Research Article
J. Food Sci. Technol. Nepal, Vol. 13 (43-53), 2022
ISSN : 1816-0727

DNA extraction and PCR optimization for DNA Barcode analysis of commercially grown coffee varieties of Nepal

Shreejan Pokharel[1], Namita Bhattarai[1], Bignya Chandra Khanal[1], Anish Basnet[2] and Gyanu Raj Pandey[2]

[1]National Biotechnology Research Centre, Nepal Agricultural Research Council, Lalitpur, Nepal

[2]Shubham Biotech Nepal Pvt. Ltd., Chitwan, Nepal

Abstract

The isolation of high quality Genomic DNA is an essential criterion for further Molecular analysis. *Coffea* genus is well known for its high amount of polyphenols, polysaccharides and other secondary metabolites that degrades the quality of the DNA isolation needed for further downstreaming processes. The present work was carried out with the goal of obtaining simple and efficient DNA isolation protocol generating high quality amplification for Barcoding. The protocol involves modifying the CTAB extraction, incorporating use of polyvinylpyrrolidone and β-mercaptoethanol yielding quality DNA with ratio (A260/280) between 1.8-2.0 indicating low contamination. The PCR conditions were optimized for high amplification on the basis of optimal concentration of $MgCl_2$ (3mM), primer (0.5 µM), Taqpolymerase (0.2 U), 50-60 ng of DNA template and cycle conditions as: initial denaturation of 94°C for 4min followed by 35 cycles of denaturation at 94° for 50 sec, annealing at 54°C for 50 sec and extension at 72°C for 80 sec, followed by final extension at 72°C for 7 min. The optimal conditions produced high amplified reproducible data. Thus, the optimized method proposed enabled a simple and fastDNA extraction and PCR amplification for Coffeegenus, may serve as efficient tool for further molecular analysis.

Key words: *Coffea*, Coffee, DNA Isolation, PCR amplification, Internal Transcribed Spacers, Maturase K,ribulose-1, and 5-bisphosphate carboxylase large subunit

Introduction

Globally, genus *Coffea* belongs to the family *Rubiaceae*. Of the 124 species of the genus *Coffea* (Davis 2011), contains the two main commercially cultivated species; *Coffea arabica* and *Coffea canephora. C. arabica* is the only tertraploid (2n =4x=44) species in the genus and is self-fertile, whereas other species are diploid (2n=2x=22) and genetically self-incompatible (Clarindo and Carvalho, 2008). *C. arabica* is derived from the hybridization of *C.canephora* and *C. eugenioides* (Lashermes et al. 1999). Coffee is mainly grown in tropical and subtropical regions and is an important cash crop in more than 60 countries in south and central America, Asia, and Africa with an acreage of over 11 million ha (Waller *et al.,* 2007).

Coffea is not indigenous plant to Nepal; its seed were first brought and planted in Nepal in 1938 A.D.With its popularity it has been one of the emerging and high value cash generating commodities for hill farmers of Nepal (Khanal, 2003).Coffee plantation covers more than 1000 ha of land with a total production of 250 mt of dry cherry (Dhakal, 2005). Any data of the first planted coffee varietyand the diversity of C*offee*s pecies in last 7 decadeare not officially recorded.

*Coffea*species Hybridize readily with one another and produce relatively fertile hybrids (Charrier 1978; Louarn 1992). Infrageneric classifications have been proposed based on morphological characters (Lebrun 1941; Chevalier 1947). However, grouping criteria have become very complex and rather confused, and to-date they are considered of low value (Bridson and Verdcourt 1985). Complementary investigations are therefore required to clarify the phylogenetic relationships among these taxa (Charrier and Berthaud 1985).

In plants, the nuclear ribosomal DNA units (rDNA) consist of the 18s, 5.8s and 36s coding regions separated by intergenic spacers. Nuclear rDNA has proven to be a powerful phylogenetic tool because of the ubiquity of rDNA throughout plant species, the development of techniques for the rapid determination of the primary nucleotide sequence, and the diverse rates of evolution within and among component subunits and spacers (Hamby and Zimmer 1992).

According to Johnson and Soltis (1994), Olmstead and Palmer (1994), the rate of nucleotide susbstitution in matK is three times higher than that of the large subunit of Rubisco (rbcL) and six fold higher than the amino acid substitution rate, which significantly presents it as a fast evolving gene. This capacity of matK gene also provides high phylogenetic signal for resolving evolutionary relationships and relatedness among plants at all taxonomic levels (Soltis and Soltis, 1998; Hilu *et al.,* 2003).

Many chloroplast, mitochondrial and nuclear genes have been utilized for studying and understanding sequence variations and evolutionary trends at the genus level (Clark *et al.,* 1995; Hsiao *et al.,* 1999). Among the genes, sequences for the rbcl gene was frequently used and analyzed by researchers in the bid to understanding plant systematics beyond the family level (Donoghue *et al.,* 1992; Chase *et al.,* 1993; Duval *et al.,* 1993). However, maturase K (matk) gene, formally known as orfk has emerged as a gene of interest with potential in plant molecular systematics and evolution because of the genes' rapid evolution at nucleotide and corresponding amino acid levels (Johnson and Soltis, 1995; Liang and Hilu, 1996; Miller *et al.,* 2006).

Quality Nucleic acid isolation is essential for further downstreaming application (PCR amplification, Restriction Digestion, Cloning, Genotyping, and Sequencing). Like many other tree plant species, coffee contains high amounts of polysaccharides, polyphenols and various secondary metabolites such as alkaloids, flavonoids and phenols which usually interfere during DNA isolation. Flavonoids, phenols, and polysaccharides bind firmly with nucleic acids during DNA isolation and interfere with subsequent reactions (Demeke and Jenkins, 2010; Varma, *et al.,* 2007). In addition to this, co-isolation of highly viscous polysaccharides along with DNA was the major problem encountered during coffee DNA isolation. Several methods have been reported for minimizing the DNA extraction steps and cost (Berthomieu and Meyer, 1991; Edwards et al., 1991). One isolation protocol may not permit optimal DNA yields in different taxa so, an efficient DNA isolation and PCR optimization is must. Researchers often modify a protocol or blend two or more different procedures to obtain DNA of the desired quality (Varma *et al.,*2007). Classical DNA extraction protocol for many plants (Doyle and Doyle, 1987) have been modified for own convenience.

Here, we report a total Genomic DNA extraction protocol originally for plants (Doyle and Doyle, 1987) and some modification to it for high quality DNAyield, optimization of PCR conditions in order to identify phylogenetic relationship of *Coffea* species by the means of ITS, MatK and Rbcl Sequences.

Materials and Methods

Plant Materials

For DNA Extraction, 23 samples (Table:1.1) of coffee were collected from Nepal Coffee Research Center, Gulmi, Nepal (Latitude : 27°56'17.85'' N to 27°5'44.87'' N; Longitude: 8° 25' 29.2''1 E to 83°25'30.20'' E) and Coffee Development Center, Aapchaur-05 ,which were maintained ex-situ in Research Center (Nepal Agricultural Research Council, Khumaltar, Nepal).

Code	Variety	Sample Type	Source (Location)	Year of Introduction (B.S.)	Young leaf Colour	Fruit Colour
C1	Gulmi local	Seed	CDC, Gulmi	2071	Green	Red (G to R)
C2	Chhetradeep	Seed	HRS, Malepatan, Pokhara	2071	Green	Red (G to R)
C3	Selection-10	Plant	HRS, Malepatan, Pokhara	2071	Green	Maroon red (G to R)
C4	Selection-12 (Cavery)	Seed	Thanapathi VDC, Thorga, Gulmi	2073	Green	Red (G to Y to R)
C5	Yellow Catura	Plant	HRS, Malepatan, Pokhara	2071	Green	Yellow (G to Y)
C6	Catimor (Red)	Plant	HRS, Malepatan, Pokhara	2071	Green	Red (G to R)
C7	Pacamara	Plant	HRS, Malepatan, Pokhara	2071	Green	Red (G to R)

C8	Tekisic	-	-	-		-
C9	Pacas	Plant	HRS, Malepatan, Pokhara	2071	Green	Red (G to R)
C10	Robusta Coffee	Seed	CDC, Gulmi	2073	Green	-
C11	Catuai Amarillo (Brazillian)	Plant	HRS, Malepatan, Pokhara	2071	Green	Yellow (G to Y_
C12	Sanramon	-	Indian	2057	Light Green	Dark Red (G to R)
C13	Catisic	-	-	-	-	-
C14	Bourbon Amarillo	Plant	HRS, Malepatan, Pokhara	2071	Green	Red (G to Y to R)
C15	Mundo Novo (Brazillian)	Plant	HRS, Malepatan, Pokhara	2071	Green	Red (G to Y to R)
C16	Caturra Amarillo	-		2059	Light Green	Yellow (G to Y)
C17	Syangja Special	Plant	Highland coffee nursery, Syangja	2071	Green	Red (G to R)
C18	Catuai Vermello	-	-	-	-	-
C19	Argakhachi local	Seed	-	2072	Green	Red (G to R)
C20	Kaski local	Seed	HRS, Malepatan, Pokhara	2071	Green	-
C21	Indonesia	Plant	DCPA, Kaski	2071	Green	Maroon red (G to R)
C22	Bourbon Vermelo	Plant	HRS, Malepatan, Pokhara	2071	Light Green	Red (G to R)
C23	Hawaii Kona	Plant	DCPA, Kaski	2071	-	-

Table 1.1 Coffee sample varieties collected from National Coffee Research Centre, Gulmi, Nepal. G = Green; Y = yellow; and R = Red.

Solutions

An extraction buffer consisting of 2% CTAB (w/v), Tris HCL pH 8.0 (0.1M); EDTA pH 8.0 (20mM); NaCl (1.4M); 2 %PVP (w/v); 1 % β-mercaptoethanol (added just before Pre warming) was prepared. Other additional solutions needed were Tween 20, Chloroform: Isoamylalcohol (24:1v/v), Ethanol (70% and 100%), and TE buffer (Tris-HCl 10 mM, EDTA 1mM, pH 8.0).

DNA Extraction

DNA Extraction technique (Doyle and Doyle, 1987) was used with some modifications to optimize the extraction process.

DNA Extraction Protocol

Freshly harvested young leaf sample (1g) was taken and surfaced cleaned with Tween 20 followed by periodic cleaning with Distilled water. Then the sample was ground in liquid Nitrogen using mortar and pestle. The pulverized leaves were immediately transferred to tube and freshly prepared prewarmed (65 C) extractionbuffer (700µl) was kept. The tubes were incubated at 65 C in water bath for 1 hr with inverting the tubes every 10 mins. Then an equal volume of Chloroform: Isoamylalcohol (24:1) was added and mixed properly by inversion for couple of minutes and centrifuged at 15,000 rpm for 15 min at Room temperature. After the phase separation the supernatant was carefully pipetted

out and transferred into a new tube. To the supernatant,repeated process of keeping Chloroform: Isoamylalcohol (24:1) of 700µl was done so that complete removal of pigments and debris can be obtained. The tube was centrifuged at 15000 rpm for 7 min. Then the supernatant was transferred to a new tube and precipitated by adding equal volume cold absolutealcohol and gently mixed by inverting and was stored at -20 C overnight. The samples were centrifuged at 10,000 rpm for 10 min. The pellets were washed with 70% ethanol twice. The pellet was air dried and re-suspended in TE buffer (1X).

DNA quantification and purity

DNA quantification and quality was assessed by using Nanodrop (Quawell Q-5000). The purity of the DNA was assessed by the absorbance($A_{260/280}$) ratio and running the DNA samples on 0.8% agarose comparing it with 1kb DNA ladder.

Gel Electrophoresis

The quality of extracted DNA was also assessed by using 1.5% agarose gel electrophoresis (Cleaver scientific, UK) in 1XTAE (50X TAE; 242gm Tris-base, 57.1 ml acetic acid (or 100% glacial acid) and 100ml of 0.5 M EDTA (pH-8.0) at 70V for 1 hr. PCR amplification products were analyzed by using 2% agarose gel at 70V for 2.5-3hr. using the same buffer system. The gel was stained with ethidiumbromide and photographed using Gel Documentation system (VWR® Genosmart 2, UK).

Primer Sequence

Table 1.2: Primer set for Barcoding analysis

Barcode	Primer	Primer sequences(5'-3')	References
ITS (ITS1-5.8s-ITS7)	*ITSL* *ITSR*	TCGTAACAAGGTTTCCGTAGGTG TATGCTTAAAYTCAGCGGG-3'	Hsiao et al. 1994
matK	*3F_Kim f* *1R_Kim r*	CGTACAGTACTTTTGTGTTTACGAG ACCCAGTCCATCTGGAAATCTTGGTTC	CBOL- PWG 2009
rbcl	*rbclF* *rbclR*	ATGTCACCACAAACAGAGACTAAAGC GAAACGGTCTCTCCAACGCAT	CBOL- PWG 2009

ITS = internal transcribed Spacers; matK = maturase K ; rbcl = ribulose-1,5-bisphosphate carboxylase large subunit

PCR Reaction

The reactions were carried out in Mygene L series thermo cycler (LongGene scientific instrument co .LTD). Reactions without DNA were used as Negative Controls. The conditions were optimized by varying parameters as directed in Table 1.3. The reaction contains about 50-60 ng of template DNA, 2x master mix (Promega Corporation, USA), 0.5 µm of single Primer (Macrogene Inc., South Korea), with additional 25mM$MgCl_2$ (Himedia laboratories Pvt. Ltd, India), 0.2mM dNTP Mix (Promega Corporation, USA), 0.2U of T*aq* polymerase (Promega Corporation, USA), 1mg/ml BSA. The thermo cycler was programmed for an initial denaturation step of 4 min at 94°C, followed by 35 cycles of 50s annealing at 54°C (for ITS, Matk and rbcl marker), extension was carried out at 72°C for 80 sec and final extension at 72°C for 7 min and hold temperature of 4°C at the end. PCR products were electrophoresed on 2% (w/v) agarose gel stained with ethidium bromide, at 70V for 3 hrs. Gels were visualized and photographed by using Gel documentation system (Genosmart v2, VWR, UK). 1kb and 100 bp ladder (Thermo scientific), was used as molecular marker for the size comparison of the visible fragments.

Table 1.3 Optimization of PCR reaction parameters for *Coffee Sp*(Table 1.1)

PCR Parameter	Tested Range	Optimum Condition	Observations
DNA Concentration (ng)	10, 20, 30, 40, 50, 60, 80, 100, 150 and 200	50-60ng	High Amplification observed from within range. No amplification on lower concentration and presence of smear in higher concentration.
$MgCl_2$ Concentration(mM)	1, 1.5, 2 , 2.5, 3, 3.5, 4	3mM	High /low concentration increases the non-specificity and no yield of product.

dNTPs Concentration (mM)	0.12 , 0.13 , 0.14 , 0.15 , 0.16, 0.2	0.16 – 0.2mM		Increase in concentration reduces the intensity and amplification.
Primer Concentration (µM)	0.1 , 0.5, 1 , 1.5, 2, 2.5 , 3, 3.5	0.5 µM		Intensity of amplified bands were same from 1-3 µM concentration. No amplification above 3.5 µM and faint amplification below 0.5 µM
T*aq* Polymerase (units)	0.1 , 0.2 , 0.3 , 0.4 , 0.5	0.2 U		Lower concentration showed faint bands and high concentration decreased the specificity.
Initial Denaturation time (min) at 94°C	2 ,3 , 4 and 5	94°C,4 min		Unreproducible amplification in higher/lower time intervals, loss of T*aq* activity leading to poor amplification.
Annealing Temperature (°C)	45, 48, 50, 52, 53, 55 and 62	*ITS*	54°C for 50sec	Low/no amplification in higher and lower temperature and time interval than optimum range.
Time Interval (s)	40,45,50,55,60 and 65	*matK*	54°C for 50sec	
		rbcL	54°C for 50sec	
Reaction volume (µl)	10 , 12,13 ,15	13µl		Determine cost of the PCR ingredients
Number of Cycles	25, 30 ,35 and 40	35		Low/no amplification in higher and lower cycles from optimum.
DMSO (Dimethyl sulfoxide)	3 , 5 , 7, 10 %	7%		High intensified bands in optimum conditions. Faint bands/ no bands on high/ low concentration than optimum.

RESULT AND DISCUSSIONS

DNA extraction and quality

The classical extraction protocol (Doyle and Doyle) resulted in the poor absorbance ratio ($A_{260/280}$) values below 1.5 and above 2.2 with brown coloration of the DNA pellets.The protocol was modified from Doyle and Doyle method (Doyle and Doyle, 1987). The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity by binding covalently with the extracted DNA making it useless for most research applications (Katterman and Shattuck, 1983; Peterson *et al.,* 1997; Porebski *et al.,* 1997). Polyphenols bind DNA and make downstream processing difficult as they get co-precipitated with the nucleic acid. Classical extraction protocol (Doyle and Doyle, 1987) was conducted which resulted in poor DNA quality.

Modification was done to the classical method for improving the DNA quality. These modification includes using CTAB and its components at higher concentration than classical Doyle and Doyle protocol (Doyle and doyle,1987) (2% CTAB (w/v), Tris HCL pH 8.0 (1M); EDTA pH 8.0 (0.5 M); NaCl (1.4M), using of PVP and β-mercaptoethanol. CTAB, a strong ionic detergent, has been employed to facilitate the separation of proteins from nucleic acids in extractions of biological materials (Abu-Romman, S., 2011). The high ionic strength of CTAB forms complexes with protein and the majority of the acidic polysaccharides, and a high concentration of NaCl aids in the removal of polysaccharides (Aljanabi *et al.,*1999). On the other hand, PVP and β-mercaptoethanol prevent oxidation of the secondary metabolites, such as phenols, during cell lysis. PVP forms hydrogen binding with the phenolic compounds, and when these compounds are centrifuged in the presence of chloroform, they accumulate in the interphase, between the organic and aqueous phases (Aljanabi *et al.,*1999). β-Mercaptoethanol prevents the polymerization of tannins, which hinder the isolation process in a manner similar to polysaccharides (Varma, *et al.,* 2007). The time between grounding of the samples in liquid Nitrogen to placement of vials into water bath plays a vital role in DNA quality as high polyphenolic contents are present in coffee sp. which oxidizes quickly giving brownish coloration which interfere the reading of $A_{260/280,}$ leading to low DNA quality for further downstreaming process. Leaves samples should be young and freshly picked; leaves stored in -80°C or liquid nitrogen several weeks prior can also be used. Consequently, high quality DNA was obtained from presented protocol with low-polysaccharide genomic DNA.

With all the modification and optimization in the modified protocol, increased the DNA yield and quality,

in particular in the $A_{260/230}$ratio ranging from 1.8-2.0. A ratio of absorbance ($A_{260/280}$) in the range 1.8-2.0 indicates a high level of purity (Pasakinskiene, I.; Pasakinskiene, 1999; Weising, K.; Nybom, H.; Wolff, K.; Kahl, G, 2005.)The excess of salts in the pellets gets removed by washing in 70% ethanol leading to quality DNA. The DNA yield obtained ranged from 500-2000 ng/µl. The presence of DNA was assessed by running the genomic DNA (Only first 17 samples were tested, extracted on same batch of 23 samples) (Table 1.1) in 0.8%agarose (figure1a) and DNA quantification was assessed by using Nanodrop (Quawell Q-5000).

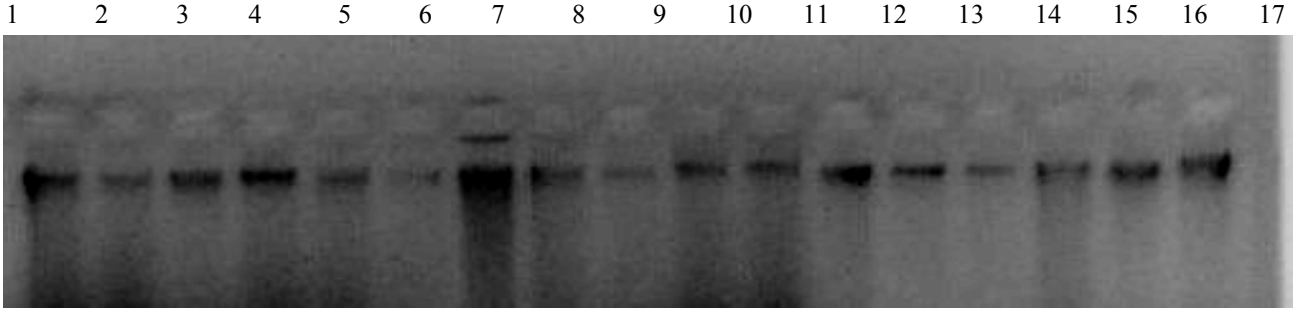

Figure 1 a) Lanes 1-17 represents the agarose gel (0.8%) of Genomic DNA isolated from first 17 species of coffee viz 1. Gulmi local, 2. Chhetradeep, 3. Selection-10, 4. Selection-12, 5. Yellow Cataura, 6. Catimor, 7. Pacamara, 8. Tekisic, 9. Pacas, 10. Robusta, 11. Catuai Amarillo, 12. Sanaramon, 13. Catisic , 14. Bourbon Amarillo, 15. Mundo Novo, 16. Caturra Amarillo, 17. Syangja Special. (Only 17 species out of 23 were ran on gel for validity of the extraction process done on the same batch). DNA isolated from this method yielded strong and reliable amplification products showing its compatibility for further downstreaming process.

PCR Reaction

Further, downstreaming process includes PCR reaction prior to sequencing and other molecular analysis. With all the components in PCR reaction, $MgCl_2$ concentration and T*aq* DNA polymerase plays the most vital role on getting good PCR product. Magnesium acts as cofactor to the Thermostable DNA polymerases during the reaction process and changing the magnesium concentration is one of the easiest reagents to manipulate with perhaps the greatest impact on the stringency of PCR (Lorenz, 2012). In general, the PCR product yield will increase with the addition of greater concentrations of Mg2+. However, increased concentrations of Mg 2+ will also decrease the specificity and fidelity of the DNA polymerase (Lorenz, 2012). Also, BSA (bovine serum albumin) and DMSO (dimethyl sulfoxide) acts as a PCR enhancer for high amplification of the PCR product. DMSO is thought to reduce secondary structure and is particularly useful for GC rich templates. A number of investigators have accordingly proposed that organic additives like DMSO enhance PCR by hydrogen bonding to the major and minor grooves of template DNA and destabilizing the double-helix (Varadaraj and Skinner, 1994; Cheng *et al.,* 1994; and references therein). DMSO at 2-10% may be necessary for amplification of some templates; however 10% DMSO can reduce Taq polymerase activity by up to 50% so it should not be used routinely. BSA has proven particularly useful when attempting to amplify ancient DNA or templates which contain PCR inhibitors such as melanin. BSA has also been reported to reduce the adhesion of the DNA polymerase to glass capillaries used in certain real-time PCR instruments. This is important for reducing the loss of reagents through adsorption to tube walls. BSA concentration of 0.01µg/µl to 0.1µg/ µl can be used. (Plante *et al.,* 2011)

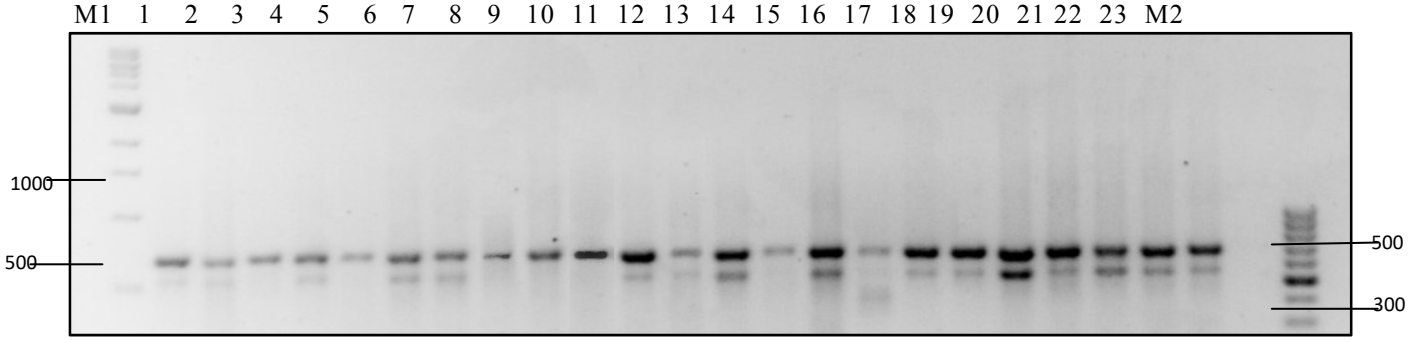

Figure 1b) M1 and M2 represents DNA ladder of 1KB and 100 bp respectively. PCR Amplification products were fractioned in 2% agarose gel. Lane 1-23 are the 23 coffee samples from Table 1.1. Amplified from genomic DNA using ITS region (ITS1-5.8s-ITS7) was amplified with primers ITSL (5'-TCGTAACAAGGTTTCCGTAGGTG-3'; Hsiao et al. 1994) and ITSR (5'-TATGCTTAAAYTCAGCGGG-3'; Hsiao et al. 1994).

Effect of leaf sample

The ITS result showed two amplified bands: one near 700 bp and other around 600 bp (fig-1b) .The full ITS region in fungi has an average length of 500 and 600 base pairs (bp) for ascomycetes and basidiomycetes, respectively, and an average length of 600 bp across all fungal lineages. (Porter and Golding, 2011).In addition, according to Zhang *et al.* (1997), the close associations between the plants and fungi are well known. According to some estimates referred by the same authors, about 80% of vascular plants host fungi, besides co-evolution between the plants and fungi has been suggested (Alexopoulos *et al.,* 1996). Many species called "endophytic" fungi live within plant tissues without causing apparent injury to the host plant, growing as symptomless parasitic fungi (Peixoto Neto *et al.,* 2004 ; Maheshwari, 2006). The primers ITSL and ITSR used in this study were designed specifically for plants but unsatisfactory results were seen (figure 1b) with non-specific amplification. The primers used in this study amplify entire ITS region (ITS1-5.8s-ITS7) it is most likely than it can amplify even fungal sequences if any fungal DNA is present by any means The universal primers for ITS region (White et al*.,* 1990) were mainly designed as from comparisons among sequences of fungi. Thus, their high specificity to these organisms is understandable, which may be even employed in clinical analysis for fungi detection (Ferrer et al., 2001). The non-specific bands observed in our result was assumed to be an amplified fungal sequence, as the untreated fungal leaf samples (Figure-2) was used for DNA isolation. Some ways to avoid fungal amplification could be the sterilization of the tissue before DNA extraction, as demonstrated by some studies (Zhang *et al.,* 1997; Guo et al., 2001). Cleaning the surface of the host tissue thoroughly (with ethanol, detergents and reducing agents), could be a simple but important manner to eliminate the phylloplane fungi and other organisms on the host surface (Guo *et al.,* 2001). With that in mind, treatment of the coffee plants with fungicide was done to remove potential fungal infection (Figure-2) and during the extraction process, surface cleaning was done by detergent Tween 20. With this implications, the result was improved with amplified bands at 700b.p which is beyond the fungal range (Porter and Golding, 2011) (Figure-1c).

Figure-2 : Comparison between fungicide treated coffee leaf and non-treated leaf respectively.

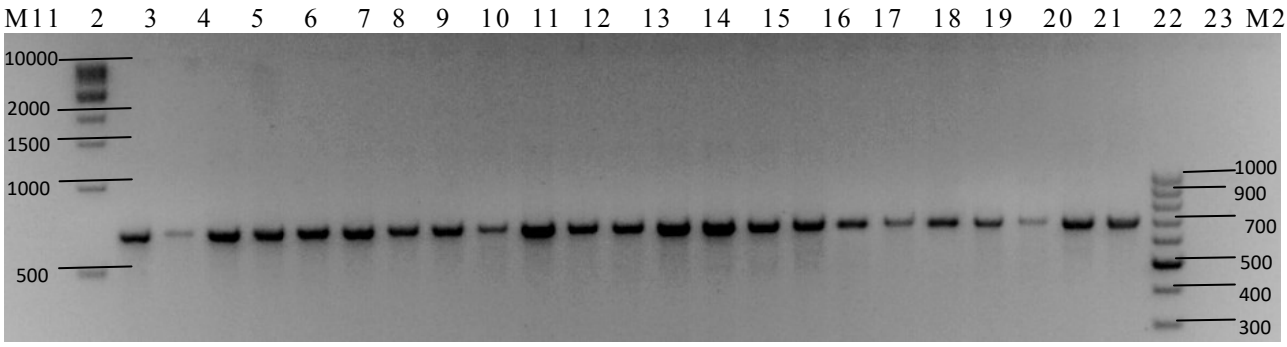

Figure 1c) M1 and M2 represents DNA ladder of 1KB and 100 bp respectively. PCR Amplification products were fractioned in 2% agarose gel. Lane 1-23 are the 23 coffee samples from Table 1.1. Amplified from genomic DNA using ITS region (ITS1-5.8s-ITS7) was amplified with primers ITSL (5'-TCGTAACAAGGTTTCCGTAGGTG-3'; Hsiao et al. 1994) and ITSR (5'-TATGCTTAAAYTCAGCGGG-3'; Hsiao et al. 1994).

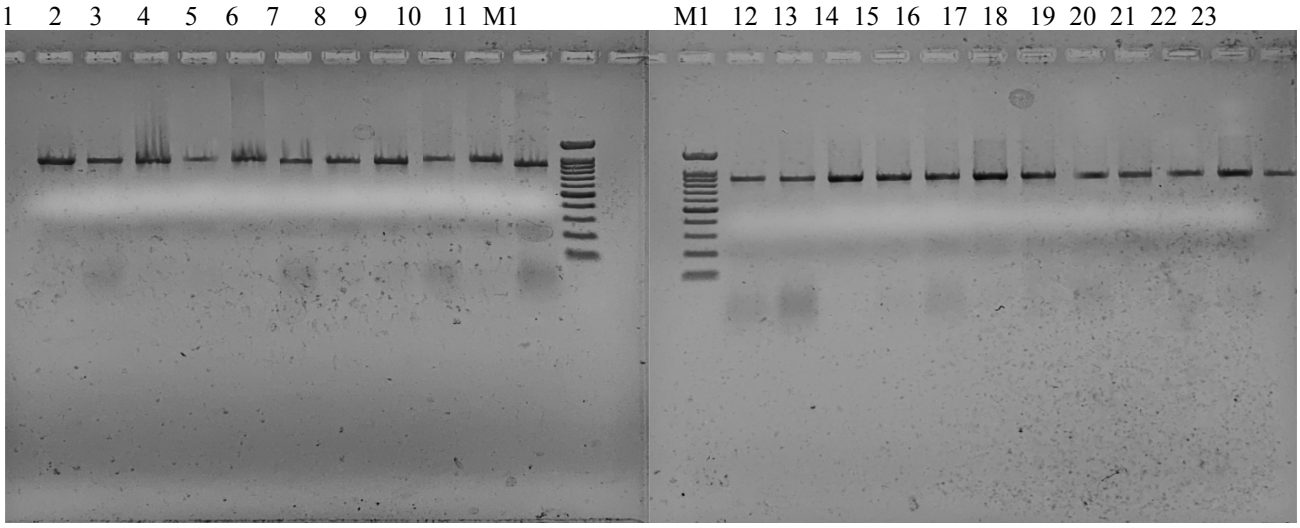

Figure 1d) PCR Amplification products were fractioned in 2% agarose gel. Lane 1-23 are the 23 coffee samples from Table 1.1. The matK region was amplified with primers *3F_Kim f* (5' CGTACAGTACTTTTGTGTTTACGAG 3') and 1R_Kim r (5'-ACCCAGTCCATCTGGAAATCTTGGTTC 3'; CBOL- PWG 2009)

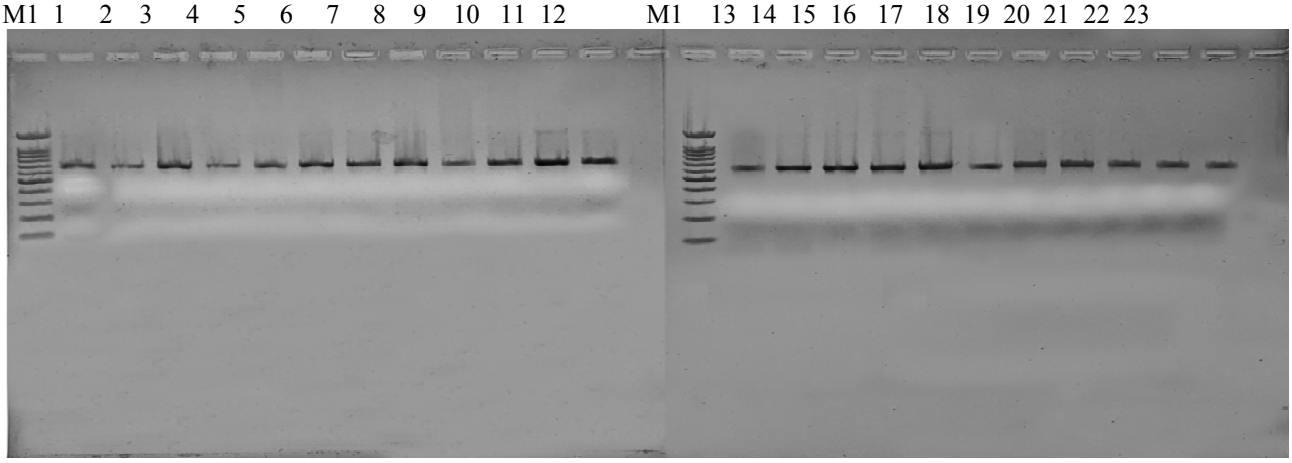

Fig 1e) PCR Amplification products were fractioned in 2% agarose gel. Lane 1-23 are the 23 coffee samples from Table 1.1. Oligonucleotide primer from rbcl region (Macrogen .Inc. , South Korea) were used for optimization of rbcl reactions. RbclF- 5'-ATGTCACCACAAACAGAGACTAAAGC-3'; RbclR- 5'-GAAACGGTCTCTCCAACGCAT-3'; CBOL- PWG 2009).

With improvised extraction protocol focused on Fungal contamination removal from leaf surface optimization was done for ITS-PCR reaction by varying different parameters like: template DNA, primer concentration, dNTPs, Magnesium Chloride, T*aq* Polymerase, temperature and time intervals during denaturation,

annealing and extension were optimized for high amplification and reproducible data. The optimized conditions for ITSprotocol are given in Table 1.3with band fragment size of~ 700 b.p (Figure-1 c). Higher or lower concentration of tested constituents beyond the optimized concentration resulting into lack of reproducibility. The modified DNA extraction protocol and the optimization of PCR conditions help in generating quality and reproducible data for further analysis. With less extraction process and reagents used in the modified protocol can be considered as fast and inexpensive giving good quality DNA.

With the optimization of the ITS region, the parameter Optimization of Matk and rbcl gene was donewith respect to ITS protocol. Good amplifications of the bands were seen for MatK-Rbcl PCRat 950 b.p and 680 b.p respectively(fig-1d and 1e). The range for Matk-Rbcl amplification were 900-1500 b.p and 650-700 b.p respectively, so the amplification were at desired range.

In conclusion, reliable DNA isolation protocol from coffee leaves and PCR optimization for 3 barcoding genes protocol can be scaled up or down depending on the samples and requirements for particular analysis. In consideration to the DNA isolation and optimization of the PCR, can be further used for Barcoding and other downstreaming applications.

Conflict of Interest

The authors declare that there are no conflict of interest regarding the publication of this paper.

Acknowledgement

The authors thankfully acknowledge National Biotechnology ResearchCentre, NARC for funding the research. They are also grateful to research intern Mr. Bishwas Ghimire, technical assistant Ms. Rima Ghimire and helper Ms. Durga Sapkota from National Biotechnology Unit for their cordial help and support during this work.

We are also grateful to the researchers Mrs. Asmita Shrestha, Ms. Ashmita Mainali, Ms. Sadikshya Rijal, Mr. Sudip Silwal, Mr. Raj Kumar Shrestha and Mr. Dipak Pandey from Shubham Biotech Nepal Pvt. Ltd. for their guidance and support during the research work.

References

Abu-Romman, S. (2011) Comparison of methods for isolating high quality DNA from sage *(Salvia)*

Albumin: The Natural Choice for Diagnostic Tests. https://albumedix.com/media/news-events/scientific-news/albumin-the-natural-choice-for-diagnostic-tests/

Aljanabi, S. M., Forget, A., Dooknn (1999) An improved and rapid protocol for the isolation of polysaccharide and polyphenol-free sugarcane DNA. *Plant Mol. BioI. Rep.* 17, 1-8.

Anthony, F., M. C. Combes, C. Astorga, B. Bertrand, G. Graziosi, and P. Lashermes. 2002. "The Origin of Cultivated *Coffea Arabica* L. Varieties Revealed by AFLP and SSR Markers." *Theoretical and Applied Genetics* 104 (5): 894–900. doi:10.1007/s00122-001-0798-8.

Berthaud J (1986) Les ressources génétiques pour l'amélioration des caféiers africains diploides. Collection "Travaux et Documents" (I 881, ORSTOM (ed), Paris.

Berthaud J. Charrier A (1988) Genetics resources of *Coffea.* In: Clarke FLl. Macrae R (eds) Coffee. vol 4: agronomy. Elsevier Applied Science. London, pp 1-42.

Berthomieu P and Meyer C. 1991. Direct amplification of plant

Berthomieu P and Meyer C. 1991. Direct amplification of plant

Berthomieu P and Meyer C. 1991. Direct amplification of plant

Berthomieu P and Meyer C. 1991. Direct amplification of plant genomic DNA from leaf and root pieces using PCR. Plant Molecular Biology 17: 555–57.

Bridson DM. I'erdcourt B (1988) Flora of tropical East Africa - 42:453-460 *Rubiaceae* (part 21. Balkema. Brookfield, Rotterdam.

Bridson DM. I'erdcourt B (1988) Flora of tropical East Africa Rubiaceae (part 21. Balkema. Brookfield, Rotterdam

CBOL Plant Working Group (2009). A DNA barcode for land plants. *Proc Natl Acad Sci USA* 6:12974–12797

Charrier A. Berthaud J (1985) Botanical classification of coffèe. In Clifford Miri, Wilson KC (eds), Coffee: botany, biochemistry and production **of** beans and beverage. Croom Helm. London, pp 13-47.

Charrier, A. & J. Berthaud, 1985. Botanical classification of coffee. In: M.N. Clifford & K.C. Willson (Eds.), Coffee Botany, Biochemistry and Production of Beans and Beverage, pp. 13–47. Croom Helm, London & Sydney .

Chevalier, A. & M. Dagron, 1928. Recherches historiques sur les débuts de la culture du caféier en Amérique. Communications et Actes de l'Académie des Sciences Coloniales, Paris.

Clarindo, W. R., and C. R. Carvalho. 2008. "First *Coffea Arabica* Karyogram Showing that This Species Is a True Allotetraploid." *Plant Systematics and Evolution* 274: 237–241. doi:10.1007/s00606-008-0050-y.

Demeke, T., Jenkins, G. R. (20 I 0) Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. *Anal. Bioanal. Chem. 396,* 1977-1990.

Dhakal, B.R. 2005. Coffee Kheti, Coffee Manual. National Tea and Coffee Development Board, Kathmandu.

Doyle, J. J. and Doyle, J. L. (1987), A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull*, **19,** 11-15. Doyle, J. J. and Doyle, J. L. (1990), Isolation of plant DNA from fresh tissue. *Focus*, **12**, 13-15.

Edwards KJ. 1998. Randomly Amplified polymorphic DNAs (RAPDs). In Molecular Tools for Screening Biodiversity – Plants and Animals, A Karp, PG Isaac and DS Ingram (eds.). Chapman and Hall, London, Weinheim, New York, Tokyo, Melbourne, Madras, p 171–179.

genomic DNA from leaf and root pieces using PCR. Plant

genomic DNA from leaf and root pieces using PCR. Plant

genomic DNA from leaf and root pieces using PCR. Plant

Hsiao C, Chatterton NJ, Asay KH, Jensen KB (1994) Phylogenetic relationships of 10 grass species: an assessment of phylogenetic utility of the internal transcribed spacer region in nuclear ribosomal DNA in monocots. Genome 37: 112-120.

J. M. Waller,M. Bigger, and R. A. Hillocks, "Coffee as a crop and commodity," in *Coffee Pests, Diseases and Their Management*, pp. 1–17, CABI, Nosworthy Way Oxford shire , UK, 2007.

Katterman FRH, VI Shattuck VI (1983). An effective method of DNA isolation from the mature leaves of *Gossypium* species that contain large amounts of phenolic terpenoids and tannins. Prep. Biochem. 13: 347-359.

Khanal, D. 2003. Coffee production technology. Bagwani Wadi. Horticulture Center, Kirtipur, Nepal. Year 3. Issue 8.

Lebrun J (1941) Recherches morphologiques et systématiques sur les caféiers du Congo. Mémoires TX1 (3), Institute Royal Colonial Belge, Bruxelles.

Lorenz, T. (2012). Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies. *Journal Of Visualized Experiments*, (63). doi: 10.3791/3998.

Matthes MC, A Daly and KJ Edwards. 1998. Amplified Fragment Length Polymorphism (AFLP). In Molecular Tools for Screening Biodiversity- Plants and Animals, A Karp, PG Isaac and DS Ingram (eds.). Chapman and Hall, London, Weinheim, new York, Tokyo, Melbourne, Madras, p 183–192.

Molecular Biology 17: 555–57

Molecular Biology 17: 555–57

Molecular Biology*oiJicinalis).* 1. *Med. Plants Res.* 5, 938-941.

P. Lashermes,M. C. Combes, J. Robert et al., "Molecular characterization and origin of the *Coffea arabica* L. Genome," *Molecular and General Genetics*, vol. 261, no. 2, pp. 259–266, 1999.

Padmalatha K. and M.N.V. Prasad. 2006. Optimization of DNA isolation and PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from Peninsular India, *African Journal of Biotechnology* **5** (3) : 230-234.

Padmalatha K. and M.N.V. Prasad. 2006. Optimization of DNA isolation and PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from Peninsular India, *African Journal of Biotechnology* **5** (3) : 230-234.

Pasakinskiene, I.; Pasakinskiene, V. Floral meristems as a source of enhanced yield and quality of DNA in grasses. Plant Cell Rep., 1999, 18, 490-492.

Peterson DG, Boehm KS, Stack SM (1997). Isolation of milligram quantities of nuclear DNA from tomato (*Lycopersicon esculentum*), a plant containing high

levels of polyphenolic compounds. Plant Mol. Biol. Rep.15: 148-153.

Plante D, Bélanger G, Leblanc D, Ward P, Houde A, Trottier YL. The use of bovine serum albumin to improve the RT-qPCR detection of foodborne viruses rinsed from vegetable surfaces. Lett Appl Microbiol. 2011 Mar; 52(3) : 239-44. doi: 10.1111/j.1472-765X. 2010.02989.x. Epub 2011 Jan 11. PMID: 21219370.

Porebski S, Baily LG, Baum BR (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol Biol Rep. 15: 8-15.

Porter TM, Golding GB (2011) Are similarity- or phylogeny-based methods more appropriate for classifying internal transcribed spacer (ITS) metagenomic amplicons? New Phytol 192: 775–782.

Varadaraj, K., Skinner, D.M., 1994. Denaturants or cosolvents improve the specificity of PCR amplification of a G 1 C rich DNA using genetically engineered DNA polymerases. Gene 140, 1–5.

Varma, A., Padh, H., Shrivastava, N. (2007) Plant genomic DNA isolation: an art or a science. *Biotechnol. J* 2, 386-392.

Weeden NF. 1992. Inheritance and reliability of RAPD markers. In'Application of RAPD technology to Plant Breeding'. Joint Plant Breeding Symposia Series. Crop Science Society of America, American Society for Horticulture Science, American Genetic Association.

Weising, K.; Nybom, H.; Wolff, K.; Kahl, G. DNA Fingerprinting in Plants: Principles, Methods and Applications, 2nd ed.; CRC Press: Boca Raton, 2005.

Wetzel DK, MJ Horak and DZ Skinner. 1999. Use of PCR-based molecular markers to identify weedy Amaranthus species. Weed Science 47(5): 518–523.

Yasuda K, A Yano, Y Nakayama and H Yamaguchi. 2002. Molecular identification of *Echinochloa oryzicola* Vasing. and E. crus-galli (L.) Beauty. using a polymerase chain reaction-restriction fragment length polymorphism technique. Weed Biology and Management 2: 11–17

♦♦