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DNA Barcoding and Phylogenetic Analysis of Indian Medicinal Plant *Piper longum*

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Abstract

Piper longum is an important medicinal plant, widely used in treating diseases in the Ayurvedic system. *Piper longum* is dioecious with male and female plants. Female *Piper longum* is more widely used than the male owing to its metabolites, making it economically valuable. This study aimed to amplify the DNA barcodes for both male and female plants using rbcL, ITS2, and psbA-trnH followed by Sanger sequencing. The phylogenetic trees were constructed using the UPGMA method from the sequence data obtained. Based on the sequence alignment, rbcL and psbA-trnH were found to be effective in distinguishing *Piper longum* between or within the species when compared to ITS2. While complete rbcL and psbA-trnH regions were amplified, ITS2 showed incomplete amplification for *Piper longum*. Hence, rbcL and psbA-trnH can be used for the successful authentication of *Piper longum.*

Keywords: *Piper longum*, rbcL, ITS2, psbA-trnH, Sanger sequencing, Phylogenetic trees

Introduction

Piper longum (Piperaceae family) commonly called “long pepper” or “Pippali” is a dioecious medicinal plant species that is widely distributed in tropical and subtropical regions of most Asian countries like India, Nepal, Sri Lanka, and Indonesia. It is very difficult to determine the sex of the plant at the earlier stage since the male and female *Piper longum* plants are morphologically very similar until the formation of spikes. Male spikes are long greenish-yellow coloured, fleshy, cylindrical, with minute flowers, and female spikes are short yellow-coloured, erect, thick, and shorter than male spikes (1). Studies have proved that the female spikes of *Piper longum* contain more valuable compounds than male spikes (2), hence female plants are preferred over male plants. Thus, the elimination of unwanted plants is necessary (3). Piperine is an important alkaloid present in the fruits of *Piper longum* which contains high medicinal properties. It also contains piperidine, piperlongumine, lignans, volatile oils, esters, organic acids, and minerals such as calcium, phosphorus, and iron (4).

The parts of the *Piper longum* plant such as spikes, roots, and stems are commonly used for pharmacological purposes. The metabolites present in the roots, spikes, and leaves of the *Piper longum* plant are used for several diseases, especially for respiratory-related problems and also for worms, dyspepsia, and amoebiasis (5).

Nowadays, traditional crops are replaced by medicinal and aromatic plants. India plays a vital role in manufacturing and exporting herbal product-based medicines globally. Mainly, the fruits and roots of *P. longum* are exported in fresh, dried, or powdered form which is much more expensive to many countries such as the USA, Canada, Philippines, Europe, Australia, and Southeast Asian countries like Singapore, Japan, Pakistan, etc. Hence the authenticity of these medicinal plants is a matter of importance (6).

Many techniques have been evolved to identify the diversity in dioecious medicinal plant species. Molecular markers such as Random Amplified Polymorphic DNA (RAPD) (7), Inter Simple Sequence Repeats (ISSR) (8), and DNA barcoding are widely used. Among them, DNA barcoding (9) is one of the most reliable methods for the identification of different types of Piper species. It uses universal primers (DNA barcodes) to amplify highly conserved regions of DNA fragments of either nuclear (ITS) or organellar (chloroplast genes such as rbcL and psbA-trnH) genomes. Compared to other traditional methods it provides more accurate results (10).

Materials and Methods

DNA isolation and quantification

The male and female *Piper longum* (long pepper) plants were collected from Aromatic and Medicinal Plants Research, Station Odakkali, Ernakulam district, Kerala. The young leaves of male and female *Piper longum* plants were collected and washed properly under running tap water and air-dried. The male and female plant leaves were weighed and ground using liquid nitrogen to a powder. To this, CTAB extraction buffer and β-mercaptoethanol were added and incubated at 60ºC for 30 minutes. This was then centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred into new centrifuge tubes and treated with RNase A and incubated at 32ºC for 20 minutes. To this, 2/3 volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 10 minutes. The above step was repeated twice. The aqueous phase was transferred to new tubes and DNA was precipitated using isopropanol. The precipitated DNA was washed with 70% ice-cold ethanol by centrifuge at 10,000 rpm for another 5 minutes. The DNA pellet was then air-dried and resuspended in TE buffer and stored in a deep freezer at -4ºC (11). The concentration and purity of the DNA samples were determined in NanoDrop.

DNA barcoding

DNA amplification was carried out using three different primers rbcL, ITS2, and psbA-trnH. The reaction mixture for PCR amplification included genomic DNA (50 ng), forward and reverse primers each (10 pmol), master mix, and nuclease-free water. The conditions for rbcL amplification are as follows; initial denaturation at 94ºC for 4 minutes followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing temperature at 55ºC for 30 seconds, extension at 72ºC for a minute and final extension 72ºC for 10 minutes. For ITS2, the amplification was set as initial denaturation at 95ºC for 3 minutes followed by 35 cycles of denaturation at 92ºC for a minute, annealing temperature at 50ºC for a minute, extension at 72ºC for 2 minutes, and a final extension at 72ºC for 10 minutes. Amplification of psbA-trnH was performed under the following conditions; initial denaturation at 95ºC for 3 minutes followed by 35 cycles of denaturation at 95ºC for 30 seconds annealing temperature at 55ºC for a minute, extension at 72ºC for 1.5 minutes and a final extension at 72ºC for 4 minutes. The amplified products were visualized by 1.2% Agarose gel electrophoresis in 1x Tris–EDTA buffer and documented using a gel documentation system (Alpha Innotech, USA). The PCR amplified products were purified and sequenced using Sanger sequencing (Agrigenome labs, Kochi, Kerala).

Sequence analysis

The amplified sequences were analyzed using the NCBI- BLAST. The phylogenetic trees

were constructed using the UPGMA method and the Maximum Composite Likelihood model for 1000 bootstraps using MEGA-X software with the most similar *Piper species* in BLAST.

Results and Discussion

Quantification of DNA

The DNA concentration and its purity are important in molecular studies for obtaining accurate results. The concentration of DNA was found to be between 500.1 ng/ µL and 144.1ng/ µL. The purity (260/280 nm) was found to be in the range of 1.89 and 1.86. The DNA samples of good quality which ranged between 1.8-1.9 were considered for PCR amplification.

DNA barcoding

The size of the amplified product for rbcL was ~600 bps for both male and female plants (Figure 1).

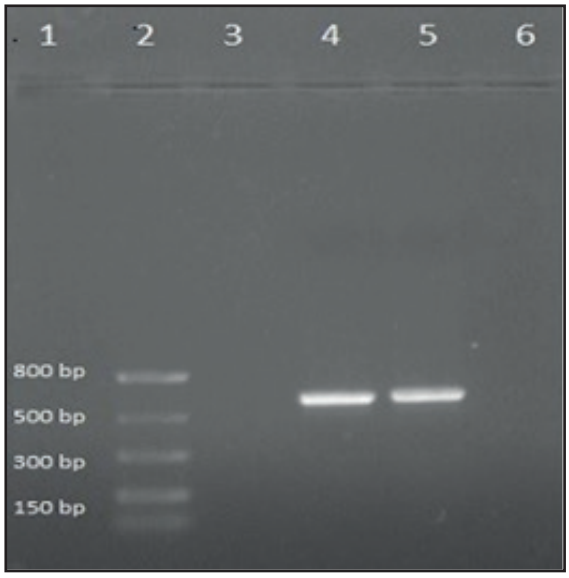

Figure 1: rbcL region amplification of Piper longum; Lane 2 is the DNA ladder, Lane 4 is the male plant, and Lane 5 is the female plant.

For ITS2, a clear band was seen in the case of the male sample of about 300-400 bps. ITS2 amplification in the female sample was unsuccessful under the given conditions (Figure 2).

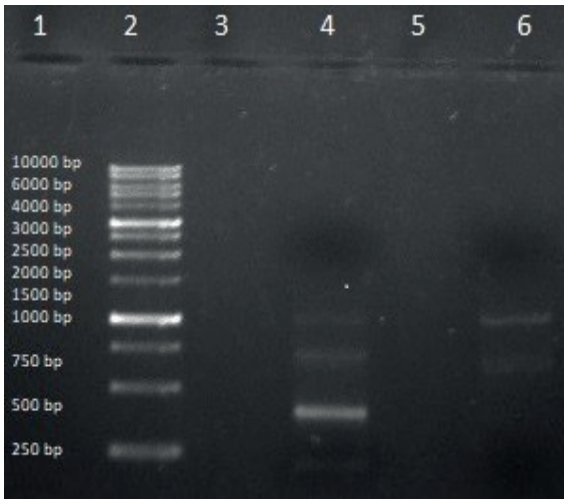

Figure 2: ITS2 region amplification of *Piper longum*; Lane 2 is the DNA ladder, Lane 4 is the male plant, and Lane 6 is the female plant.

For psbA-trnH, a clear intact band in both male and female samples showed the presence of DNA which was of band size ~500 bps (Figure 3).

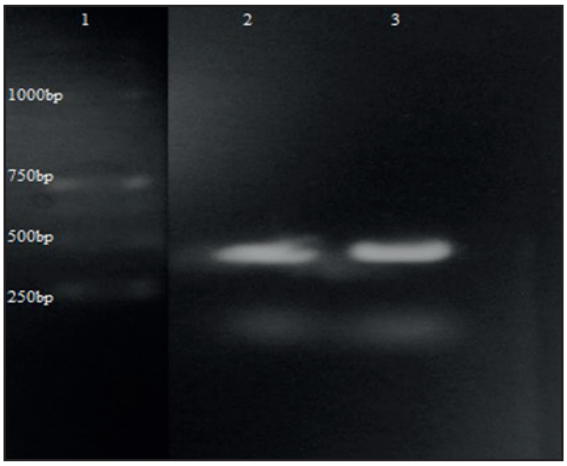

Figure 3: psbA-trnH region amplification of *Piper longum*; Lane 1 is the DNA ladder, Lane 2 is the male plant, and Lane 3 is the female plant.

Both rbcL and psbA-trnH regions have shown clear and consistent bands for both male and female plants. However, in the case of ITS2, amplification was successful only in the

male sample. Studies have shown that the ITS region shows more variation than chloroplast DNA markers. The polymorphic behaviour of ITS could be due to either introgression from hybridization or could be the result of homoploid speciation (12). Studies have revealed that ITS is successful for Piper species but still, it is yet to prove that ITS2 shows good amplification for *Piper longum* plants. Also, in this study, ITS2 was successfully amplified for the male plant only. Thus, it is early to consider ITS2 to be used as a tool to distinguish between male and female *Piper longum* thereby helping in selecting economically important plants.

BLAST

The sequences obtained by Sanger Sequencing were reverse complemented in BioEdit software and analyzed using Blastn. BLAST for rbcL sequences of the male and female plants revealed maximum similarity to *Piper longum* with 95.07% and 94.66% respectively, and for psbA-trnH, both male and female plants showed the maximum similarity to *Piper nigrum* with 95.38% and 95.96% respectively. BLAST using ITS2 showed no similarity with any other species.

Pairwise Sequence alignment-emboss needleman

Pairwise alignment between the male and female *Piper longum* samples showed 89.40% similarity for rbcL and 93.4% for psbA-trnH. A maximum similarity between the male and female sequences was shown by psbA-trnH (Table 1).

Table 1: Pairwise sequence alignment for sequence similarity identification using EMBOSS Needle algorithm for male and female *Piper longum* plants with rbcL, ITS2, and psbA-trnH

DNA Barcodes/ primers	Length sequences	% of identity	% of similarity	Gap penalty	Extended gap	Gaps	Score
rbcL	624	89.4 (558/624)	89.4 (558/624)	10.0	0.5	30/624	2539.0
ITS2	627	33.8 (212/627)	33.8 (212/627)	10.0	0.5	347/627	324.5
psbA-trnH	331	93.4 (309/331)	93.4 (309/331)	10.0	0.5	11/331	1710.0

Phylogenetic analysis

The UPGMA-based phylogenetic trees were constructed using MEGA-X software for both rbcL and psbA-trnH sequences. From the phylogenetic tree for rbcL, the male *Piper longum* plant was found to be closely related to *Piper nigrum* while the female *Piper longum* was closely related to *Piper attenuatum* by sharing a common ancestor respectively (Figure 4).

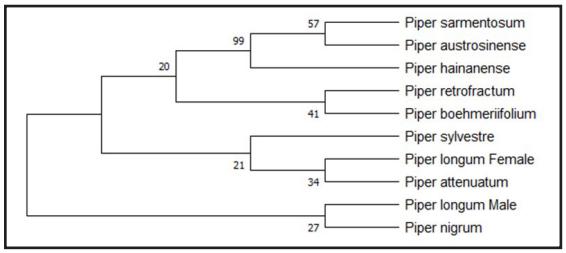

Figure 4: Phylogenetic tree constructed using UPGMA tree method of male and female *Piper longum* plant for rbcL

For psbA-trnH, both the male and female Piper longum plants have formed a cluster indicating a shared common ancestor which is evident from the highest sequence similarity between male and female Piper longum as seen in BLAST analysis (Figure 5).

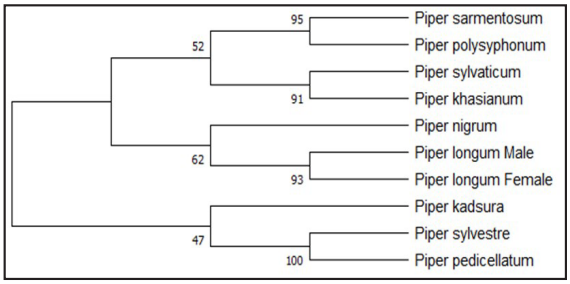

Figure 5: Phylogenetic tree constructed using UPGMA tree method of male and female *Piper longum* plant for psbA-trnH

Conclusion

In this study, the DNA barcoding of *Piper longum* has been successful using rbcL, ITS2, and psbA-trnH. The *Piper longum* samples have shown amplification for all the barcodes used. Among the DNA barcodes, rbcL and psbA-trnH have shown results for both male and female *P. longum* whereas ITS2 has shown significant results for only male *P. longum*. The phylogenetic study has revealed the similarity of *Piper longum* with other Piper species. Thus, the DNA barcodes for both male and female *P. longum* were successfully generated for rbcL and psbA-trnH for *Piper longum*.

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