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Optimization of PCR conditions to amplify mitochondrial COI gene fragments of wildlife species in Kenya

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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 c oxidase I (COI) gene fragments of some of the targeted wildlife species in Kenya; buffalo,common zebra, grant's gazelle,warthog and common eland and domestic samples purchased from the market as 'beef', 'goat'or 'mutton'. DNA from five wildlife species and one hundred of domestic samples were extracted for PCR amplification. Various trials and combinations were tested to determine the best conditions of PCR mixtures and annealing temperatures to obtain the best PCR products for sequencing purposes. Four selected target factors for enhancing PCR, annealing temperature, concentration of primer pair, amount of *D*ream Taq™ PCR Master Mix (2x) (Fermentas) and PCR cycle duration, were optimized by keeping the amount of DNA template (2µL) and concentration of PCR buffer, MgCl2 4mM and dNTP mixture constant (Fermentas). All genes were successfully amplified, giving the correct fragment lengths of 700 base pair (bp), as assigned for both forward and reverse primer. The optimal conditions were determined to be: 0.5µl (5pmoles) for each primer, 25µl of DreamTaq ™ PCR Master Mix (2x), 30 s of both denaturation and annealing cycles and annealing temperature of 56.5°C. PCR products obtained under these conditions produced excellent bands.

INTRODUCTION

In recent years, geneticists have discovered the usefulness of molecular data and its deployment in several studies such as phylogenetics, population studies, DNA bacoding and molecular systematics [1,2&3]. 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 [4]. 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 [5]. Exploration and understanding of the PCR principles itself can help ensure a good PCR product with less cost and time. Because of the 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 [6]. Several changes can be made to the PCR composition in order to optimize the reaction [7]. Some parameters that should be of concern in order to facilitate the optimization process are chemical concentrations (MgCl2, dNTP mix, PCR buffer, and *Taq* polymerase), primers used and PCR conditions that include the highest annealing temperature, optimal cycle number and amplification duration [8]. A typical PCR consists of three stages: denaturation stage (1-2 min); primer annealing (hybridization) stage (1-2 min), and an extension stage (1-2 min) for several cycles [9].

A study, based on PCR method, was conducted on five Kenyan wildlife species and hundred domestic samples purchased from the market in Kajiado county in Kenya to optimize the right PCR conditions for successful cytochrome oxidase I (COI) gene amplification. In Kenya, drought, escalating poverty, prevailing food shortages and global financial crisis, has lead to a rise in the 'bushmeat' trade [10]. Commercial bushmeat trade is threatening to wipe out Kenya's precious wildlife resource in some areas. A variety of species are poached for illegal bushmeat regularly ranging from Insects, Rodents and Birds to small and medium sized antelopes like Duikers, Gazelles and Impala to big game like Zebra, Giraffe, Eland and Buffaloes [10]. Various animal species are affected by bushmeat poaching differently in different localities and preference is mainly based on availability. The harvesting rates of wild herbivores across Africa such as buffalo (*Syncerus caffer*), giraffe (*Giraffa camelopardalis*), impala (*Aepyceros melampus*), topi (*Damaliscus lunatus*) and wildebeest (*Connochaetes taurinus*), zebra (*Equus burchelli*), warthog (*Redunca redunca*) eland (*Tragelaphus oryx*) in parts of their ranges are alarming [11 &12].

The use of molecular approaches offers promise as an effective tool for monitoring poaching and commercial trade in endangered species, especially when investigating semi-processed or morphologically indistinguishable wildlife products [13]. Mitochondrial DNA (mtDNA) is regarded as an important tool in the study of evolutionary relationships among various taxa due to its conserved protein-coding regions, high variability in non-coding sequences, and lack of recombination [14]. Sequence divergence accumulates more rapidly in mtDNA than in nuclear DNA due to a faster mutation rate and lack of repair system, meaning that it often contain high levels of informative variation [15]. DNA barcoding has become a promising tool for the rapid and accurate identification of various taxa, and it has been used to reveal unrecognized species in several animal groups. Animal DNA barcodes (600- to 800-bp segments) of the mitochondrial cytochrome oxidase I (COI) gene have been proposed as a means to quantify global biodiversity. DNA barcoding has the potential to improve the way researchers relate to wild biodiversity [16]. Furthermore, the introduction of DNA barcoding has highlighted the expanding use of COI as a genetic

marker for species identification [17].

A DNA barcode is defined as one or more short gene sequences taken from a standardized portion of the genome used to identify species. The use of such short DNA sequences for biological identification with the vital goal of quick and reliable species-level identifications applies to all forms of life. Currently, the concept of a universally recoverable segment of DNA that can be applied as an identification marker across species has been most successfully applied to animals [18]. At least, three criteria must be met to identify a gene region as appropriate for a DNA barcode these include: significant species-level genetic variability and divergence; short sequence length to facilitate DNA extraction and amplification, and universal PCR primers. For most groups of animals, a portion of the mitochondrial gene for COI has been identified as a species-level barcode. COI has been shown to fit the three criteria in the great majority of animal taxa to which it has been applied [19].

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 the COI gene are presented herewith.

MATERIALS AND METHODS

Sampling area

Study samples were collected from Ol pejeta Ranch in Laikipia, Marula Ranch and Annex KWSTI in Naivasha in Kenya. The samples were blood and tissue samples of 25 individuals from 5 wild species of the Bovidae family (Table1). The selection of wild species represents the commonly targeted wild meat species in Kenya. Known tissue samples were obtained by biopsy darting of free-ranging animals, carried out by a qualified KWS veterinarian involved in the project. The samples were from both male and female and were stored in 50-ml plastic tubes containing 70% alcohol at room temperature.

Sampling butcheries for bush meat test

Meat samples were purchased from various butcheries in Kajiado County and its environs (Isinya, Kitengela and Kiserian) by stratified random sampling method. Samples were collected either from shopping centers or informal settlements (Table2). Samples presented by the seller as either 'beef', 'goat' or 'mutton' was purchased. Also some 'mutura' and 'samosa' were bought. The samples were aseptically excised in 0.5g sections and stored in 50-ml plastic tubes containing 70% alcohol at 4°C temperature prior to analysis.

DNA extraction

Genomic DNA extraction from tissue and blood samples was extracted using the ZR Genomic DNA™- Tissue Mini Prep kit according to the manufacturer's instructions. The isolated DNA was quantified using a Bio photometer machine. For further estimation of DNA quantity and extraction blank, 2 µL was loaded on 0.8% agarose gel stained with 5µL ethidium bromide (concentration 10mg/ml) at 100 V for 30 min and visualized on UV transilluminator (Uvidoc).

Oligonucleotide primers and PCR

Annealing temperature of the universal primer was first estimated by using the equation of T_m=4(G+C) + 2(A+T) suggested by Fermentas manufacturer, where G, C, A, T represent the number of respective nucleotides in the primer. Primer and estimated annealing temperature (*T*m) details are listed in (Table3). PCR chemicals used in this study were obtained from Fermentas (Canada). DNA amplifications by PCR were initially carried out using PCR chemical concentrations (Table4) suggested by the Fermentas manufacturer in a Gene Amp_ PCR system 9700 (Applied Biosystems) machine before further optimization stages. Initial PCR was performed in a total mixture of 50-µL containing approximately 2µL DNA template, 0.4 µL of each primer (Reverse and Forward), 22.2 µL of water, nuclease free, and 25 µL of Dream Taq™ PCR Master Mix 2x (is a ready-to-

Table 2. Details of the market survey samples in Kajiado County and its environs

Towns	Zones	Number of samples
Kajiado	Shopping center	13
	Informal settlement	5
Isinya	Shopping center	6
	Informal settlement	6
Kitengela	Shopping center	16
	Informal settlement	22
Kiserian	Shopping center	21
	Informal settlement	11

Table 1. Details of the animals samples used in this study

Common name	Scientific name	Sub-family	Sample type	No. of samples
Buffalo	*Syncerus caffer*	*Bovinae*	Biopsy	5
Common eland	*Taurotragus oryx*	*Bovinae*	Biopsy and blood	4
Warthog	*Phacochoerus africanus*		Blood and tissues	3
Grant's gazelle	*Gazella granti*	*Antilopinae*	Blood	8
Plain zebra	*Equus quagga*		Blood and tissues	7

Table 3. Details of forward and reverse primer used for PCR amplification

Primer	Sequence 5'-3'	Bases	Amplicon size	G+C (%)	Estimated T_m	Reference
VFId-t1	TCTCAACCAACCA CAAGAATGG	22	700bp	45	64	Ivanova, *etal* 2007
VRId-t1	TAGACTTCTGGGT GGCCAAAACA	23	700bp	48	65	Ivanova, *etal* 2007

Table 4. Initial and final concentration used in PCR optimization used in this study

Reagents	Initial concentration	Final concentration
Reverse primer	0.4µl	0.5 µl
Forward primer	0.4 µl	0.5 µl
Water, nuclease-free	22.2 µl	22 µl
Template DNA	2 µl	2 µl
Dream Taq™ PCR Master Mix (2x)	25 µl	25 µl
Total volume	50 µl	50 µl

Table 5. Summary of optimum conditions for amplification of COI genes for individual samples.

Reagents	Concentration
Template DNA	2 µl
Reverse primer	0.5 µl
Forward primer	0.5 µl
Water, Nuclease-free	22. µl
Dream Taq ™ Master Mix (2X)	25 µl
Total volume	**50µl**
Stages	**PCR profile**
Denaturation	94°C for 30s
Primer annealing	56.5°C for 30s

use solution containing Dream Taq™ DNA polymerase, optimized Dream Taq™ buffer,$MgCl_2$ and dNTPs). Initial PCR consisted of initial denaturation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 30 s, gradient ranged annealing temperature for 30 s and 72°C for 1 min, and incubation at 72°C for 10 min. Negative controls, which contained all elements of the reaction mixture except DNA template, were assigned as a real time measure for potential contamination. Successful bands were detected on 0.8% agarose gel in 1X TBE buffer.

PCR optimization assays

For optimization, the concentrations of PCR buffer, MgCl2, dNTP mixture, and amount of genomic DNA were kept constant. The first parameter assayed for the optimization stage was the annealing temperature of primer. PCR was performed using the suggested concentration together with 2µL genomic DNA. The best annealing temperature was then used for PCR amplification of other samples in study. The primer concentrations used in PCR was then optimized by 0.1 µL decrement and increment from the previous initial primer concentration used (0.4 µl). The best primer concentration was 0.5 µL, the sharpness of the DNA band and the absence of primer dimers on the agarose gel was observed.

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 [20]. The most important part of PCR is the optimum *Tm*. The estimated *Tm* calculated by primer sequences often varies from the actual *Tm*. An approach to detect the highest and correct annealing temperature of primer can be conducted by ranging the temperature 5°C below and above the estimated *Tm* [21]. The requirement of an optimal PCR *Tm* is to amplify a specific locus without any nonspecific by-products. The best primer annealing temperature in the study is 56.5°C and the fragment gene length for both wildlife species and domestic sample was 700 base pair (bp) (Figure1 and 2). *T*m experiments were conducted in a Gene Amp_ PCR system 9700 (Applied Biosystems) machine with temperature that ranged from 54° to 65°C. 56.5°C was the optimal *T*m, which is approximately 8.5°C less than the estimated 65°C. At 54° to 55°C, faint single bands were detected while multiple bands were observed between 57° to 59°C and no band was detected at 60°C to 65°C. 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 [22]. The highest *T*m that produced the best visible product on agarose gel was chosen to obtain the highest specificity of the annealing of the primer to its perfect matched template [20]. [23] reported that temperatures at or above the *T*m may ensure better specificity at the expense of sensitivity.

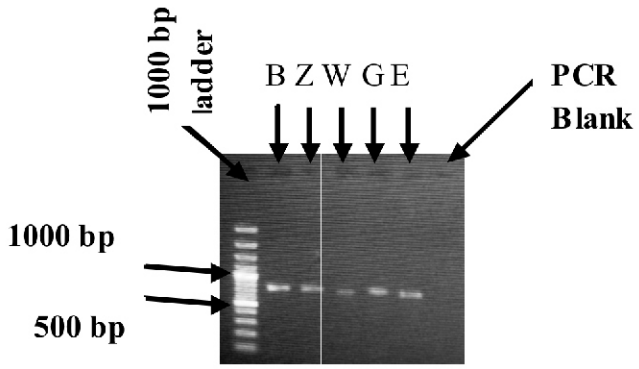

Figure 1. : Agarose gel electrophoresis of wild animals PCR products photograph, B= Cape buffalo= Plain zebra=Warthog =Grant's gazelle and E= Common eland

Primer analysis and final concentration

The DNA template and oligonucleotide primers must be considered in greater detail [24]. Optimization stages for primers are essential because the efficacy and sensitivity of PCR depend largely on the efficiency of primers [25]. The primer used in this study was designed as universal primer for Ungulates and have been used in several previous studies [26&27].

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 [28]. The forward and reverse GC% of the universal primer used was 45 and 48% respectively. According to [28], primers should have GC content between 45 and 60%. Though, the primer pair with the highest GC% does not necessarily require the highest *T*m but 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 0.6 µl and the best possible DNA band with the least or no primer dimers indicated the ideal primer concentration (Figure1 and 2). According to [28], 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 [23].

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 [20]. The initial PCR profile suggested by the manufacturer with the shortest possible cycle duration was set as the starter for gene amplification. The initial thermal cycling step consisted of initial denaturation at 95°C for 15min, 35 cycles (denaturation at 94°C

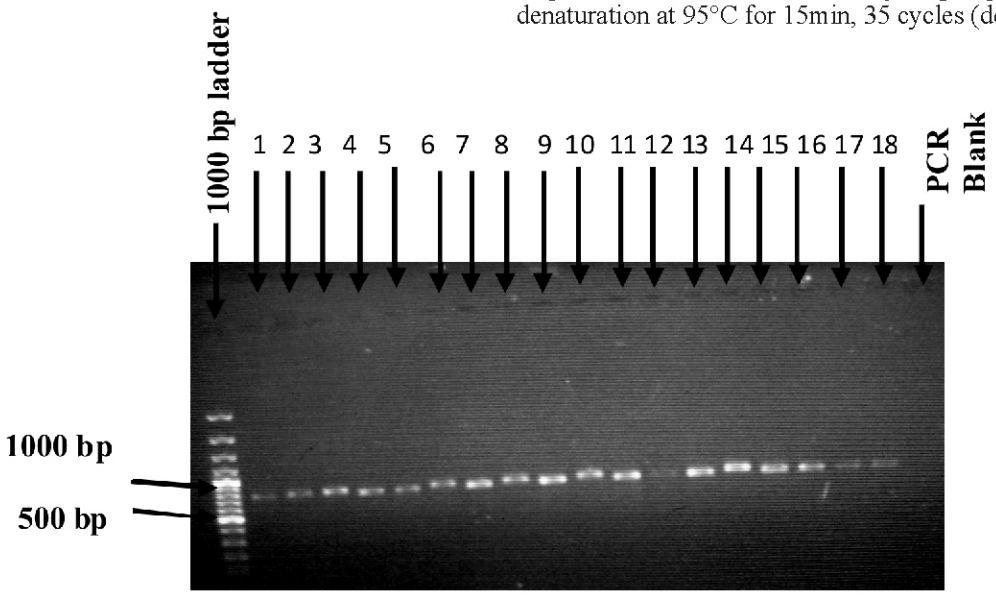

Figure 2. : Agarose gel electrophoresis domestic PCR products photograph.

for 30s, primer annealing at different temperature for 30s and primer extension at 72°C for 1 min) followed by final extension single stage at 72°C for 10 min. Cycle duration assays were conducted to investigate the effective cycle time for COI gene and the implication of increasing cycle duration to PCR products. It is important that the template is efficiently denatured in order to provide single-stranded templates for PCR. 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 [20]. In this study, two duration assays with parallel duration time increment between denaturation and primer annealing stage were conducted by increasing 15 s (30 s, and 45 s), and the best duration was 30 s for both denaturation and primer annealing. The results were indicated by bands on agarose gel (Figure 1 and 2).

CONCLUSION

In conclusion, optimization of PCR proved to be an important approach to obtain the best positive results. Table 5 shows final optimum parameters used for amplification of COI gene for five Kenyan wildlife species samples targeted for bushmeat and hundred domestic samples collected from Kajiado County and its environs. Results in this study also indicate that, by optimizing the four parameters discussed above, the amount of constant parameters (DNA template, PCR buffer, MgCl2, and dNTP mixture) seemed to be optimum for COI gene PCR cocktail studied with the final volume of 50 µL. Many studies have demonstrated a range of PCR optimization stages for mitochondrial cytochrome oxidase I gene and this review could be considered as one of those guides for designing any PCR optimization assay.

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