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Improvement of DNA Barcode Amplification Using Gradient PCR

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Abstract
DNA barcoding employs sequence variation in short, standardized gene regions as a tool to discriminate species and has many applications in plant authentication. Success amplification through PCR plays a vital role in DNA barcode library construction and sequencing. This study aims to improve determine the optimal annealing temperature for DNA barcode amplification. In this study, eight DNA barcode regions including ITS, matK, rbcL, rpoC1, ycf1b, trnH-psbA, atpF-atpH and psbK-psbI were amplified by gradient PCR to assess and determine the proper annealing temperature. Our results indicated that the PCR yield and specificity for ITS, matK, rbcL, rpoC1 and ycf1b were optimized using gradient PCR. 58°C was required for optimal primer binding temperature in ITS regions while the other regions involved lower annealing temperature, ranging from 49.1°C to 54.2°C. These findings illustrated that an appropriate annealing temperature contributed significantly for PCR success, which is a key step for sequencing quality.

Keywords: *Amplification efficiency, annealing temperature, DNA barcodes, gradient PCR, plant authentication*

List of abbreviations: *Atp - adenosine triphosphate synthase, DNA - deoxyribonucleic acid, ITS - internal Transcribed Spacer, matK - maturase K, rbcL - ribulose carboxylase large subunit, PCR - polymerase chain reaction, psbA: D1 protein of photosystem II, rpoC1 - RNA polymerase subunit beta, trnH: histidine accepting tRNA, ycf1b - hypothetical protein RF1.*

INTRODUCTION

The concept of DNA barcode indicates such standard DNA sequences with the length between 400-800 bp that was first introduced by Hebert *et al.* (2003). These sequences are highly conservative and can be used for the identification of an organism. Because of focusing on DNA sequences, which are stable during all living stages and little influenced by environmental factors, this technique is an appropriate solution for rapid and accurate identification and authentication (Kress, 2017). Inland plants, studies of DNA barcode are more complicated compared to those in animals, this phenomenon based on the low substitution rate in the plant genome. So far, several loci in both nucleus and chloroplast genome were considered as potential candidates for universal DNA barcodes (CBOL, 2009).

In order to construct the DNA barcode database, three main stages are involved: (1) DNA extraction, (2) amplification of target regions and (3) DNA sequencing (Kress, 2017). The amplification procedure is a vital step that influences further analysis, amplicons with high specificity will enhance the accuracy of the sequencing process. It is acceptable that the optimal annealing temperature will increase the hybridization between primers and their specific sequence (Rychlik *et al.*, 1990). This study aims to select optimum annealing temperatures for the amplification of eight barcoding regions (ITS, *mat*K, *rbc*L, *psb*K-*psb*I, *trn*H-*psb*A, *atp*F-*atp*H, *ycf*1b, and *rpo*C1) in two fruiting plants representatives, *Durio zibethinus* and *Citrus maxima*.

MATERIALS AND METHODS

Isolation of total DNA

The procedure was modified from the protocol of Rogers and Bendich (1988). Healthy leaves were cut into small pieces and grind into powder by using liquid nitrogen and iron balls in 2.0 mL tube. Leaves powder then added by 1 mL Extraction Buffer [100 mMTris.Cl, pH8; 50 mM EDTA, pH8; 0.5 M NaCl; 0.4% (w/v) PVP40, 0.07% (v/v) β-mercaptoethanol] and 50 µL of 10% (w/v) SDS. The tubes were inverted several

times and then incubated at 65°C for 30 minutes. Centrifuge the tube at 12,000 rpm for 10 minutes and carefully transfer 800 µL supernatant into a new tube containing 800 µL isopropanol. Samples were frozen at -20°C for at least 3 hours. Isolate the precipitated DNA by centrifugation at 12,000 rpm for 10 minutes and discard the supernatant. Each tube was added with 400 µL of TE 1X [10 mM Tris.Cl; 2.5 mM EDTA] and incubated with 10 µL of RNAse at 37°C in 10 minutes for RNA elimination, the solution was then added 400 µL of 2% CTAB (Cetyltrimethylammonium bromide) and incubated at 65°C for 15 minutes. The following step was an addition of 800 µL chloroform: isoamylalcohol (24:1) and inverted several times. After centrifugation at 12.000 rpm for 5 minutes, 600 µL of the upper phase was transferred gently to another tube containing 1.200 µL absolute ethanol and incubated 20 minutes. Centrifuge the tubes at 12,000 rpm for 10 minutes and discard the supernatant. The residue NaCl was washed out by centrifugation with 70% ethanol twice. The ethanol was discarded and DNA was dried for 10 minutes at 45°C in a vacuum centrifuge concentrator. Extracted DNA was stored in 100 µL TE 0.1X at -20°C. The quality and intact DNA were evaluated by 1% agarose gel electrophoresis.

Amplification of barcode regions

The primer sequences that used in this study were listed in Table 1 and the thermal cycles of those were showed in Table 2.

Table 1: Primer sequences of eight barcoding regions used is this study

Primers	Nucleotide sequences (5'-3')
ITS	ITS1: TCCGTAGGTGAACCTGCGG
	ITS4: TCCTCCGCTTATTGATATGC
*mat*K	matK-390F: CGATCTATTCATTCAATATTTC
	matK-1326R: TCTAGCACACGAAAGTCGAAGT
*atp*F-*atp*H	atpF: ACTCGCACACACTCCCTTTCC
	atpH: GCTTTTATGGAAGCTTTAACAAT
*rbc*L	rbcLaF: ATGTCACCACAAACAGAGACTAAAGC
	rbcLaR: GTAAAATCAAGTCCACCRCG
*psb*K-*psb*I	psbKF: TTAGCCTTTGTTTGGCAAG
	psbIR: AGAGTTTGAGAGTAAGCAT
*trn*H-*psb*A	psbA3F: GTTATGCATGAACGTAATGCTC
	trnHF05R: CGCGCATGGTGGATTCACAATCC
*ycf*1b	ycf1bF: TCTCGACGAAAATCAGATTGTTGTGAAT
	ycf1bR: ATACATGTCAAGTGATGGAAAA
*rpo*C1	rpoC1_2F: GGCAAAGAGGGAAGATTTCG
	rpoC1_4R: CCATAAGCATATCTTGAGTTGG

(Boldsystems.org)

Table 2: Thermal cycles for amplification of 8 DNA barcode regions in this study

Loci	Initial denaturation	35 cycles			Final extension
		Denaturation	Annealing	Extension	
ITS	95˚C 5 mins	95˚C 30 s	52.1-58˚C 30 s	72˚C 1 min	72˚C 7 mins
matK	94˚C 1 min	94˚C 30 s	41.3-54.9˚C 40 s	72˚C 40 s	72˚C 5 mins
atpF-atpH	94˚C 4 mins	94˚C 30 s	48.1-54˚C 40 s	72˚C 40 s	72˚C 5 mins
psbK-psbI					
rbcL	94˚C 4 mins	94˚C 30 s	52.1-58˚C 30 s	72˚C 1 min	72 ˚C 10 mins
trnH-psbA					
rpoC1	94˚C	94˚C	49.1-55°C	72°C	72°C
	4 mins	40 s	40s	1 min	10 mins
ycf1b	94˚C 4 mins	94˚C 30 s	49.1-55˚C 40 s	72˚C 1 min	72˚C 10 mins

Each reaction was carried out with the volume 50 µL including 25 µL ddH_2O, 20 µL MyTaq mix 2X (Bioline, England), 1 µL for each forward and reverse primer and 3 µL DNA template (100 ng/µL). The thermal cycles were reviewed from Fazekas *et al.* (2012). Gradient temperatures were conducted by a decrease and increase from the reference protocols.

PCR products analysis

The amplified products were evaluated by 2% agarose gel electrophoresis at 50 V in Run One Cell (Embi Tec, USA). Gel patterns were detected by Safeview under UV light (Gel Doc XR system, Bio-rad, USA).

RESULTS AND DISCUSSION

Amplification of ITS, *mat*K, *rbc*L and *rpo*C1

The gel patterns illustrated that the amplification process was successful. All amplicons from ITS, *mat*K, *rbc*L, and *rpo*C1regions were visualized on the gel background. In general, the band of interest was amplified at different temperature ranges in both the DNA template from durian and pomelo; however, the nonspecific bands also appeared. The increment of annealing temperature minimized effectively the false binding between primer and DNA template.

In the case of the ITS region (Fig. 1A), which is the non-coding sequence in the nucleus genome, the target band size was approximately 700-800 bp. In the annealing stage with low temperature, the inaccurate hybridization between primer and background DNA was reflected through the occurrence of extra bands. The positions of these bands were varied between durian and pomelo DNA. They located below the specific band in the former case while above in the latter one. However, non-target bands were nearly disappeared at 58°C, so the specificity was improved following the increase of annealing temperature in both two species, only one band was observed at this temperature. Thus, it was reasonable to identify 58°C as a suitable primer binding temperature for amplification of the ITS sequence.

The result of the amplified reaction for the *mat*K gene was different between durian and pomelo. In the case of durian DNA, the yield of PCR product was achieved through the high-intensity band and the size of the PCR products was 800-900 bp (Fig. 1B). However, the formation of multiple bands, which were shorter than the band of interest, lead the amplification was less specific. On the other hand, PCR efficiency and specificity of the *mat*K gene were confirmed for pomelo with a wide range of temperatures, from 41.3 to 54.2°C. Gel patterns from the *rbc*L gene and *rpo*C1 spacer gave the potential result when the only specific band was amplified at all primer binding temperature ranges (Fig. 1C and 1D). This effortless amplification may due to the short length of two barcoding regions, from 500-600 bp. In both two representatives, the band intensity and specificity shared the similarity between the lowest and highest annealing temperature.

Amplification of *ycf*1b gene and three spacers; *atp*F-*atp*H, *psb*K-*psb*I and *trn*H-*psb*A

The *trn*H-*psb*A spacer was not successfully amplified in *Durio zibethinus* due to the occurrence of multiple bands that shared similar intensity. On the other hand, this region was quite easy to amplify in *Citrus maxima*, which produced a clear band with a length of about 500 bp (Fig. 1E). Another point should be considered is gradient PCR was ineffective to reduce unwanted band, which could be observed at all temperature ranges.

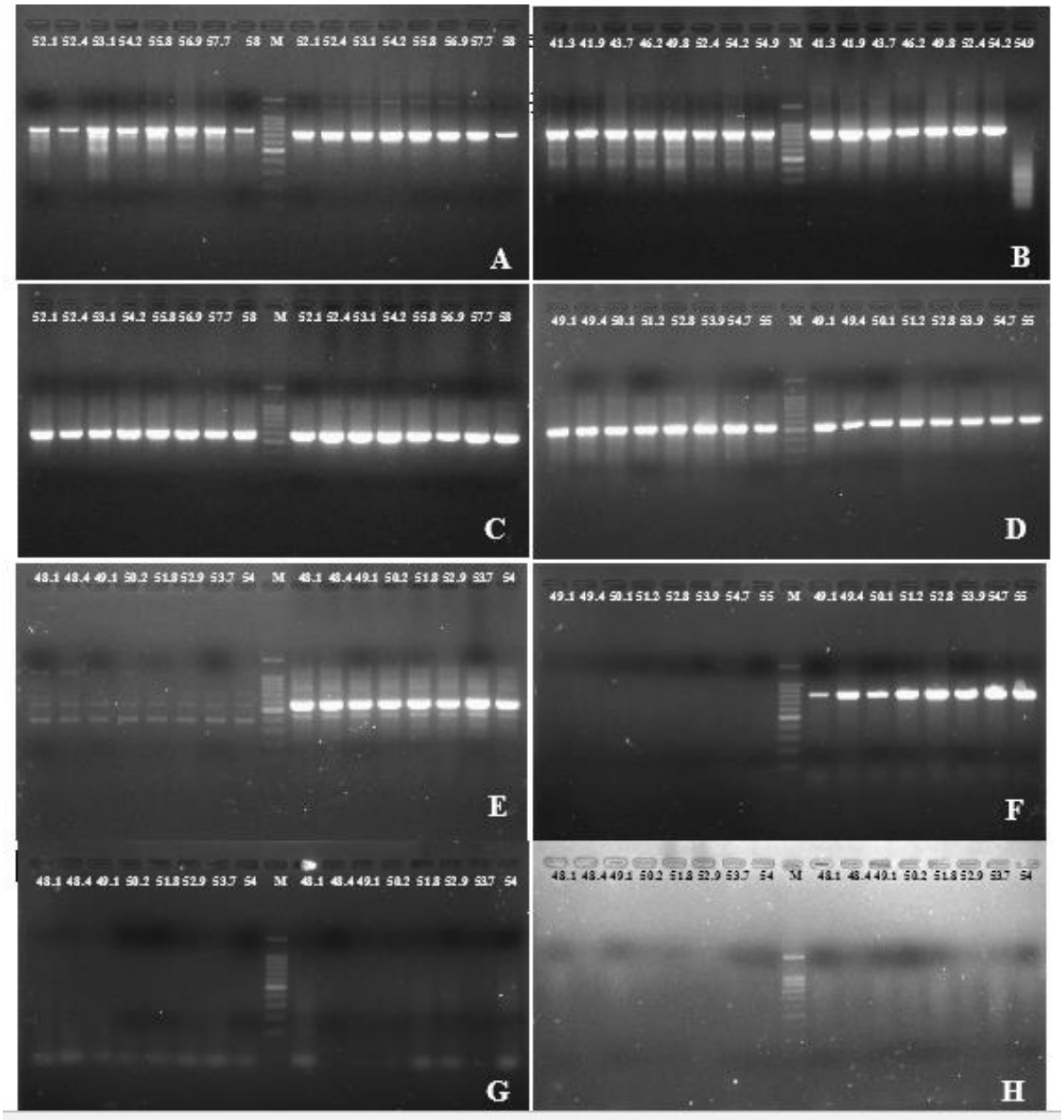

Fig. 1: Gel patterns for the amplification of eight DNA barcode regions of *Durio zibethinus* (on the left of the DNA ladder) and *Citrus maxima* (on the right of the DNA ladder) at different annealing temperatures on 2% agarose gel. M: 100bp ladder.

A: ITS, B: *mat*K, C: *rbc*L, D: *rpo*C1, E: *trn*H-*psb*A, F: *ycf*1b, G: *atp*F-*atp*H, H: *psb*K-psbI

Our two representatives were less related to each other, *Durio zibethinus* belongs to Malvales order while *C. maxima* is the member of Sapindales. Therefore, that far relationship might lead to PCR products were only visualized in the case of *C. maxima* instead of *Durio zibethinus* Moreover, the band of *ycf*1b in *C. maxima* was bright, clear and specific at all tested temperature points (Fig. 1F), so the PCR success was increased. For the two rest barcoding regions, *psb*K-*psb*I and *atp*F-*atp*H, no amplicons were formed (Fig. 1G and 1H). The results illustrated the less universality of such DNA sequences in *Durio zibethinus* and *C. maxima*, so the PCR success was decreased. The data for the amplification of eight barcode regions were summarized in Table 3.

Table 3: Parameters from amplicons for eight DNA barcode regions by gradient PCR

Barcode regions	*Durio zibethinus*			*Citrus maxima*		
	product length (bp)	formation of nonspecific bands	optimal annealing temperature	product length (bp)	formation of nonspecific bands	optimal annealing temperature
ITS	700-800	Yes	58°C	700-800	Yes	58°C
*mat*K	800-900	Yes	54.2°C	800-900	Yes	54.2°C

*rbc*L	500-600	No	52.1°C	500-600	No	52.1°C
*rpo*C1	500-600	No	49.1°C	500-600	No	49.1°C
*trn*H-*psb*A	NA	Yes	NA	500-600	Yes	NA
*ycf*1b	NA	NA	NA	800	No	49.1°C
*atp*F-*atp*H	NA	NA	NA	NA	NA	NA
*psb*K-*psb*I	NA	NA	NA	NA	NA	NA

NA: Not available

The fidelity and specificity of each loci were different between durian and pomelo. This result showed that amplification and sequencing of the *mat*K barcoding region were difficult due to high sequence variability in the primer binding sites (Hollingsworth *et al.*, 2011). This phenomenon recommended that the lower temperature could be applied because of higher temperature will decrease the PCR yield as well as a negative impact on *Taq* polymerase (Cha and Thilly, 1993). The data also confirmed the universality of *rcb*L and *rpo*C1 primers for successful amplification. In term of trnH-psbA intergenic spacer, the low PCR effectiveness was also confirmed by the result from other studies (Siew *et al.*, 2018), when these authors sequenced chloroplast DNA regions (*trn*L-*trn*F, *atp*B-*rbc*L, and *trn*H-*psb*A) from 124 durian varieties in Malaysia. A similar size band was received in the study carried on *C. maxima* in Hangzhou, China (Li *et al.*, 2019). Another point should be considered is gradient PCR was ineffective to reduce unwanted bands, which could be observed at all temperature ranges. *ycf1*b is the second largest gene in the chloroplast genome (5,709 bp in *Nicotiana tabacum*) and too variable between species, so it is difficult to design a universal primer for all plant species (Dong *et al.*, 2012; Dong *et al.*, 2015).

CONCLUSION

The impact of gradient PCR was different between eight DNA barcode candidates in *Durio zibethinus* and *C. maxima* due to the variable of target sequences. The optimal annealing temperatures for primer binding of ITS, *mat*K, *rbc*L, *rpo*C1 and *ycf*1b were determined. ITS achieved the PCR efficiency and specificity at 58°C while lower temperatures were involved for other regions; 54.2°C was showed as the suitable annealing temperature for *mat*K, 52.1°C was required for *rbc*L and 49.1°C was selected for optimal band expression of *rpo*C1 and *ycf*1b. For the improvement of specific hybridization between *trn*H-*psb*A and DNA template, further solutions should be considered such as touchdown PCR or primer redesigning.

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REFERENCES

i. *Cha, R.S., and Thilly W.G., 1993. Specificity, efficiency, and fidelity of PCR. PCR Methods and Applications. 3(3):18-29.*
ii. *CBOL Plant Working Group, 2009. A DNA barcode for land plants. Proceedings of the National Academy of Sciences of the United States of America. 106(31):12794-12797.*
iii. *Dong, W., Liu, J., Yu, J., Wang, L., and Zhou, S., 2012. Highly variable chloroplast markers for evaluating plant phylogeny at low taxonomic levels and for DNA barcoding. PloS one. 7(4):1-9.*
iv. *Dong, W., Xu, C., Li, C., Sun, J., Zuo, Y., Shi, S., Cheng, T., Guo, J. and Zhou, S., 2015. ycf1, the most promising plastid DNA barcode of land plants. Scientific Report. 5: 8348: 1-5.*
v. *Fazekas, A.J., Kuzmina, M.L., Newmaster, S.G., and Hollingsworth P.M., 2012. DNA barcoding methods for land plants. In: Kress, W.J and Erickson, D.L. eds. DNA barcodes, methods and protocols. Humana Press, New York, USA. pp. 223-252.*
vi. *Hebert, P.D., Cywinska, A., Ball, S.L., and Dewaard, J.R., 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences. 270(1512): 313-321.*
vii. *Hollingsworth, P.M., Graham, S.W., and Little, D.P., 2011. Choosing and using a plant DNA barcode. PloS one. 6(5): 1-13.*
viii. *Kress, W.J., 2017. Plant DNA barcodes: Applications today and in the future. Journal of systematics and evolution. 55(4): 291-307.*
ix. *Li, Y., Zhou, S., Tong, X., Wang, L., and Xiang, T., 2019. Analysis of the Origin of Citrus changshan-huyou by DNA Barcode. International Journal of Agriculture and Biology. 22(5): 973-978.*

x. *Rogers, S.O., and Bendich, A.J., 1988. Extraction of DNA from plant tissues. In: Gelvin, S.B., Schilperoort, R.A., and Verma, D.P.S. Eds. Plant molecular biology manual. Springer, Dordrecht. pp: 73-83.*

xi. *Rychlik, W.J.S.W., Spencer, W.J., and Rhoads, R.E., 1990. Optimization of the annealing temperature for DNA amplification in vitro. Nucleic acids research. 18(21): 6409-6412.*

xii. *Siew, G.Y., Ng, W.L., Salleh, M.F., Tan, S.W., Ky, H., Mohammed, A., Noorjahan, B., Tan, S., and Yeap, S. K., 2018. Assessment of the Genetic Variation of Malaysian Durian Varieties using Inter-Simple Sequence Repeat Markers and Chloroplast DNA Sequences. Pertanika Journal of Tropical Agricultural Science. 41(1):321-332.*