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DNA Based Identification of Species of *Piperaceae* Family Plants Using Bar-HRM Analysis

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Abstract:- To evaluate the standardized short regions of the genome of plant or animal species DNA barcoding technique was developed. This method is very useful to identify novel genes of known species. In online numerous sequence, databases are stored world-wide. To identify the species this is one of the methods used and it saves cost and time by omitting the sequencing step. The use of available barcode data to design optimized primers for further analysis, such as high-resolution melting analysis (HRM).This study identified the species that share similar external morphological features, using hybrid method Bar-HRM rather than conduct traditional taxonomic identification that requires major parts leaf, flower, the fruit of the specimens. Primer pairs derived from chloroplast regions *(ITS1, ITS2,* and *rbcl)* were used in the Bar-HRM. The method developed here was proven to be effective in distinguishing between the identification of different species in *Piperaceae* family plants. The extracted DNA from tested samples yielded a specific amplification product with the *ITS1, ITS2* and *rbcl* primer. *ITS1* region is proved to be the most promising universal DNA barcode for the plant family of *Piperaceae*.

Keywords:- *Piperaceae; Bar-HRM; rbcL; ITS2; ITS1; DNA Barcoding*

Abbreviations:- *HRM- High Resolution Melting Curve; DNA- Deoxyribonucleic Acid ;CTAB- Cetyltrimethylammonium Bromide; PCR- Polymerase Chain Reaction:*

I. INTRODUCTION

Pepper plants belong to the family Piperaceae and order Piperales, pepper plants are commercially important because of its the source of black and white pepper[1]. Piperaceae comprises about 5 genera, of which the most important genera are piper which consisting of 2000 species and peperomia consists of 1600 species. The plants grow as herbs, vines, shrubs, and trees and are widely distributed throughout the tropics and subtropics[2].

The plants have many medicinal properties, fruits and roots are used to treat many respiratory tract diseases such as cough, bronchitis, asthma, etc. These plants also used as a counter-irritant and analgesic when applied to muscular pains and inflammation. Internally it is used as carminative as a sedative in insomnia and epilepsy as a general tonic and haematinic [3].

One of the Ayurvedic drug `Trikatu' is prepared using Indian long pepper and whose constituents and piperin are reported to possess bioavailability enhancing activity which increases the efficacy of the co-administered Ayurvedic formulations or medicaments [4]. An Ayurvedic drugs `Mrtyunjayarasavati' used for chronic sinusitis and `AnandBhairavaRas', used for the treatment of amoebiasis[5].

The thicker parts of the stem are cut and dried and used as an important drug (Piplamul) in the Ayurvedic and Unani systems. Similar crude drugs (1) P.peepuloides, (2) P. retrofractum[6,7].

Many techniques are evolved to identify the species of the Piperaceae family in that DNA barcoding is one of the most acceptable methods.

A new technique developed to identify species is DNA barcoding which uses universal primers to amplify small, conservative ends of DNA fragments[8]. Compare to other traditional methods it provides more accurate results[8]. Chloroplast sequences have been used for the identification of species, the sequence used is usually short in length and is known as DNA Barcodes [9]. DNA-barcoding technology has been increasingly used in the food and forensic fields for species identification [10].

An automated analytical molecular technique High resolution melting analysis (HRM) which is used to measure the rate of double standard DNA dissociation to single standard DNA with increasing the temperature[11,12]. Increased precision and resolution of instrument and development of saturated dyes helps the use of HRM for genotyping the SNP, SSR markers for methylation analysis, as an alternative to gel electrophoresis and for quantitative detection of adulterants [13]. In this present study, we are going to identify the Bar-HRM curve in the Piperaceae family.

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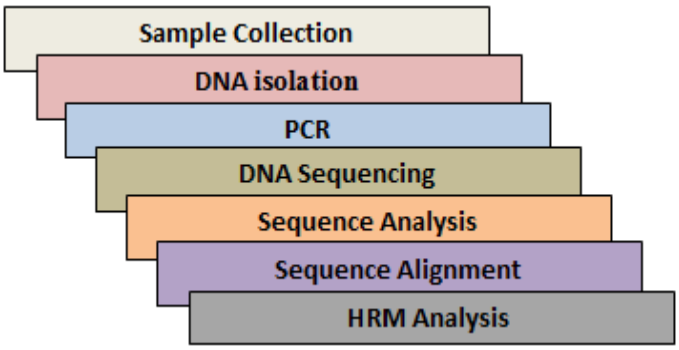
II. MATERIALS AND METHODS

A. Materials

Piperaceae family samples are collected from different geographical regions was through prior permission from the respected authority. Genomic DNA was isolated from 40 to 50 mg dried material using chemicals such as 3% C-TAB extraction buffer contains 3 % CTAB, 1.4 M NaCl, 20 mM EDTA,100 mMTris pH 8, 1-2 % PVP polyvinylpyrrolidone 40, 0.2 % Beta-mercaptoethanol.

In the present study, we analyzed the sequences of ***Piperaceae*** family plants through PCR and sequencing by barcode regions for the HRM component.

B. Methodology



C. DNA Isolation Protocol:

Piperaceae family plant material of 40-50 mg is homogenized by using 500 µl of CTAB DNA Extraction Buffer. Vortex and mix the homogenate thoroughly.incubate the homogenate to a 60°C water bath for 30 minutes. centrifuge the homogenate for 5 minutes at 14,000g Transfer supernatant to a new tube. Add 5 µl of RNase solution A and incubate at 32°C for 20 minutes. Add an equal volume of chloroform/isopropyl alcohol (24:1). Vortex for 5 seconds then centrifuges the sample for 1 min. at 14,000 x g to separate the phases. Transfer the upper aqueous phase to a new tube. Precipitate the DNA by adding 0.7 volume of cold isopropanol and incubate at -20°C for 15 minutes. Centrifuge the sample at 14,000 x g for 10 minutes. Decant the supernatant without disturbing the pellet and subsequently wash with 500 µl ice-cold 70% ethanol. Decant the ethanol. Remove residual ethanol by drying in a Speed Vac. Dry the pellet long enough to remove alcohol, but without completely drying the DNA. Dissolve DNA in 20 µl TE buffer (10 mMTris, pH 8, 1 mM EDTA). The pellet may need warming in order to dissolve.

D. Polymerase Chain Reaction:

To detect and differentiate ***Piperaceae*** family plants, To amplify a conserved region of the DNA polymerase PCR assay was performed.15 µl reaction mixtures included 1X assay buffer, 50ng genomic DNA, dNTP 0.2 mM, each primer 1 Pico mole, $MgCl_2$ 1.5 mM and Taq DNA polymerase 1 unit. The PCR program was as follows: initial denaturation 95℃ - 10mins, cycle denaturation, 95℃ - 1 min, annealing 60℃ – 45° C with 1 min (touch down), extension 72℃ - 12min; final extension 72℃ - 10 min; no of cycles 35. The final PCR product from the assay is visualized using Agarose gel electrophoresis.

E. Sanger Sequencing:

➢ *Standard Sequencing Protocol:*

The sequencing reaction was performed in a 10 µl scale using the Big-Dye Terminator,10 pmol of various primers, and 50–1500 ng of template DNA one experimental run. The thermal cycle was used for the amplification: 96 °C for 1 min, followed by 40–50 cycles of 96 °C for 10 s, 50–58 °C for 5 s and 60 °C for 150 s.

We outsourced samples for the sequencing and obtained results are analyzed using the NCBI Blast tool for the identification of ***Piperaceae*** plant species.

F. HRM-PCR Amplification And Data Analysis

The HRM-PCR reaction mixture (13 µL) contained 50 ng of genomic DNA, HRM- master mix (7uL), Forward primer(1uL), Reverse primer (1uL), Nuclease-free Water(3uL).

➢ *HRM Conditions*

Pre-denaturation at 95^0 C for 10min, Amplification at 95^0 C for 1min, 60^0-45^0 C for 10sec, 72^0 C for 20 sec, HRM melt curve at 95^0 C for 1 minutes, 40^0 C for 1 minutes, 60^0 C for 1 sec, 95^0 C for Continuous, cooling 40^0 C 10 seconds.

III. RESULTS

PCR amplification of all collected samples showed good results with the ***ITS1*** **and** ***rbcl*** primers (primers used ***ITS1, ITS2,*** **and** ***rbcl***)

Sanger sequencing is used to identify species level of all the collected samples and analyzed sequence results showed in table 1

HRM analysis of each species showed the different melting temperature shown in table 1

Image 1, 2 and 3 show the Bar-HRM curves of Normalization, Temperature shift and differential plots of both ***ITS1, ITS2,*** **and** ***rbcl***

From the above analysis, the ***ITS1*** region is the most promising universal DNA barcode for the identification of plant family ***Piperaceae***.

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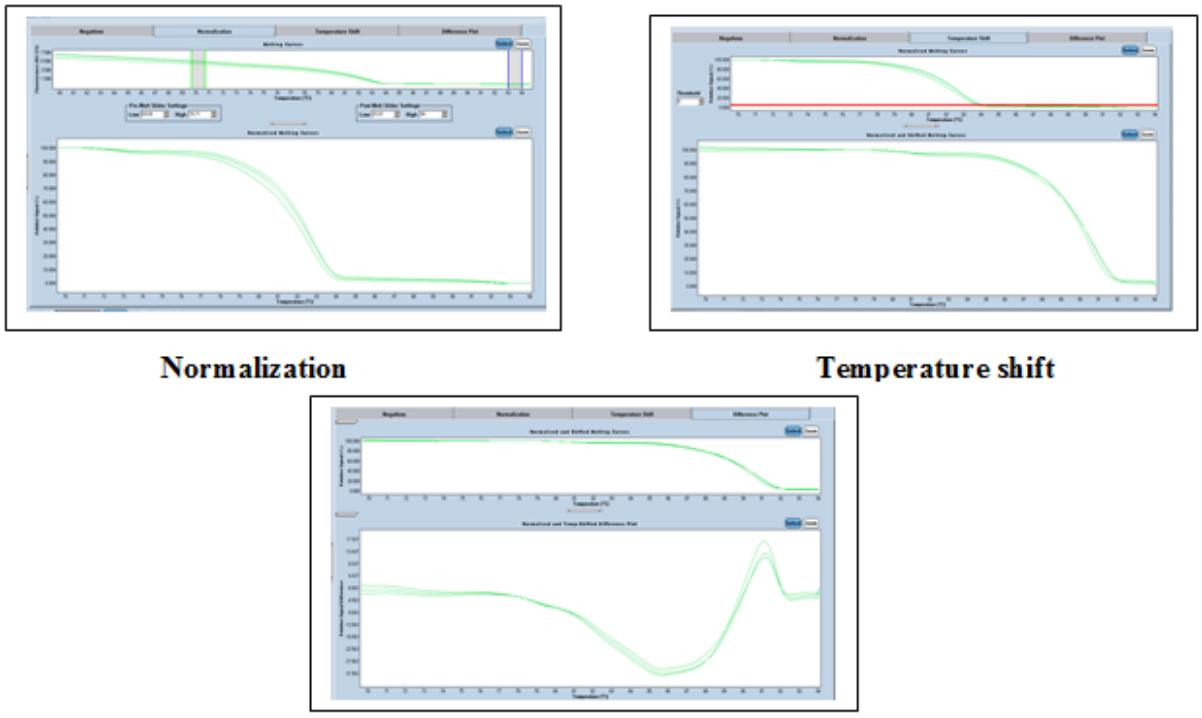

Fig 1:- Shows the Bar-HRM Results with DNA extracted from *Piper nigrum* collected from Kerala and amplified by *ITS1*

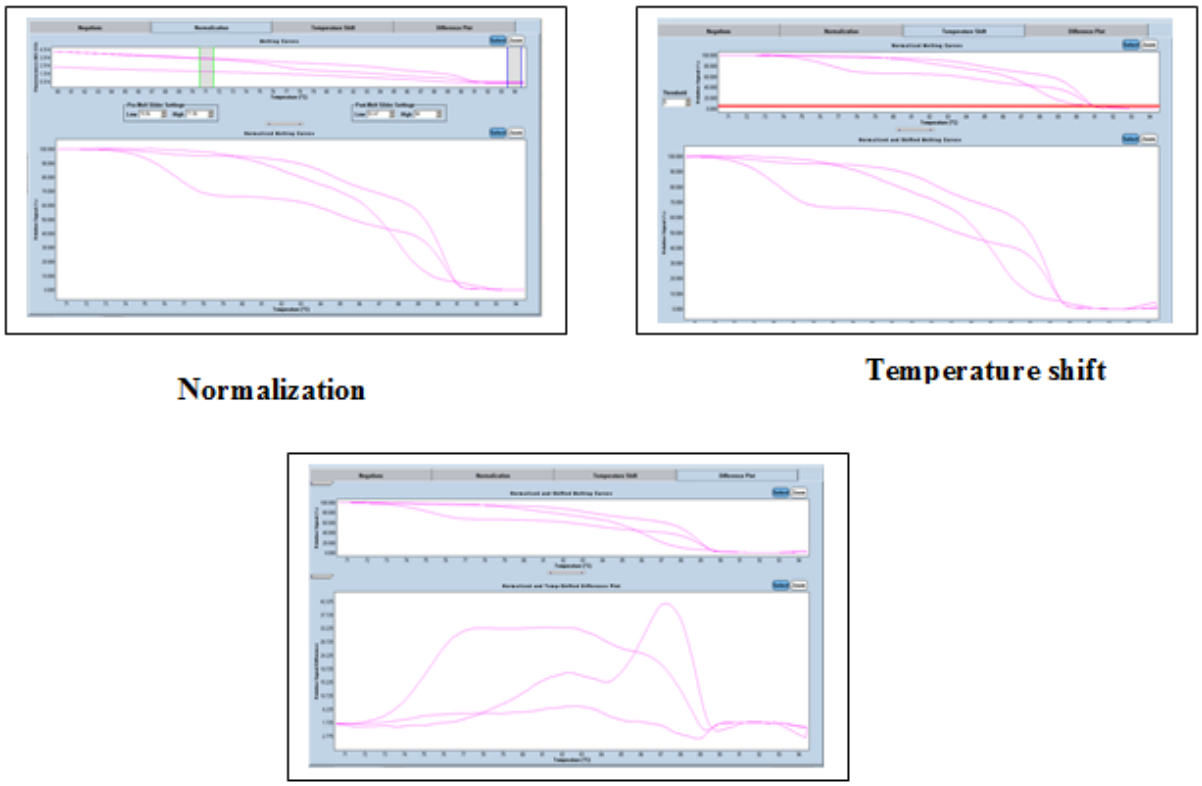

Fig 2:- Shows the Bar-HRM Results with DNA extracted from *Piper nigrum* collected from Kerala and amplified by *ITS2*

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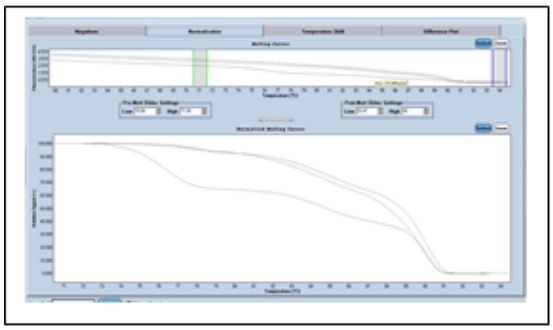
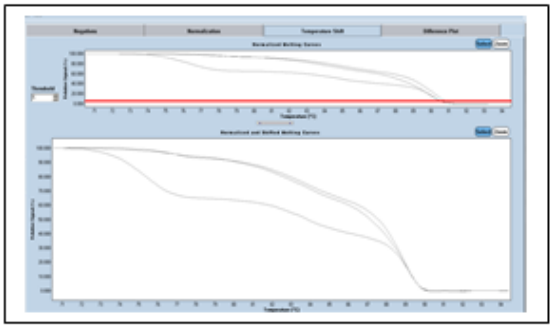
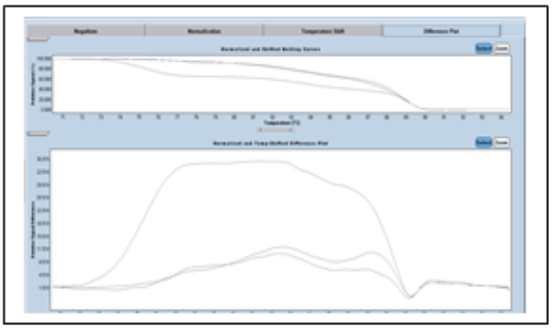


Normalization



Temperature shift



Differential plot

Fig 3:- Shows the Bar-HRM Results with DNA extracted from *Piper nigrum* collected from Kerala and amplified by *RBCL*

Sl. No.	Sample	Place of collection	Primers	Gene	Blast Hit	HRM	Avg Tm
1.	White pepper	Puttur	*ITS1*	*ITS1*	***Piper nigrum***	✓	93.47±0.5
2.	Black pepper	Puttur	*ITS1*	*ITS1*	***Piper nigrum***	✓	93.01±0.5
3.	Long pepper	SDUMC-Kolar	*ITS1*	*ITS1*	***Piper longum***	✓	93.41±0.5
4.	Black pepper	Kerala	*ITS1*	*ITS1*	***Piper nigrum***	✓	93.01±0.5
5.	Black pepper	Hbhalli	*ITS1*	*ITS1*	***Piper nigrum***	✓	93.01±0.5
6.	Black pepper	Coorg	*ITS1*	*ITS1*	***Piper nigrum***	✓	93.01±0.5
7.	White pepper	Puttur	*ITS2*	*ITS2*	***Piper nigrum***	×	….
8.	Black pepper	Puttur	*ITS2*	*ITS2*	***Piper nigrum***	×	….
9.	Long pepper	SDUMC-Kolar	*ITS2*	*ITS2*	***Piper longum***	×	….
10.	Black pepper	Kerala	*ITS2*	*ITS2*	***Piper nigrum***	×	….
11.	Black pepper	Hbhalli	*ITS2*	*ITS2*	***Piper nigrum***	×	….
12.	Black pepper	Coorg	*ITS2*	*ITS2*	***Piper nigrum***	×	….
13.	White pepper	Puttur	*RBCL*	*RBCL*	***Piper nigrum***	×	….
14.	Black pepper	Puttur	*RBCL*	*RBCL*	***Piper nigrum***	×	….
15.	Long pepper	SDUMC-Kolar	*RBCL*	*RBCL*	***Piper longum***	×	….
16.	Black pepper	Kerala	*RBCL*	*RBCL*	***Piper nigrum***	×	….
17.	Black pepper	Hbhalli	*RBCL*	*RBCL*	***Piper nigrum***	×	….
18.	Black pepper	Coorg	*RBCL*	*RBCL*	***Piper nigrum***	×	….

Table 1:- Shows the blast results of Sanger sequencing and HRM analysis of different melting temperatures.

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IV. DISCUSSION

In the present study, we have developed the most accurate method for the identification of plant species of ***Piperaceae*** family plants which are commonly available and closely related and share common morphological features using Bar-HRM analysis. For the identification of different species in the piperace family the developed method was very effective. The DNA extracted from tested samples yielded a specific amplification product with the ***ITS1, ITS2*** **and** ***rbcl*** primers. , out of these primers, the ***ITS1*** region is proved to be the most promising universal DNA barcodes for the plant family ***Piperaceae.***

Hebert et al. in 2003 studied 36 piper species using three different DNA barcoding marker regions to support a previous hypothesis of genetic distance values showing a significant variance in sequences between species and a comparatively small variance within species. Note that the economic and planted species, ***Piper beetle*** had the highest intraspecific genetic distance values of 0.386 for the ***matK*** region, which may have been due to the presence of human growth factors [14].

Hao et al. in 2013 studied 27 pairs of species of piper plants and reported that The interspecific genetic distances for the ***matK*** region were effective for the identification of different species. they proved that ***matK*** had high species identification reliability and suggested that this region should be used for the identification of ***Piper*** species along with the ***ITS*** region[15].

To the group, the species into their taxonomic positions ***ITS2*** is also used and the practical approach of DNA barcoding is to identify plant species by nontaxonomists and also it is useful for evolutionary studies [16,17]. The ***ITS2*** has also been proved to differentiate different species levels in ***fabaceae***[18]. ***ITS2*** showed the success rate of discriminating 78 % and 100 % at the species and genus levels respectively in the Rosaceae family [18]. ***ITS2*** region was routinely used for authenticating Chinese herbal medicine [16,19].

V. CONCLUSION

The feasibility of DNA barcoding was studied by evaluating the different potential barcode candidates across the medicinally important family ***Piperaceae.*** To discriminate closely related species and also for the phylogenetic analysis in ***Piperaceae ITS1*** is an important region to be analyzed Out of ***rbcl*** **and** ***ITS2. ITS1*** was found excellent barcode gene. This developed barcoding technology can also be applied to solve taxonomic confusions in the ***Piperaceae*** family at the generic and species level. To identify and authenticate the herbal species from their substitute.

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