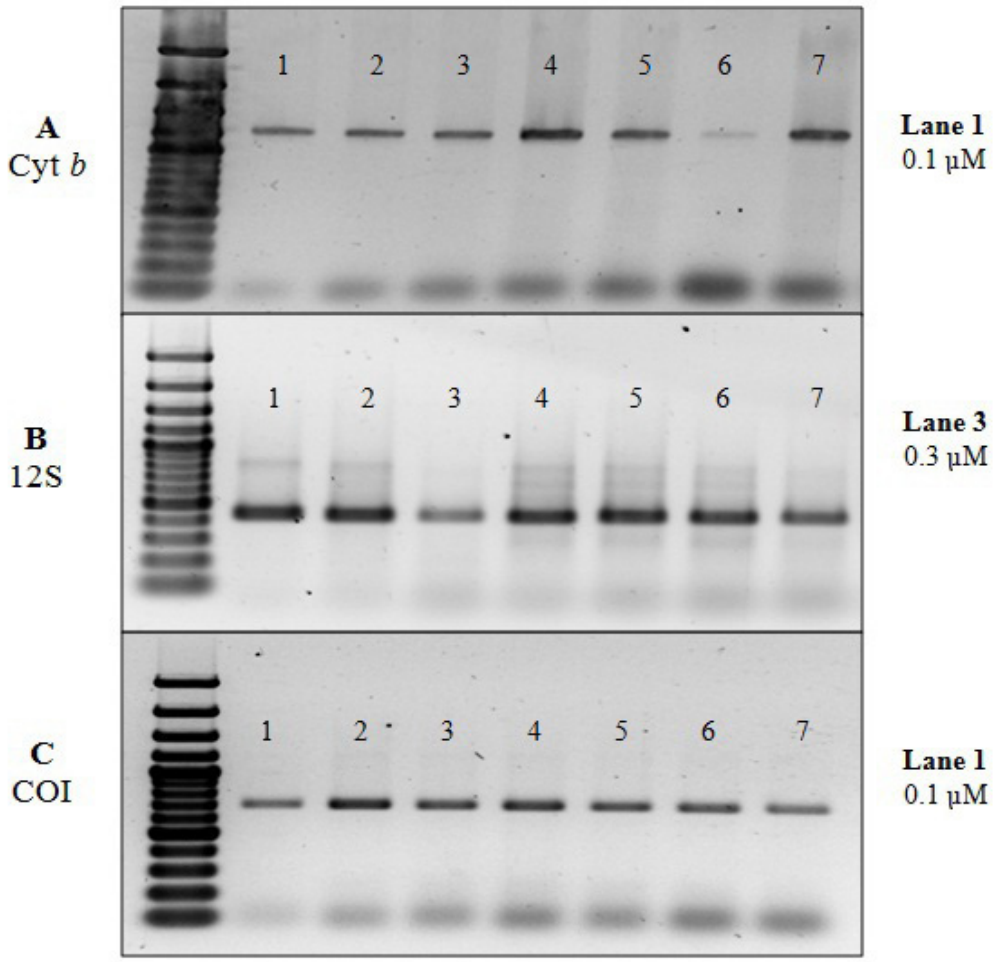


**Figure 2.** Optimization of primer concentration for Cyt *b*, 12S and COI genes. Each lane on agarose gel indicates different primer concentrations: *lane 1* = 0.1 µM; *lane 2* = 0.2 µM; *lane 3* = 0.3 µM; *lane 4* = 0.4 µM; *lane 5* = 0.5 µM; *lane 6* = 0.6 µM; *lane 7* = 0.7 µM. The best concentration chosen considers the exact desired band with fewest primer dimers, 0.1 µM for both Cyt *b* and COI and 0.3 µM for 12S.

## Profile modification of PCR cycle duration

It is useful to try to use the shortest effective time in order to retain the highest DNA polymerase activity in the reaction (McPherson and Moller, 2006). The initial PCR profile suggested by the manufacturer with the shortest possible cycle duration was set as the starter

for each gene amplification. The initial thermal cycling profile consists of 4 min of initial denaturation at 95°C, 30 cycles of 30 s of denaturation at 95°C, 30 s of primer annealing at each primer pair's $T_m$ and 1 min of primer extension at 72°C, followed by a 7-min final extension single stage at 72°C (Table 4). Cycle duration assays were conducted to investigate the effective cycle time for each gene and the implication of increasing cycle duration to PCR products and DNA sequences. It is important that the template is efficiently denatured in order to provide single-stranded templates for PCR. Ernie-Muneerah et al. (2005) stated that the optimum annealing time is needed as it is the time when the primer anneals to DNA for amplification. Generally about 30-60 s is reported in methods and the shorter the better (McPherson and Moller, 2006).

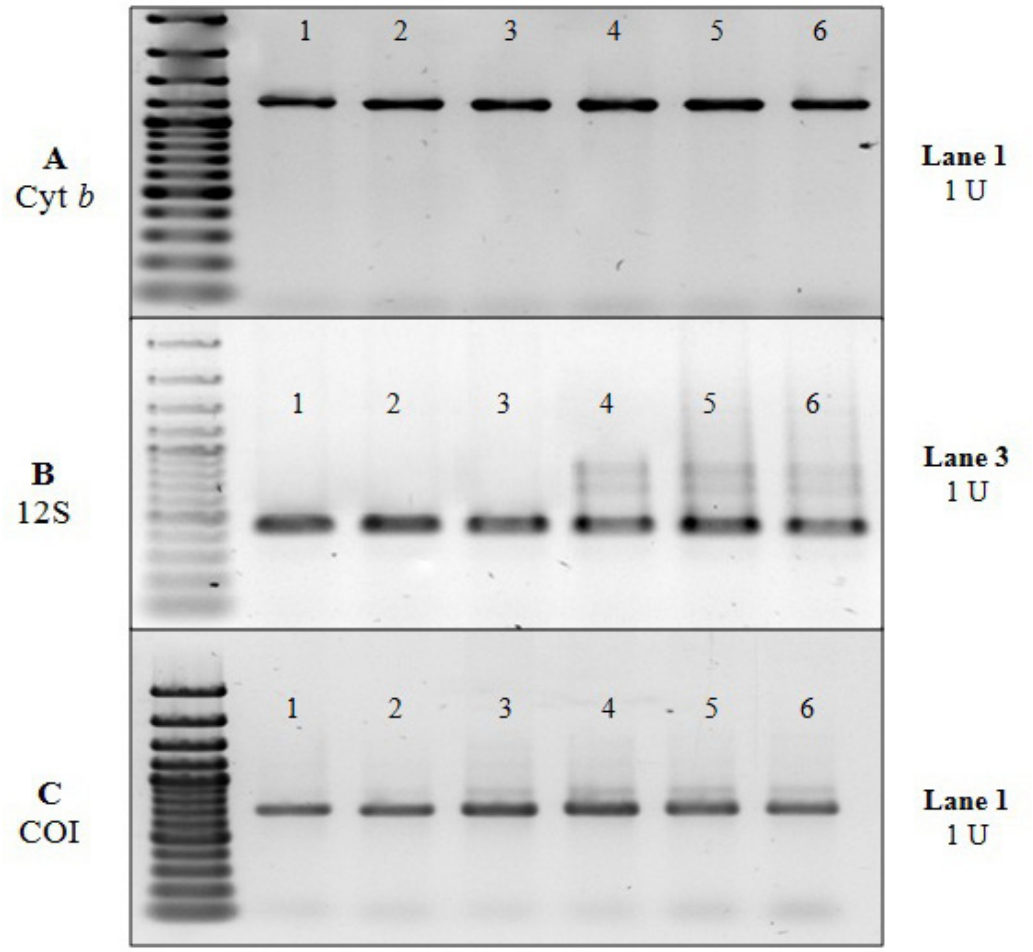


**Figure 3.** Optimization of *Taq* polymerase unit (U) in PCR mixures for Cyt *b*, 12S and COI. *Lane 1* to *lane 6* correspond to *Taq* unit of 1, 2, 4, 6, 8, and 10 U, respectively. Results indicated that 1 U *Taq* in 25 µL PCR mixure was sufficient to amplify each gene successfully.

**Table 4.** Details of PCR cycle duration assays performed and the results obtained.

| Assays | Profile | Cycle duration | Cyt *b* | 12S | COI |
|---|---|---|---|---|---|
| Assay 1 | Initial denaturation | 4 min | Vivid DNA bands on agarose gel with good DNA chromatogram sequences | Strong smearing pattern observed on agarose gel | Clear DNA bands on agarose gel with poor DNA chromatogram sequences |
| | Denaturation | 30 s | | | |
| | Annealing | 30 s | | | |
| | Extention | 1 min | | | |
| | Final extension | 7 min | | | |
| Assay 2 | Denaturation | 45 s | Not performed | Mild smearing pattern observed on agarose gel | Clear DNA bands on agarose gel with poor DNA chromatogram sequences |
| | Annealing | 45 s | | | |
| Assay 3 | Denaturation | 1 min | Not performed | Clear DNA bands on agarose gel with good DNA chromatogram sequences | Clear DNA bands on agarose gel with good DNA chromatogram sequences |
| | Annealing | 1 min | | | |

In this study, three duration assays with parallel duration time increment between denaturation and primer annealing stage were conducted by increasing 15 s (30 s, 45 s and 1 min assays) for duration of both in each assay. Results were indicated by bands on agarose gel and DNA chromatogram sequences (Figure 4). The starter PCR profile was observed to be sufficient to amplify the Cyt *b* region and provide reliably good DNA chromatogram sequences. The starter profile also produced good visible results of desired DNA bands on agarose gel for the COI gene. However, the COI DNA chromatogram sequences did not show good base peaks (Figure 5). For the 12S gene, the starter profile showed smeared DNA bands on agarose gel. The second assay using 45 s for both manipulated stages was conducted to amplify COI and 12S genes. Results for COI appeared to have a similar pattern as previous assay of DNA bands and DNA chromatogram sequences (Figure 5). For the 12S gene, reduced smearing of DNA was observed on agarose gel. Third assays (1 min) provided good DNA bands on agarose gel and excellent DNA chromatogram sequences for both 12S and COI.

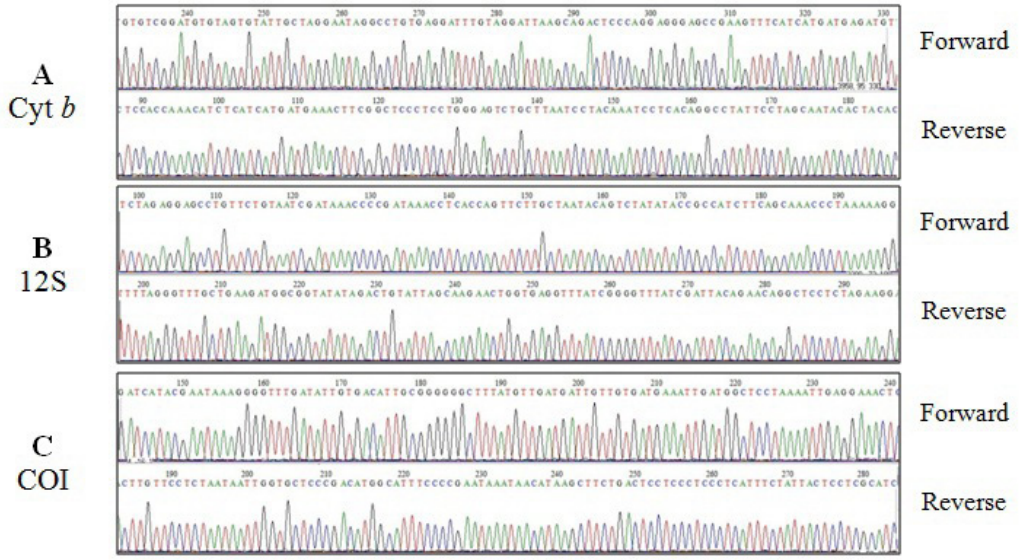


**Figure 4.** DNA chromatograms of Awani sample for Cyt *b*, 12S and COI genes. DNA sequenced from purified PCR products with the final cocktail of optimum parameters. All chromatograms show good base peaks for both forward and reverse primers of each gene.

These results showed that increasing the duration of the denaturation and primer annealing cycles can improve the result of amplified products on agarose gel visibly (Adlimoghadam et al., 2008) and DNA sequences as well. Time increment also appears to be one of the possible optimization approaches to overcome the DNA smearing problem. Smearing can be caused by the present of high molecular weight products rich in single-stranded DNA or if the level of starting template is too high (Roux, 1995). Previously, it was believed that each of the three stages in the cycle requires a minimum amount of time to be effective while too much time at each stage can be both wasteful (time waste) and deleterious to the DNA polymerase (Coen, 1991). Even though in general, higher $T_m$ and shorter time allowed for annealing and

extension stages improved specificity of PCR (Cha and Thilly, 1993), it should also be pointed out that it is necessary to increase the duration of each stage including the denaturation stage for effective amplification (Kwok et al., 1990), as has been shown in this study.

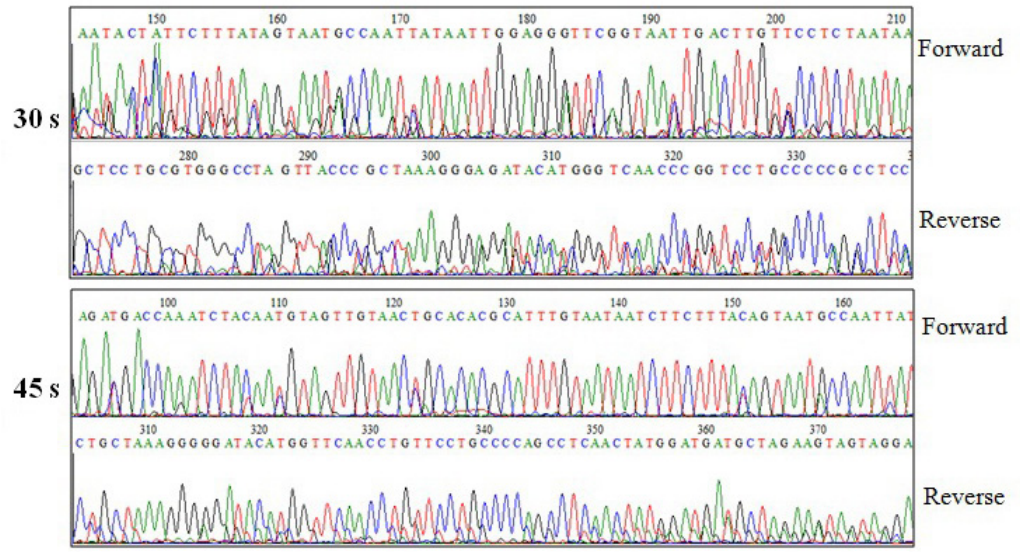


**Figure 5.** Poor base peaks on COI chromatograms due to insufficient duration of denaturation and primer annealing stages of PCR cycles. Increasing the cycle duration led to improvement of DNA sequence quality.

## DNA chromatograms and ClustalW2 scores

In this study, two approaches were used in order to evaluate the effectiveness of PCR optimization assays towards quality of DNA sequences. DNA chromatogram was used as an indicator for DNA sequence purity by base peaks and the ClustalW2 score was used to calculate the matched values between forward primer with its complementary reverse primer of each primer pair. All seven samples for Cyt *b*, 12S and COI had good DNA chromatograms with 46 bases of average waterfall interference at the beginning of each primer. ClustalW2 scores fell between 87 to 94% for Cyt *b*, 86 to 93% for 12S and 88 to 92% for the COI gene. Based on the high scores, all seven Malayan gaur samples were considered as highly purified DNA (Table 5).

## CONCLUSION

In conclusion, optimization of PCR proved to be an important approach to obtain the best positive result especially in research that involves DNA sequences as the final data type. Table 5 shows final optimum parameters used for amplification of Cyt *b*, 12S and COI genes for Malayan gaur samples. Results in this study also indicate that, by optimizing the four parameters discussed above, the amount of constant parameters (DNA template, PCR buffer,

**Table 5.** Summary of optimum conditions for amplification of Cyt *b*, 12S and COI genes and ClustalW2 scores for individual samples.

| Parameters | Optimum conditions | | |
|---|---|---|---|
| | Cyt *b* | 12S | COI |
| Reagents | Concentration | | |
| PCR buffer | 1X | 1X | 1X |
| dNTP mix | 200 µM | 200 µM | 200 µM |
| $MgCl_2$ | 2.5 mM | 2.5 mM | 2.5 mM |
| Primer (forward) | 0.1 µM | 0.3 µM | 0.1 µM |
| Primer (reverse) | 0.1 µM | 0.3 µM | 0.1 µM |
| Taq polymerase | 1 U | 1 U | 1 U |
| DNA template | 50 ng/µL | 50 ng/µL | 50 ng/µL |
| Stages | PCR profiles | | |
| Denaturation | 94°C for 30 s | 94°C for 1 min | 94°C for 1 min |
| Annealing | 58.4°C for 30 s | 56.1°C for 1 min | 51.3°C for 1 min |
| Samples | ClustalW2 scores (%) | | |
| Awani | 91 | 86 | 88 |
| Waja | 88 | 91 | 88 |
| Seroja | 90 | 89 | 92 |
| Nino | 90 | 90 | 90 |
| Sarum | 87 | 86 | 89 |
| BGH 8 | 93 | 89 | 90 |
| BGH 17 | 94 | 93 | 90 |

$MgCl_2$, and dNTP mixture) seemed to be optimum for each gene PCR cocktail studied with the final volume of 25 µL. Many studies have demonstrated a range of PCR optimization stages and this review could be considered as one of those guides for designing any PCR optimization assay.

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## REFERENCES

Abd-Eslam K (2003). Bioinfomatic tools and guideline for PCR primer design. *Afr. J. Biotechnol.* 2: 91-95.

Adlimoghadam A, Hedayati MH, Siadat SD, Ahmadi MH, et al. (2008). Optimization of PCR conditions for detection of human brucellosis from human serum samples. *Res. J. Microbiol.* 3: 352-358.

Ang KC, Leow JW, Yeap WK, Hood S, et al. (2011). Phylogenetic relationships of the Orang Asli and Iban of Malaysia based on maternal markers. *Genet. Mol. Res.* 10: 640-649.

Avise JC (2004). Molecular Markers, Natural History, and Evolution. 2nd edn. Sinauer Associates Inc., Sunderland.

Bataille M, Crainic K, Leterreux M, Durigon M, et al. (1999). Multiplex amplification of mitochondrial DNA for human and species identification in forensic evaluation. *Forensic Sci. Int.* 99: 165-170.

Cai X, Chen H, Lei C, Wang S, et al. (2007). mtDNA diversity and genetic lineages of eighteen cattle breeds from *Bos taurus* and *Bos indicus* in China. *Genetica* 131: 175-183.

Cha RS and Thilly WG (1993). Specificity, efficiency, and fidelity of PCR. *PCR Methods Appl.* 3: S18-S29.

Coen DM (1991). The Polymerase Chain Reaction. Current Protocol in Molecular Biology. Green Publishing, Wiley Interscience, New York.

Conry PJ (1989). Gaur (*Bos gaurus*) and development in Malaysia. *Biol. Conserv.* 49: 47-65.

Dieffenbach CW, Lowe TMJ and Dveksler GS (1995). General Concepts for PCR Primer Design. In: PCR Primer, A Laboratory Manual (Deiffenbach CW and Dveksler GS, eds.). Cold Spring Harbor Laboratory Press, New York, 67.

Ernie-Muneerah MA, Ahmad N, Ang KC, Zainal ZZ, et al. (2005). Polymerase chain reaction (PCR) optimization for COII rRNA gene fragment of Malaysian Leaf Monkeys. *J. Wildl. Parks.* 22: 71-76.

Evans MF (2009). The polymerase chain reaction and pathology practice. *Diagn. Histopathol.* 15: 344-356.

Gibson UE, Heid CA and Williams PM (1996). A novel method for real time quantitative RT-PCR. *Genome Res.* 6: 995-1001.

Gunson R, Gillespie G and Carman F (2003). Optimisation of PCR reactions using primer chessboarding. *J. Clin. Virol.* 26: 369-373.

He Q, Marjamaki M, Soini H, Mertsola J, et al. (1994). Primers are decisive for sensitivity of PCR. *Biotechniques* 17: 82-87.

Hebert PD, Cywinska A, Ball SL and deWaard JR (2003). Biological identifications through DNA barcodes. *Proc. Biol. Sci.* 270: 313-321.

Innis MA, Gelfand DH, Snlnsky JJ and White TJ (1990). PCR Protocols, A Guide to Methods and Applications. Academic Press Inc., New York.

Irwin DM, Kocher TD and Wilson AC (1991). Evolution of the cytochrome b gene of mammals. *J. Mol. Evol.* 32: 128-144.

Knowlton N and Weight LA (1998). New dates and new rates for divergence across the Isthmus of Panama. *Proc. Roy. Soc. Lond. B.* 256: 2257-2263.

Kocher TD, Thomas WK, Meyer A, Edwards SV, et al. (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. U. S. A.* 86: 6196-6200.

Kwok S, Kellogg DE, McKinney N, Spasic D, et al. (1990). Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. *Nucleic Acids Res.* 18: 999-1005.

Lalam N (2006). Estimation of the reaction efficiency in polymerase chain reaction. *J. Theor. Biol.* 242: 947-953.

Lardeux F, Tejerina R, Aliaga C, Ursic-Bedoya R, et al. (2008). Optimization of a semi-nested multiplex PCR to identify *Plasmodium* parasites in wild-caught *Anopheles* in Bolivia, and its application to field epidemiological studies. *Trans. R. Soc. Trop. Med. Hyg.* 102: 485-492.

Lavoue S and Sullivan JP (2004). Simultaneous analysis of five molecular markers provides a well-supported phylogenetic hypothesis for the living bony-tongue fishes (Osteoglossomorpha: Teleostei). *Mol. Phylogenet. Evol.* 33: 171-185.

Lim LS, Ang KC, Mahani MC, Shahrom AW, et al. (2010). Mitochondrial DNA polymorphism and phylogenetic relationships of proto Malays in Peninsular Malaysia. *J. Biol. Sci.* 10: 71-83.

Linz U, Delling U and Rubsamen-Waigmann H (1990). Systematic studies on parameters influencing the performance of the polymerase chain reaction. *J. Clin. Chem. Clin. Biochem.* 28: 5-13.

Matthee CA and Davis SK (2001). Molecular insights into the evolution of the family Bovidae: a nuclear DNA perspective. *Mol. Biol. Evol.* 18: 1220-1230.

McPherson MJ and Moller SG (2006). PCR. 2nd edn. Taylor & Francis Group, New York.

Md-Zain BM, Morales JC, Hassan MN and Jasmi A (2008). Is *Presbytis* a distinct monophyletic genus: inferences from mitochondrial DNA sequences. *Asian Primates J.* 1: 26-36.

Md-Zain BM, Lee SJ, Lakim M and Ampeng A (2010a). Phylogenetic position of *Tarsius bancanus* based on partial cytochrome *b* DNA sequences. *J. Biol. Sci.* 10: 348-354.

Md-Zain BM, Mohamad M, Ernie-Muneerah MA, Ampeng A, et al. (2010b). Phylogenetic relationships of Malaysian monkeys, Cercopithecidae, based on mitochondrial cytochrome c sequences. *Genet. Mol. Res.* 9: 1987-1996.

Nguyen TT, Genini S, Bui LC, Voegeli P, et al. (2007). Genomic conservation of cattle microsatellite loci in wild gaur (*Bos gaurus*) and current genetic status of this species in Vietnam. *BMC Genet.* 8: 77.

Rolfs A, Schuller I, Finckh U and Weber-Rolfs I (1992). PCR: Clinical Diagnostic and Research. Springer-Verlag Berlin, Heidelberg.

Rosli MK, Zakaria SS, Syed-Shabthar SM, Zainal ZZ, et al. (2011). Phylogenetic relationships of Malayan gaur with other species of the genus *Bos* based on cytochrome b gene DNA sequences. *Genet. Mol. Res.* 10: 482-493.

Roux KH (1995). Optimization and troubleshooting in PCR. *PCR Methods Appl.* 4: S185-S194.

Sahir O (2001). Breeding performance of the Malaysian captive gaur. *J. Wildl. Parks.* 19: 25-32.
Saiki RK (1989). The Design and Optimization of the PCR. In: PCR Technology - Principles and Applications for DNA Amplification (Erlich HA, ed.). Stockton Press, New York, 116.
Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, et al. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.
Simon C, Paabo S, Kocher T and Wilson AC (1990). Evolution of Mitochondria1 Ribosomal RNA in Insects as Shown by the Polymerase Chain Reaction. In: Molecular Evolution. UCLA Symposia on Molecular and Cellular Biology (Clegg M and O'Brien S, eds.). New Series, A.R. Liss Inc., New York, 235-244.
Tabone T, Mather DE and Hayden MJ (2009). Temperature switch PCR (TSP): robust assay design for reliable amplification and genotyping of SNPs. *BMC Genom.* 10: 580.
Vun VF, Mahani MC, Lakim M, Ampeng A, et al. (2011). Phylogenetic relationships of leaf monkeys (*Presbytis*; Colobinae) based on cytochrome b and 12S rRNA genes. *Genet. Mol. Res.* 10: 368-381.
Wallace RB, Shaffer J, Murphy RF, Bonner J, et al. (1979). Hybridization of synthetic oligodeoxyribonucleotides to phi chi 174 DNA: the effect of single base pair mismatch. *Nucleic Acids Res.* 6: 3543-3557.
Wang Y, Prosen DE, Mei L, Sullivian JC, et al. (2004). A novel strategy to engineer DNA polymerases for enhanced processivity and improved performance *in vitro*. *Nucleic Acids Res.* 32: 1197-1207.
Zainudin R, Shukor MN, Norhayati A, Md-Zain BM, et al. (2010). Genetic Structure of *Hylarana erythraea* (Amphibia: Anura: Ranidae) from Malaysia. *Zool. Stud.* 49: 688-702.