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Amplification of barcoding genes from Sri Lankan Turmeric plants.

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Abstract

Sri Lanka has many varieties of turmeric plants. They are morphologically identified with the observable differences, but they were not tested fully for any molecular differences. The present research was to check any size differences between *rbcL* and *ycf1b* genes in two varieties of Sri Lankan turmeric plants that could be detected by gel electrophoresis. Genomic DNA was extracted using CTAB method and the samples (*Curcuma domestica* MT 23, *Curcuma domestica* MT 32) were PCR amplified. The PCR program for the *rbcL* gene were as follows, initial denaturation at 95°C for 5 minutes, denaturation at 94 °C for 1 minute, primer annealing at 55 °C for 30 seconds, extension at 72 °C for 1 minute and finally, final extension at 72 °C for 10 minutes. The PCR program for *ycf1b* gene were as follows, initial denaturation at 95 °C for 5 minutes, denaturation at 95 °C for 30 seconds, primer annealing at 49°C for 30 seconds, extension at 72 °C for 30 seconds and finally, final extension at 72 °C for 7 minutes. Annealing temperatures were used accordingly for the primers. The agarose gel electrophoresis with TAE buffer showed bands proving the presence of genomic DNA and also the PCR was successful. Gel electrophoresis which was done using TBE gel revealed amplicon length polymorphism between the two samples used. At the used experimental conditions, it proved that both the varieties have the same molecular size of *rbcL* gene, but different molecular size of *ycf1b* gene. This simple and cost-effective method could be used as an alternative or a prior step to

sequencing of PCR products. As well as in supportive of sequencing results.

Keywords: Sri Lankan Turmeric, *Curcuma domestica*, *rbcL* gene, *ycf1b* gene

1. Introduction

Sri Lanka is an island nation which exhibits remarkable biological diversity and considered to be the richest country in the Asian region in terms of species concentration. For many types of flora, the country provides favorable conditions such as ecological, climatic, soil and topographical variability.

Home gardens are considered as the heart of the agricultural biodiversity. They are widespread throughout the country, existing in dry zones to wet zones.[1] On the rainfall distribution, Sri Lanka has been traditionally classified into three agricultural zones. They are the wet zone, intermediate zone and dry zone.

The following figure (Figure 1) shows the agricultural zones of Sri Lanka and the topography of Sri Lanka.

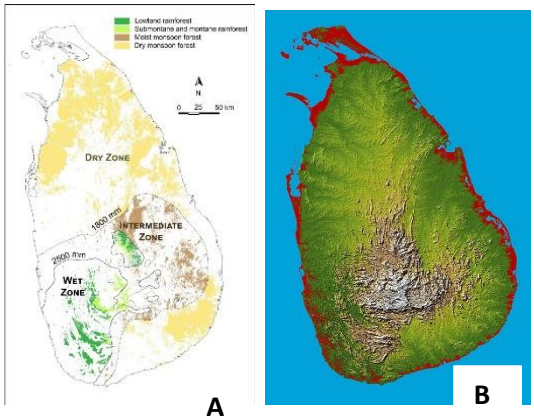

Figure 1. A) Agricultural zones in Sri Lanka[2] B) Topography of Lanka.[5]

The scientific name for turmeric is *Curcuma domestica*. Turmeric is a plant in the ginger family, which is also known as Zingiberene. Turmeric is native to Southeast Asia, and is grown commercially in that region. Its rhizome is used as a culinary spice and as a traditional medicine.[3]

Turmeric is also a common spice and a major ingredient in curry powder. Curcumin is the major component of turmeric. The activities of turmeric are commonly attributed to curcuminoids, which means curcumin and its similar substances. Curcumin also gives the yellow color to turmeric. It is the main active ingredient in turmeric. Curcumin has powerful anti-inflammatory effects, and it is a very strong antioxidant. Turmeric has a lot of health benefits. Mainly it contains bioactive compounds with medicinal properties.[3,4]

As mentioned, before there are three main agricultural zones in Sri Lanka, and turmeric is only grown in wet and intermediate zones. It is grown as a single crop and as an intercrop under coconut trees. major growing districts for turmeric are Kurunegala, Gampaha, Kalutara, Kandy, Matale and Ampara.[5]

In Sri Lanka, there are thirty-two morphological varieties described under turmeric as of 2023. *Curcuma domestica* MT 32 and *Curcuma domestica* MT 23 were researched in this project. the M stands for multiplication and T stands for turmeric in this research. (Through Personal Communication with Intercropping and Betel Research Station, Narammala, Sri Lanka.).

DNA barcoding is a system used for species identification focused on the use of short, standardized genetic region.[6] For the estimation of biodiversity, conservation of species and for ecological analysis, precise characterization of organisms is an essential fact. Typically, this has been performed by considering the visible morphological characters, but in recent years this is not universally acceptable because of cryptic morphological similarities between the species. With the advent of new technologies, “DNA barcoding” which is based on PCR was introduced to the world. This technology has emerged as an excellent tool in the unambiguous identification and improvement of their livestock and their byproducts used in several commercial entities such as food, medicine etc.[4] Figure 3 shows some examples of DNA barcoding species.

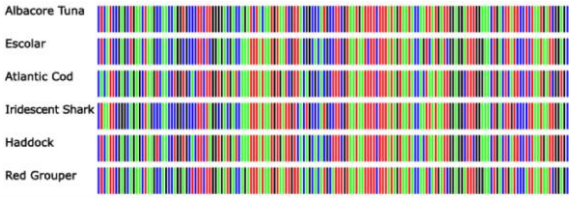

Figure 2. Examples for DNA barcoding species.[7]

In DNA barcoding most botanists use chloroplast coding regions such as *rbcL* and *mat K* with the *trnH- psbA* intergenic regions. There are several barcoding genes in turmeric. They are *mat K*, *rbcL*, *trnH-psbA*, *tmL-F* and ycf.[8]

Initiatives are taking place around the world to generate DNA barcodes for all groups of living organisms and to make these data publicly available in order to help understand, conserve, and utilize the wo’ld's biodiversity. For land plants, the core DNA barcode markers are two sections of coding regions within the chloroplast[9]. Other than these two chloroplast genes *ycf1b* is also identified as a chloroplast gene and it is the most variable plastid genome region, and this gene can serve as a core barcode of land plants.[10]

There are several accessions of turmeric with yield differences and differences in chemical composition which cultivated in Sri Lanka (Through personal communication with Intercropping and Betel Research, Narammala, Sri Lanka However, they were not proven for their differences at the molecular level. Therefore, it is important that they should be checked for any variations present among them. This is important because if the variations are being identified, it can be used for new medicinal inventions and also in food industry.

ycf1b and *rbcL* barcode genes of Sri Lankan turmeric plants were PCR amplified and checked for successful amplification, the

possible differences between amplified products were evaluated using gel electrophoresis.

2. Methodology

2.1 Sample Collection. The plant samples for this study were collected from the Intercropping and Betel research station. The information of the 2 different plant types selected are mentioned in table 1 and their morphological appearance is given in figure 3.

Table 1. Plant species of the project.

Plant species	Scientific name	Variety	Plant part used for DNA extraction
Sri Lankan turmeric plants	*Curcuma domestica*	MT 23 and MT 32	Middle part of the rhizome

Figure 3. A) *Curcuma domestica* MT 23, B) *Curcuma domestica* MT 32

2.2 Preparation of equipment. The motor and pestles used for DNA extraction was surface disinfested with 70% of ethanol and wiped with tissues, then wrapped with demy papers and were surface sterilized in the hot air oven for 2 hours at 180°C. Pipette tips, falcon tubes, Microcentrifuge tubes and distilled water were sterilized by autoclaving at 120°C under 15psi for 20 minutes. The samples were washed, and the skin was peeled off before they were used for measuring.

2.3 Plant genomic DNA extraction using CTAB method. A mass of 200 mg of both the samples were weighted into 2 different plastic holders. CTAB was preheated in the shaking incubator for 30 minutes. After adding 1ml of CTAB solution to the sterile motor, started to grind the samples with the pestle. A total of 4 ml of CTAB solution was added to the samples in order to achieve the required level of homogenization. Then the samples were incubated at 65 °C for 30 minutes in the shaking incubator. The samples were taken out and kept until it cooled down to the room temperature. After the samples were cooled down, 330 µl of 5 M potassium acetate was added and mixed it by inverting. Samples were incubated in an ice box for 45 minutes. 600 µl of chloroform-isoamyl alcohol was added. The samples were centrifuged at 12,000 g (RCF) at 4 °C for 15 minutes. Afterwards the supernatant was transferred into a separate 1.5 ml of Microcentrifuge tube. 600 µl of 30% PEG was added to the supernatants and mixed by inverting. The samples were incubated in the freezer overnight. The samples were again centrifuged at 12,000 g (RCF) at 4 °C for 15 minutes. The supernatant was removed while leaving the white DNA pellet. 500 µl of 70% ethanol was added to the samples while disturbing the pellet. The tubes were mixed by using the vortex. Again, the samples were centrifuged at 12,000 g (RCF) at 4 °C for 15 minutes. The supernatant was removed and left the white DNA pallet in the tube. Then pipetted out any residual ethanol and let the pallet air-dry. Finally, 20 µl of PCR water was added and let the samples dissolved and stored the samples at -20 °C. Afterwards spot gel method was used to check the concentration of the samples.

2.4 DNA quantification by spectrophotometer. Before measuring the absorbance values of the samples, the original DNA samples were diluted. The samples were made up to 125µl of a volume (100x diluted). The machine was calibrated, and the diluted samples were filled one by one. Then the computer was set to the optimum conditions and the samples were run. When samples were changed from the cuvettes, they were emptied and washed with distilled water.

2.5 Amplification of genomic DNA using PCR. The PCR components were taken out from the

-20°C refrigerator and was thawed on ice and given a quick spin prior to preparing the master mix. The master mix was prepared and the PCR reaction mixtures were made inside the laminar hood. Table 2 show the components and volumes of the master mix for *rbcL* primer and *ycf1b* primer.

Table 2. Components of the master mix for *rbcL* primers and *ycf1b* primers.

Components	Volume	X2	X3
PCR water	2.8 µl	5.6 µl	8.4 µl
2x buffer	5 µl	10.0 µl	15 µl
rbcL forward primer	0.3 µl	0.6 µl	0.9 µl
rbcL reverse primer	0.3 µl	0.6 µl	0.9 µl
Taq polymerase	0.6 µl	1.2 µl	1.8 µl
DNA template	1 µl	2 µl	3 µl
Total volume	10.0 µl	20.0 µl	30.0µl

A volume of 9µl of master mix was added into each tube and 1µl of DNA template from each sample was added into 2 different PCR tubes. The remaining PCR tube, which was the negative control tube, 1µl of PCR water was added instead of DNA templates.

The labeled PCR tubes were added to the PCR machine and the programs were adjusted accordingly. Following tables (Table 3 and Table 4) show the programs of the two primers.

Table 3. Stages, temperatures, and time for the PCR program for *rbcL* primer.

Stages	Temperatures	Time
Initial denaturation	95°C	5minutes
Denaturation	94°C	1minutes
Annealing	55°C	30seconds
Extension	72°C	1minutes
Final extension	72°C	10minutes

Table 4. Stages, temperatures, and time for the PCR program for *ycf1b* primer.

Stages	Temperatures	Time
Initial denaturation	95°C	5minutes
Denaturation	95°C	30seconds
Annealing	49°C	30seconds
Extension	72°C	30seconds
Final extension	72°C	7minutes

2.6 Agarose gel electrophoresis using TAE buffer. The 1% agarose in TAE gel was prepared. The PCR products were mixed with the loading dye before loading them into the gel. From each sample 3 µl was mixed with the loading dye. After mixing it well, the samples were loaded into the gel wells. The loading order was the DNA ladder, *Curcuma domestica* MT 23, *Curcuma domestica* MT 32 and finally the negative control. After loading the samples, the gel was run under the voltage of 100V for 15 minutes and 65V for 45 minutes. The PCR products were visualized following gel electrophoresis. Then the results were observed by the computer system using image lab software.

2.7 Preparation of TBE buffer. TBE buffer contains Tris, Boric acid and 0.5M Na_2EDTA. The buffer was prepared from 10x diluted buffer for an amount of 200 ml solution. The needed volumes for the solution are shown in the following table (Table 5).

Table 5. Volumes needed to prepare the TBE buffer.

Components	Needed volumes
Tris	21.6 g
Boric acid	11 g
0.5M Na_2EDTA	8 ml

2.8 Gel electrophoresis using TBE buffer. To prepare 2% of agarose gel, 3 g of agarose powder was needed. A separate conical flask was taken and 3 g of agarose powder was added into it. 150 ml of 0.5x TBE buffer was added to

the same conical flask. Then swirled gently for the powder and the buffer to mix. The solution was then heated in the microwave until the powder was dissolved in the buffer completely and no bubbles were formed. Then the solution was kept to cool down and added 3 µl of ethidium bromide. Swirled the solution gently without forming any bubbles. Then the solution was poured into the gel tray which was prepared before and kept for it to set. The samples were loaded as the same way when the samples were loaded using TAE buffer. The gel was run under a voltage of 120V.

3. Results

3.1 Plant genomic DNA extraction.

The spot gel image of the extracted DNA is indicated in figure 6. In the gel image it showed as bands and the image are shown in figure 7.

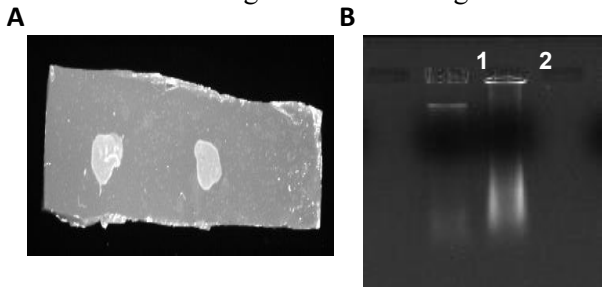

Figure 4. A) Image of the spot gel. [S1(sample 1: *Curcuma domestica* MT 23), S2 (sample 2: *Curcuma domestica* MT 32). B) Image of the gel. Lane 1: *Curcuma domestica* MT 23), lane 2: *Curcuma domestica* MT 32)].

3.2 Quantification of PCR products using Spectrophotometer. Samples were diluted with 1/100 dilution factor; the total volume was made up to 125µl.

Table 6. Quantification results of extracted genomic DNA.

Sample	A[260]	A[280]	Ratio	Nucleic Acid/diluted sample (µg/ml)	Nucleic Acid/concentrated sample (µg/ml)
Curcuma domestica MT23	0.0019	0.0006	0.9175	0.0293	2.93
Curcuma domestica MT 32	0.0002	0.0013	1.0591	0.1050	10.5

3.3 Visualization of PCR products.

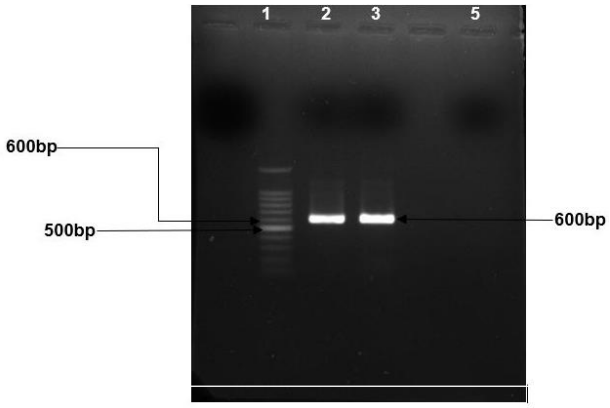

Figure 5. Gel image of PCR products with *rbcL* primers using TAE buffer. (Lane 1: 100bp ladder),(lane 2: *Curcuma domestica* MT 23), (lane 3: *Curcuma domestica* MT 32). (lane 5: Negative Control).

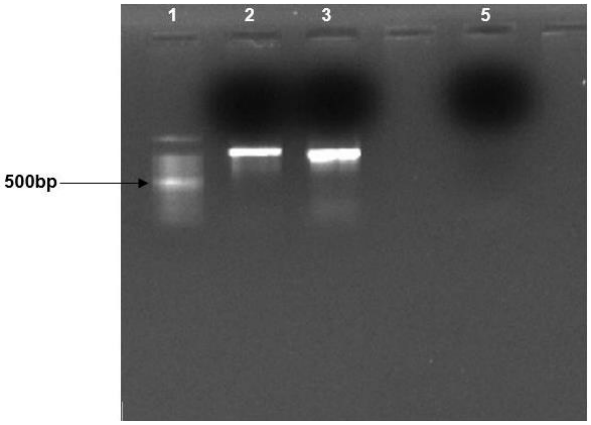

Figure 6. Gel image of the PCR products with *ycf1b* primers using TAE buffer. (lane 1:100bp ladder), (lane 2: *Curcuma domestica* MT 23), (lane 3: *Curcuma domestica* MT 32), (lane 5: Negative control.)

3.4 Visualization of PCR products using TBE buffer.

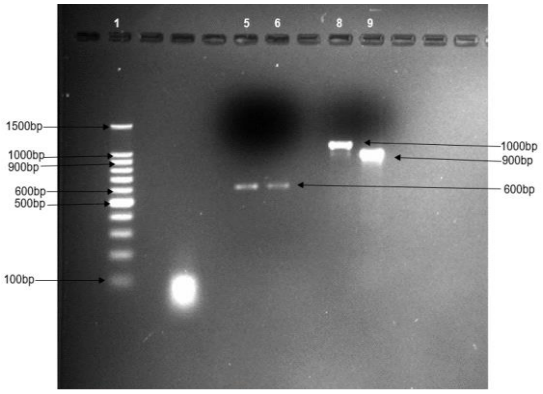

Figure 7. Gel image of PCR products using TBE buffer. (lane 1:100bp ladder), (lane 5: *Curcuma domestica* MT 23(*rbcL*), (lane 6: *Curcuma domestica* MT 32(*rbcL)*, (lane 8: *Curcuma domestica* MT 23(*ycf1b*). (lane 9: *Curcuma domestica* MT 32 (*ycf1b*).

4. Discussion.

When analyzing genes by PCR amplification, it usually depends on relatively small volumes of DNA. In this research, CTAB method was used to extract the genomic DNA from turmeric plants. Plant tissues have two classes of biomolecules which are polysaccharides and polyphenols. These biomolecules vary significantly between species and are problematic when isolating DNA. These two biomolecules can be reduced by using the CTAB method.[11] According to the spot gel image of figure 4A, the extracted plant samples contain genomic DNA in sufficient amounts. This is shown by the intense spots on the spot gel. For further confirmation, the samples were run in a gel electrophoresis, this is shown by figure 4B. According to the figure 4B, the samples contain genomic DNA by showing bands.

Polymerase chain reaction (PCR) is a common laboratory technique which is use to make many copies of an interested DNA fragment.[12] One of the essential components of PCR is *Taq* polymerase. Annealing temperatures are important when considering each primer, the temperatures can be different from one primer to another, and this is important, because if it is not in the optimal temperature non-specific products can be formed and also the yield of products can be reduced.[14] In this research, *rbcL* and *ycf1b* primers were used and the annealing temperatures for them accordingly were 55°C and 49°C. 2x PCR buffer was included with $MgCl_2$ and dNTPs. For *ycf1b*, the annealing temperatures can be used for further researches as it showed good bands without any primer dimers.

Agarose gel electrophoresis is the most effective way to separate DNA fragments in different sizes. When the current is applied, the DNA backbone is negatively charged, therefore, the negatively charged DNA was migrated from the negative end to the positive end. The results were visualized under the gel documentation system. It was shown as bands according to their molecular weights as shown in figure 5 and figure 6.

Even though as the normal routine TAE buffer was used at first, for more clarification the samples were run in an agarose gel with TBE buffer. TBE buffer is needed for a higher resolution for small DNA fragments. Furthermore, the samples were run in an agarose gel with TBE buffer too. As in the figure 7 shows the bands were very clear when the gel was observed under the UV light. The same figure shows, the PCR products were amplified using the *rbcL* primers in the same line. Both the varieties of Turmeric contain *rbcL* and both have the same molecular size *rbcL* gene, but the PCR products that contain *ycf1b* primers are not in the same line. Both the varieties of Turmeric contain *ycf1b* gene, but do not contain the same molecular size of *ycf1b* gene.[16]

5. Conclusion

In this study we, demonstrated the molecular differentiation of *rbcL* and *ycf1b* genes on the basis of amplicon length polymorphism revealed by agarose gel electrophoresis. This method will be very useful for accurate identification of plant genes' molecular sizes. This technique is simple and cost effective and could be used as an alternative or a prior step to sequencing of PCR products, The objectives which were proposed were successfully achieved. Further work includes obtaining the DNA sequence of the PCR products of the *ycf1b* primers.

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