Molecular identification of medicinal plants with amplicon length polymorphism using universal DNA barcodes of the *atpF–atpH*, *trnL* and *trnH–psbA* regions

Vishwa Vijay Thakur¹ · Sharad Tiwari^{1,2} · Niraj Tripathi¹ · Gyanendra Tiwari³

Received: 12 September 2018 / Accepted: 19 April 2019 / Published online: 25 April 2019
© King Abdulaziz City for Science and Technology 2019

Abstract

Amplification success and species discrimination efficiency of universal DNA barcode primers (*trnH–psbA*, *trnL*, *ycf1b*, *atpF–atpH*, *matK* and *rbcL*) was evaluated in 46 representative medicinal plant species of 28 families on agarose gel. The results showed that amplicons length polymorphism revealed by the primers *atpF–atpH*, *trnH–psbA* and *trnL* can simultaneously discriminate all the 46 species under study precisely. Some of the plant species included in this study are used as potential adulterants to other plant species. We were able to successfully discriminate the plant species from their potential substitute. *Vitex negundo* is an adulterant of *Ocimum sanctum* which has been successfully discriminated by all these three markers. Another example of adulteration between *Bacopa monnieri* and *Centella asiatica* was successfully discriminated by *atpF–atpH*, *trnL* and *trnH–psbA* on the basis of variability in amplicon length of these two medicinal herbs. Further, *Cassia tora* and *Cassia fistula* are also adulterants for each other and variability in amplicon length between these two species was revealed by *atpF–atpH* and *trnH–psbA* markers. A colour code distance matrix based on amplicon length polymorphism was designed to select primers which can effectively discriminate plants species on the basis of their amplicon length. Discrimination of plant species with the universal markers on agarose gel is a noble and inexpensive approach as it does not require sequencing of amplicons. This procedure will provide a way for the development of diagnostic markers to identify adulteration not only in herbal drug formulations but also in food material.

Keywords PCR amplicons · Barcoding primers · Adulteration · Plastid genome · Agarose gel electrophoresis

Introduction

Herbal medicines are getting generous consideration in global health sectors, for their health benefits and commercial importance. The flora of India is made up of 7–8% of global biodiversity with colossal assets of medicinal plants. They comprise more than 8000 medicinal plants species, about 960 of which have been used in herbal product market for trade. The medicinal plant market in India is growing at a

rapid pace. As per estimates, it is one of the fastest growing industries with an estimated annual growth rate of 7%, and is expected to be 40% hike by 2020 (Anonymous 2018). These herbal plants are rich source of biologically active natural compounds, which have enormous potential for discovery of new drugs. The modern pharmaceutical industry actively engaged in identification of novel constituents of medicinal plants for formulation of new drugs. In addition, there are various types of herbal blends sold as herbal teas, nutraceutical and cosmetics related to health and personal care. Due to continuously growth in the herbal market, the global demands of medicinal plants dramatically increased (Giang and Otsuka 2018; Efferth and Greten 2012). In addition, many medicinal plants species are threatened to extinction due to results of habitat destruction, climate change and over exploitation to fulfil the global demands. In these conditions, chances of adulteration become very high because of the supply shortage and high market demand. The herbal industry experiences deliberate or indeliberate substitution and/or

✉ Sharad Tiwari
shtiwari@gmail.com

¹ Biotechnology Centre, Jawaharlal Nehru Agriculture University, Jabalpur 482004, India

² Department of Plant Breeding and Genetics, Jawaharlal Nehru Agriculture University, Jabalpur 482004, India

³ College of Agriculture, Jawaharlal Nehru Agriculture University, Ganjbasoda, Vidisha 464221, India

adulteration of therapeutic herbs with related or divergent species. Some time event of adulteration are also observed because of many related herbs have identical or different common names. For instance, *Bacopa monnieri* a medicinally important plant belonging to the family Schrophulariaceae also known by its vernacular name Brahmi. It contains various types of saponins such as bacosides A, B, C and D. These bacosides are memory chemicals and used as a brain tonic. *Centella asiatica* belonging to family apiaceae is a common adulterant and sold in the market by same name Brahmi. However, both herbs are belonging to different family and contain different constituents (Yadav et al. 2012).

The entry of adulterant in herbal product reduced therapeutic potential of original herbal medicine and some time causes serious health concern for consumers (Newmaster et al. 2013). Earlier, local physicians (Vaidyas and Hakims) were responsible for herb identification, procurement, storage, formulation and its distribution. However, during urbanization with the lost contact with nature has resulted in lack of ability to identify most of the medicinal plants. The customary techniques for identification incorporate organoleptic strategies (by the senses: taste, sight, smell, and touch), naturally visible and microscopic systems and chemical profiling such as TLC, HPLC–UV, HPLC–MS (Soni et al. 2013). However, these above mentioned methods are often failing in identifying closely related species correctly as the effect of physiological and storing conditions may alter the result of chemical profiles or biochemical markers. In addition, none of these methods can conveniently identify species of medicinal plants that are phylogenetically close (Tehen et al. 2014). Further, identification of medicinal plants based on traditional taxonomic approach cannot be applied for identifying powdered or processed samples. Therefore, there is an urgent need to develop globally acceptable approach for tracing adulterant and authentication of herbs used in herbal product. In this context, species verification at the DNA level assures greater reliability in comparison to RNA and protein because DNA is a steady macromolecule that is not influenced by environmental effect and is found in all tissues. Hence, advancement in DNA-based markers is critical for identification of plants as well as confirmation of herbal ingredients in products.

In recent days, molecular-based identification systems are widely used to identify biological specimen as a specific species. Among those, DNA barcoding relies on standardized methods, sequencing and analyses (El-Atroush et al. 2015). Since the first report of DNA barcoding (Hebert et al. 2003), it has been widely used in animals for recognizing species and taxonomic clarification. A small segment of the mitochondrial *cytochrome c oxidase 1* (*COI* or *cox1*) gene sequence is successfully used as a universal barcode in animals but this gene is not suitable for plants as its rate of nucleotide substitution is very low in plant mitochondrial

genome (Mower et al. 2007). To overcome this problem, plastid DNA sequences have been targeted to established DNA barcodes for plants. The Plant Working Group of Consortium for the Barcode of Life (CBOL Plant Working Group 2009) evaluated the performance of seven plastid barcode loci (*psbK-psbI*, *atpF-atpH*, *trnH-psbA* spacers, and *matK*, *rbcL*, *rpoB*, and *rpoC1* genes) and suggested *rbcL* and *matK* as standard DNA barcode regions in addition recommended *ITS* and *trnH-psbA* as supplementary barcode loci. Many researchers (Taberlet et al. 2009; Borsch et al. 2003; Ridgway et al. 2003; Tsai et al. 2006; Spaniolas et al. 2010), have suggested *trnL* intron as a plant barcode region and highlighted its utility with degraded DNA.

Although DNA barcoding is proven useful for identification of plants (Sikdar et al. 2018) but the process of sequencing is costly and time taking which hinders the wider application of this technique in developing countries (Osathanunkul et al. 2016). Hence, there is a need to develop reliable, cost effective, rapid and simple technique for proper identification of plant's taxa. Variation in amplicon length of proposed universal DNA barcode primers is well documented by Vijayan and Tsou (2010). If these amplicon length polymorphisms of universal DNA barcode primers are used, it is possible to discriminate several taxa circumventing the need of sequencing. Consequently, the present study was aimed to develop an easy and reliable DNA discrimination of various medicinal plant taxa on the basis of simple PCR amplification and agarose gel electrophoresis. For this purpose, we screened the variation in amplicon length of universal DNA barcode primers and this amplicon length polymorphism was used to identify various plant taxa with medicinal value. In the present investigation, six universal primers *trnH-psbA*, *trnL*, *ycf1b*, *atpF-atpH*, *matK* and *rbcL* were evaluated to investigate variation in amplicon length in 46 different medicinal species including some of their closely related species. The utility of amplicon length polymorphism to discriminate herbal medicines from potential substitute have also been highlighted in this study.

Materials and methods

Plant material

Leaves from 46 plant species covering 28 families (Table 1) were collected from different regions of Central India. The leaf samples were individually placed in a plastic pouch and stored at -80°C until processed for DNA extraction.

DNA extraction

The leaf samples were immersed in liquid nitrogen and crushed using sterile mortar and pestle to get a fine powder.

Table 1 List of medicinal plant species with their local name and collection site used for examine universality in PCR amplification of all DNA barcode loci

Family	Scientific name	Local name	Collection site	Latitude/longitude
Apocynaceae	<i>Rauvolfia serpentina</i>	Sarpagandha	Jabalpur	23°10'N/79°59'E
	<i>Catharanthus roseus</i>	Sadabahar	Jabalpur	23°10'N/79°59'E
Solanaceae	<i>Solanum nigrum</i>	Makoy	Jabalpur	23°10'N/79°59'E
	<i>Datura metel</i>	Datura	Amarkantak	22°40'N/81°45'E
Lamiaceae	<i>Ocimum sanctum</i>	Tulsi	Jabalpur	23°10'N/79°59'E
	<i>Coleus forskohlii</i>	Patharchur	Amarkantak	22°40'N/81°45'E
	<i>Mentha viridis</i>	Pudina	Lucknow	26°55'N/80°59'E
Asclepiadaceae	<i>Tylophora asthmatica</i>	Antamula	Dindori	22°57'N/81°41'E
	<i>Calotropis procera</i>	Akon	Jabalpur	23°10'N/79°59'E
	<i>Gymnema sylvestre</i>	Gudmar	Jabalpur	23°10'N/79°59'E
Fabaceae	<i>Clitoria trenata</i>	Aparajita	Jabalpur	23°10'N/79°59'E
	<i>Cassia tora</i>	Chakvad	Mandla	22°43'N/80°35'E
	<i>Pongamia pinnata</i>	Karanja	Amarkantak	22°40'N/81°45'E
	<i>Terminalia chebula</i>	Harra	Amarkantak	22°40'N/81°45'E
	<i>Cassia fistula</i>	Amaltash	Amarkantak	22°40'N/81°45'E
	<i>Caesalpinia crista</i>	Latakaranja	Dindori	22°57'N/81°41'E
Acanthaceae	<i>Andrographis paniculata</i>	Kalmegh	Jabalpur	23°10'N/79°59'E
	<i>Justicia adhatoda</i>	Adusa	Amarkantak	22°40'N/81°45'E
Santalaceae	<i>Santalum album</i>	Safed Chandan	Amarkantak	22°40'N/81°45'E
Plantaginaceae	<i>Bacopa monnieri</i>	Brahmi	Jabalpur	23°10'N/79°59'E
Apiaceae	<i>Centella asiatica</i>	Manduk Parni	Jabalpur	23°10'N/79°59'E
Phyllanthaceae	<i>Phyllanthus niruri</i>	Bhui amla	Jabalpur	23°10'N/79°59'E
Xanthorrhoeaceae	<i>Aloe barbadensis</i>	Ghritkumari	Jabalpur	23°10'N/79°59'E
Nyctaginaceae	<i>Boerhavia diffusa</i>	Punarnava	Jabalpur	23°10'N/79°59'E
Asparagaceae	<i>Asparagus racemosus</i>	Satavar	Jabalpur	23°10'N/79°59'E
Zingiberaceae	<i>Curcuma longa</i>	Haldi	Amarkantak	22°40'N/81°45'E
Acoraceae	<i>Acorus calamus</i>	Buch	Amarkantak	22°40'N/81°45'E
Convolvulaceae	<i>Argyrea nervosa</i>	Vidhara	Amarkantak	22°40'N/81°45'E
Asteraceae	<i>Cenratherum anthementium</i>	Vanjeera	Amarkantak	22°40'N/81°45'E
	<i>Eclipta alba</i>	Bhringraj	Amarkantak	22°40'N/81°45'E
Costaceae	<i>Costus speciosus</i>	Keokand	Raipur	21°15'N/81°41'E
Vitaceae	<i>Cissus quadrangularis</i>	Harjod	Raipur	21°15'N/81°41'E
Piperaceae	<i>Piper longum</i>	Pippali	Amarkantak	22°40'N/81°45'E
Combretaceae	<i>Terminalia bellirica</i>	Baheda	Amarkantak	22°40'N/81°45'E
	<i>Terminalia arjuna</i>	Arjun	Dindori	22°57'N/81°41'E
Verbenaceae	<i>Clerodendrum serratum</i>	Bharangi	Amarkantak	22°40'N/81°45'E
	<i>Vitex negundo</i>	Nirgundi	Mandla	22°43'N/80°35'E
Scrophulariaceae	<i>Scoparia dulcis</i>	Jangli zeera	Mandla	22°43'N/80°35'E
Menispermaceae	<i>Tinospora cordifolia</i>	Giloy	Amarkantak	22°40'N/81°45'E
Malvaceae	<i>Sida rhombifolia</i>	Bariyar	Amarkantak	22°40'N/81°45'E
Dioscoreaceae	<i>Discorea floribanda</i>	Yam	Amarkantak	22°40'N/81°45'E
Cucurbitaceae	<i>Bryonia laciniosa</i>	Shivlingi	Dindori	22°57'N/81°41'E
Poaceae	<i>Echinochloa frumentacea</i>	Sawa	Rewa	24°32'N/81°18'E
	<i>Echinochloa crusgalli</i>	Sawa	Rewa	24°32'N/81°18'E
	<i>Setaria italica</i>	Kakun	Rewa	24°32'N/81°18'E
	<i>Panicum miliaceum</i>	Ragi	Rewa	24°32'N/81°18'E

DNeasy plant mini kit (Qiagen) was used for DNA isolation. Quality of the extracted DNA was determined using

gel electrophoresis and spectrophotometer. Isolated plant genomic DNA was stored at -20°C for further use.

Polymerase chain reaction

A total volume of 10 µL of PCR reaction mixture contained the following: 1 µL of 10×PCR buffer (Genei), 200 µM of each deoxynucleotide, 1.5 mM MgCl₂, 1 U *Taq* polymerase (Thermo Fisher Scientific, USA), 5 pmol primer (Integrated DNA Technologies, USA, Table 2), 1 µL of genomic DNA and the rest was adjusted with sterile distilled water. PCR amplification was performed with a thermal cycler (Agilent) programme at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by final extension step at 72 °C for 5 min. PCR conditions were similar for all the primer pairs except the annealing temperature (*T*) for *matK* (58 °C).

Agarose gel electrophoresis and data analysis

Agarose gel (1%) using 1× TAE buffer containing 0.5 µg/mL ethidium bromide was used for electrophoresis of PCR products. Gel images were obtained using bio-imaging system (Syngene, USA). The amplicon sizes of PCR products resulting from the primer pairs of the specific barcoding gene were determined using a 100 bp ladder (Fermentas, USA). Amplicon length variation was screened by creating a 1, 0 matrix. Similar amplicon length was scored as 1 and variation in amplicon size was scored as 0. On the basis of amplicon length, all the studied plant taxa were subjected to NTSYSp version 2.02 (Rohlf 1997) for grouping.

Results

Primer universality

We observed complete amplification with all the universal primers for PCR amplification of 46 plant species representing a wide coverage of these 28 families signifying their uniform effectiveness in amplifying intense bands. All the primers amplified desired size range. In terms of absolute discriminatory power estimate on the basis of amplicons length polymorphism, *atpF–atpH*, *trnH–psbA* and *trnL*

(Fig. 1) were found to be suitable for species discrimination while, *rbcL*, *matK* and *ycf1b* failed to discriminate species under study. During this investigation, three primers in combination were able to discriminate all the medicinal plants species under study successfully with each other except *Terminalia chebula* to *Aloe barbadensis*, *Andrographis paniculata* to *B. monnieri* and *Echinochloa frumentacea* to *Echinochloa crusgalli*.

Evaluation of amplicon length polymorphism

Amplicon length polymorphism revealed by *atpF–atpH* barcode regions

The primer pair *atpF–atpH* produced 17 differently sized amplicons categorizing all the 46 species into 17 groups (Fig. 2a). These groups are based on sizes of amplicons produced. Among all 46 species, 7 species (*Cassia fistula*, *Datura metel*, *Ocimum sanctum*, *Coleus forskohlii*, *M. virdis*, *Sida rhombifolia* and *P. milliaceum*) produced unique amplicons with each and formed individual group. Further, six groups contained two species in each with amplicons of identical size. One group consisted of six species whereas; remaining three groups contained seven species in each.

Amplicon length polymorphism revealed by *trnL* barcode regions

The primer *trnL* separated the entire 46 specimens in 10 groups according to amplicon size (Fig. 2b). Group one was the largest among all the groups consisting of 22 plant species sharing a similar sized amplicon followed by group ten (9 species) and group five (5 species). The primer pair *trnL* amplified four unique amplicons and clearly differentiated four species from others. Four species individually represented a single species each namely group two (*Justicia adhatoda*), group four (*Tylophora asthmatica*), group seven (*Argyrea nervosa*) and group nine (*Scoparia dulcis*). Three groups contained two species in each group namely three (*D. metel* and *Boerhavia diffusa*), six (*Acorus calamus* and

Table 2 DNA barcoding primers used for analysis

Primer	Forward 5'–3'	Reverse 5'–3'	References
<i>matK</i>	CGTACAGTACTTTTGTGTTTACGAG	ACCCAGTCCATCTGGAAATCTTGGTTC	CBOL Plant Working Group (2009)
<i>rbcL</i>	ATGTCACCACAAACAGAGACTAAAGC	GAAACGGTCTCTCCAACGCAT	CBOL Plant Working Group (2009)
<i>atpF–atpH</i>	ACTCGCACACACTCCCTTTCC	GCTTTTATGGAAGCTTTAACAAT	Wang et al. (2010)
<i>trnL</i>	CGAAATCGGTAGACGCTACG	GGGGATAGAGGGACTTGAAC	Taberlet et al. (1991)
<i>ITS2</i>	ATGCGATACTTGGTGTGAAT	GACGCTTCTCCAGACTACAAT	Chen et al. (2010)
<i>trnH–psbA</i>	CGCGCATGGTGGATTACAATCC	GTTATGCATGAACGTAATGCTC	Kress et al. (2005)
<i>Ycf1b</i>	TCTCGACGAAAATCAGATTGTTGTGAAT	ATACATGTCAAAGTGATGGAAAA	Dong et al. (2015)

Fig. 1 Electrophoretic banding pattern of medicinal plant species using **a** atpF–atpH, **b** trnH–psbA, and **c** trnL primers

Cenrathium anthementium) and group eight (*Costus speciosus* and *S. rhombifolia*).

Amplicon length polymorphism revealed by trnH–psbA barcode regions

On the basis of amplicons amplified by *trnH*–*psbA* primer, all the 46 plants were clustered into 9 groups (Fig. 2c). Most

Fig. 2 Grouping of medicinal plant species according to amplicon sizes amplified with **a** *atpF*–*atpH*, **b** *trnL*, and **c** *trnH*–*psbA* primers

of the groups consisted of three or more species, however, two groups (seven and eight) were individually confined to only one species *C. longa* and *T. arjuna*, respectively.

Amplicon length polymorphism within families

The present investigation not only discriminated 28 families but also revealed the variation between various species within a family. Variability in amplicon length between plants species belonging to same family was revealed by the three primer pairs targeting *atpF*–*atpH*, *trnL* and *trnH*–*psbA*. Out of 28 families understudy, 10 families were represented

by more than 1 plant species and remaining 18 by only 1 species. The two plant species *R. serpentina* and *C. roseus* belonging to the Apocynaceae family were differentiated from each other on the basis of amplicon length polymorphism by *atpF*–*atpH* and *trnH*–*psbA* primers (Fig. 3). Two plant species belonging to the family Solanaceae (*S. nigrum* and *D. metel*) amplified different sized amplicons using all the three primers. Three plant species namely *O. sanctum*, *C. forskohlii* and *M. viridis* belonging to the family Lamiaceae also exhibited polymorphism by *atpF*–*atpH* and *trnH*–*psbA* and these both primers were able to differentiate them. However, amplicon size of *M. viridis* amplified by primer *trnL*

Fig. 3 Colour code distance matrix of discrimination ability of DNA barcode primers among medicinal plant species

varied from remaining two species within this family and was able differentiate only *M. viridis* from rest of two species. Three plant species from Asclepiadaceae were also discriminated by the primers *atpF-atpH* and *trnL* by amplifying longer amplicons in *T. asthmatica* as compared to *C. procera* and *G. sylvestre*. On the other hand, *trnH-psbA* amplified similar sized amplicons in *T. asthmatica* and *C. procera* while longer in *G. sylvestre*. Five species, *C. trenata*, *C. tora*, *P. pinnata*, *C. fistula* and *C. crista* of family Fabaceae were also differentiated according to amplicon sizes generated by *atpF-atpH* and *trnH-psbA* primers. Two species *A. paniculata* and *J. adhatoda* of the family Acanthaceae were discriminated by all the three primers. Two plant species *C. anthemintum* and *E. alba* belonging to family Asteraceae also amplified diverse sized amplicons with all the three primers. Two members *C. serratum* and *Vitex negundo* of the family Verbenaceae also showed variability in length of amplicons rising from *atpF-atpH* and *trnH-psbA* primers.

Among four medicinally important millet species (*E. frumentacea*, *E. crusgalli*, *S. italica* and *P. miliaceum*) belonging to the family Poaceae, only *S. italica* and *P.*

miliaceum were successfully discriminated from each other by *atpF-atpH* and *trnH-psbA* primers. Three medicinally important tree species (*T. bellirica*, *T. chebula* and *T. arjuna*) belonging to the family Combretaceae were discriminated from each other by *atpF-atpH* primer. *trnL* primers discriminated *T. chebula* and *trnH-psbA* discriminated *T. arjuna* from the other two species. Primer *trnL* produced similar sized amplicon for *T. arjuna* and *T. bellirica* where as *trnH-psbA* failed to show any variations in amplicon length of *T. bellirica* and *T. chebula*.

During the present investigation, 18 families representing a single species (Table 1) were differentiated using *atpF-atpH* and *trnH-psbA* primers. In addition, *B. diffusa*, *A. racemosus*, *C. longa*, *A. calamus* and *A. nervosa* were discriminated by *trnL* primers. They amplified four unique amplicon and clearly differentiated four species from others. Four groups individually represented a single species *J. adhatoda*, *T. asthmatica*, *A. nervosa* and *S. dulcis*. Three groups contained two species *D. metel* and *B. diffusa*, *A. calamus* and *C. anthemintum*, *C. speciosus* and *S. rhombifolia*.

Amplicon length polymorphism to identify adulteration/substitution

In this study, some plant species are used as potential adulterants to other plant species. Using this molecular technique, we can successfully discriminate the plant species from their potential substitute. *V. negundo* is an adulterant of *O. sanctum* which has been successfully discriminated by all these three markers. Another example of adulteration between *B. monnieri* and *C. asiatica* was successfully discriminated by *atpF-atpH*, *trnL* and *trnH-psbA* on the basis of variability in amplicon length of these two medicinal herbs. Further, *C. tora* and *C. fistula* are also adulterants for each other and variability in amplicon length between these two species was revealed by *atpF-atpH* and *trnH-psbA* markers.

Application of amplicon length polymorphism

After analysis of amplicon length polymorphism showed by these three markers among the entire 46 species, we developed a colour code distance matrix. In matrix, these three markers were represented by three different colours, i.e. green for *atpF-atpH*, red for *trnL* and blue for *trnH-psbA*. The absence of amplicon length polymorphism between two species is left blank. In the present analysis, three primers in combination were able to discriminate all the medicinal plants species under study successfully with each other except few (Fig. 3).

Discussion

Identification of plant species based on amplicon length variability which can be visibly envisaged on agarose gel and does not necessitate sequencing; therefore, it is a decent and inexpensive approach to differentiate plant species (Sikdar et al. 2018). This method can provide the way to sort adulteration in herbal drug materials in a normal facility molecular biology laboratory because of no need of sequencing of amplicons. Various molecular markers are available in the literature for identifying plant species; however, one marker alone is not sufficient to attain certain results (Dong et al. 2012). The correct identification of plant species in herbal drug industry is critical for quality control. The present study is the first report on medicinal plant species identification using universal DNA barcode primers based on amplicon sizes separated on agarose gel only. The advantages of this technique are simplicity, broad spectrum potential, and circumventing the need of sequencing. The fundamental initiative at the back of this study was to make use of the amplicon length variability demonstrated by these primers to discriminate the medicinal plant species. Initially,

a total of seven primer pairs were screened for their amplification across all the plants taxa included in the present study. All the primers exhibited 100% PCR amplification efficiency confirming the universality of these primers. Further, among all DNA barcoding primers only three primer pairs (*atpF-atpH*, *trnH-psbA* and *trnL*) were able to produce amplicon length polymorphism with studied plant species and rest of the primers amplified only monomorphic alleles. Similar results were obtained in a recent study conducted to differentiate 24 representative species of Fabaceae family on the basis of amplicon length polymorphism (Sikdar et al. 2018) where, *rbcL* and *matK* produced monomorphic band. To identify the specific species, we have developed a colour code matrix which may also be used for discriminating valuable medicinal plant species from their potential adulterants.

The primer pair *atpF-atpH* amplify the intergenic spacer between the two genes *atpF* and *atpH* representing two subunits of ATP synthase genes namely CFO I and CFO III (Drager and Hallick 1993). Variations in amplicon size of this intergenic region observed during the present investigation are quite similar to the DNA barcoding results of South African Kruger National Park flora (Lahaye et al. 2008), where amplicon length variations also rose due to this very intergenic region. Similarly, variation in the amplicon length was observed in the study conducted on 31 species of family Lamiaceae (Wang et al. 2010).

The *trnL* marker of intron is being frequently used for a long time and its small loop is a major strength of this primer in species identification (Taberlet et al. 2007). The choice of the *trnL* intron as a target for identifying plant taxa as the rate of evolution of this region in land plants is well known, it is adequately variable at inter-species and highly conserved at intra-species level and a high number of insertions/deletions and discriminative single-base polymorphisms among species make the *trnL* (UAA) intron an ultimate region for identification of several plant taxa (Quandt et al. 2004; Spaniolas et al. 2010; Taberlet et al. 2007). James and Schmidt (2004) used the variability in amplicon size of *trnL* primers to discriminate various food crops. In a recent study, D'yachenko et al. (2015) observed differences in the amplicon size produced by *trnL* region among different legume species. The amplicons produced in the study were able to differentiate *Phaseolus vulgaris*, *Cicer arietinum* and *Trifolium repens* from other legume species. Using the amplicon length polymorphism exhibited by this marker, we also identified an array of plants belonging to same or different family.

Among different universal primers, one of the most widely used plastid marker is *trnH-psbA* and this variable intergenic spacer is very proficient to amplify genomic regions of most of the angiosperms (Shaw et al. 2007; Vijayan and Tsou 2010). In previous investigations, variability in amplicon length arising due to *trnH-psbA* marker,

ranged from 296 to 1120 bp (Chase et al. 2007). This marker contains *rps19* gene or pseudogene which is responsible for unexpected variations in amplicon length (Chang et al. 2006). In a similar study, Monkheang et al. (2011) obtained amplicon length polymorphism among 14 species of *Senna* using *trnH-psbA* primer. Due to the presence of inversions or mononucleotide repeats at the *trnH-psbA* locus, problems were observed in obtaining high-quality bidirectional sequences and alignment of sequences in certain taxa (Dong et al. 2012). However, our aim was to develop a sequencing free method for identification of several plant taxa using the variability in amplicon length amplified by this primer and it also revealed polymorphism across several plant taxa under present investigation.

Among the studied medicinal species, *V. negundo* is an adulterant of *O. sanctum* in herbal preparations (Kumar 2014) and belongs to different families with different pharmacological bioactive compound. Another combination of adulteration is *B. monnieri* and *C. asiatica* (Kumar 2014); *B. monnieri* is used for various nervous disorders and is an important ingredient of many traditional as well as modern medicines. Its most common substitute is *C. asiatica*; there is so much of confusion in herbal market that both the plants are known as ‘Brahmi’ although they belong to different families. Sometimes, *E. alba* is also used as adulterant to increase the volume of the drug formulation based on Brahmi (Yadav et al. 2012). Further, *C. tora* and *C. fistula* of family Fabaceae are also adulterants for each other (Seth and Thaker 2015). All the above mentioned herbal remedies were successfully discriminated from its potential adulterants on the basis of amplicon size as revealed by the three promising markers. Some of the plant species were not discriminated from each other as *T. chebula* to *A. barbadensis*, *A. paniculata* to *B. monnieri* and *E. frumentacea* to *E. crusgalli*. Therefore, further efforts are required to develop such primer combinations which can be used for the discrimination of mentioned species.

Conclusions

In this study, we demonstrated the discrimination ability of three primers namely *atpF-atpH*, *trnH-psbA* and *trnL* on the basis of amplicon length polymorphism on agarose gel electrophoresis. The amplicon length polymorphism revealed by these primers in combination can effectively discriminate several medicinally important plants from each other. This method will be very useful for accurate identification of herbal medicine as well as various food products in uncovering adulterants. This technique is simple, cost effective and less time consuming in comparison to various other contemporary molecular techniques.

Acknowledgements ST acknowledges grant from Madhya Pradesh State Agriculture Marketing Board, Bhopal, India.

Author contributions VVT and ST planned the work; GT and VVT collected the experimental materials; and VVT and NT conducted the experiments. ST, VVT and NT have contributed equally in analysis and writing of the manuscript.

Compliance with ethical standards

Conflict of interest On behalf of all the authors, the corresponding author states that there is no conflict of interest.

References

- Anonymous (2018) Annual report, 2017–18. Ministry of Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homeopathy, India
- Borsch T, Hilu K, Quandt D, Wilde V, Neinhuis C, Barthlott W (2003) Noncoding plastid *trnT-trnF* sequences reveal a well resolved phylogeny of basal angiosperms. *J Evol Biol* 16:558–576. <https://doi.org/10.1046/j.1420-9101.2003.00577.x>
- CBOL Plant Working Group (2009) A DNA barcode for land plants. *Proc Natl Acad Sci USA* 106:12794–12797. <https://doi.org/10.1073/pnas.0905845106>
- Chang CC, Lin HC, Lin IP, Chow TY, Chen HH, Chen WH (2006) The chloroplast genome of *Phalaenopsis aphrodite* (Orchidaceae): comparative analysis of evolutionary rate with that of grasses and its phylogenetic implications. *Mol Biol Evol* 23:279–291. <https://doi.org/10.1023/A:1006319803002>
- Chase MW, Cowan RS, Hollingsworth PM, van den Berg C, Madriñán S, Petersen G (2007) A proposal for a standardized protocol to barcode all land plants. *Taxon* 56:295–299
- Chen S, Yao H, Han J, Liu C, Song J et al (2010) Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS One* 5:e8613. <https://doi.org/10.1371/journal.pone.0008613>
- D'yachenko EA, Filyushin MA, Pronin EP, Kochieva EZ (2015) Variability of the *trnL* Plastid Gene's Intron in the Faboideae Species (Fabaceae). *Russ J Genet* 5:220–226. <https://doi.org/10.1134/S2079059715030053>
- Dong W, Liu J, Yu J, Wang L, Zhou S (2012) Highly variable chloroplast markers for evaluating plant phylogeny at low taxonomic levels and for DNA barcoding. *PLoS One* 7:e35071. <https://doi.org/10.1371/journal.pone.0035071>
- Dong W, Chao XC, Li C, Sun J, Zuo Y, Shi S, Cheng T, Guo J, Zhou S (2015) *ycf1*, the most promising plastid DNA barcode of land plants. *Sci Rep* 5:8348. <https://doi.org/10.1038/srep08348>
- Drager RG, Hallick RB (1993) A novel *Euglena gracilis* chloroplast operon encoding 4 ATP synthase subunits and 2 ribosomal-proteins contains 17 Introns. *Curr Genet* 23:271–280. <https://doi.org/10.1007/BF00351506>
- Efferth T, Gretten HJ (2012) Medicinal and aromatic plant research in the 21st century. *Med Aromat Plants* 1:e110. <https://doi.org/10.4172/2167-0412.1000e110>
- El-Atroush H, Magdy M, Werner O (2015) DNA Barcoding of two endangered medicinal plants from Abou Galoom protectorate. *Life Sci J* 12:101–109. <https://doi.org/10.7537/marslsj120915.14>
- Giang PM, Otsukab H (2018) New compounds and potential candidates for drug discovery from medicinal plants of Vietnam. *Chem Pharm Bull* 66:493–505. <https://doi.org/10.1248/cpb.c17-00628>
- Hebert PDN, Cywinska A, Ball SL, DeWaard JR (2003) Biological identifications through DNA barcodes. *Philos Trans R Soc Lond B: Biol Sci* 270:313–321. <https://doi.org/10.1098/rspb.2002.2218>

- James D, Schmidt A (2004) Use of an intron region of a chloroplast tRNA gene (*trnL*) as a target for PCR identification of specific food crops including sources of potential allergens. *Food Res Int* 37:395–402. <https://doi.org/10.1016/j.foodres.2004.02.004>
- Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH (2005) Use of DNA barcodes to identify flowering plants. *Proc Natl Acad Sci USA* 102:8369–8374. <https://doi.org/10.1073/pnas.0503123102>
- Kumar SP (2014) Adulteration and substitution in endangered ASU medicinal plants of India: a review. *Int J Med Arom Plants* 4:56–73
- Lahaye R, Savolainen V, Duthoit S, Maurin O, van der Bank M (2008) A test of *psbK-psbI* and *atpF-atpH* as potential plant DNA barcodes using the flora of the Kruger National Park as a model system (South Africa). *Nature Proc*
- Monkheang P, Sudmoon R, Taneet T, Noikotr K, Bletter N, Chaveerach A (2011) Species diversity, usages, molecular markers and barcode of medicinal *Senna* species (Fabaceae, Caesalpinioideae), Thailand. *J Med Plant Res* 5:6173–6181
- Mower JP, Touzet P, Gummow JS, Delph LF, Palmer JD (2007) Extensive variation in synonymous substitution rates in mitochondrial genes of seed plants. *BMC Evol Biol* 7:135. <https://doi.org/10.1186/1471-2148-7-135>
- Newmaster SG, Grguric M, Shanmughanandhan D, Ramalingam S, Ragupathy S (2013) DNA barcoding detects contamination and substitution in North American herbal products. *BMC Med* 11:222–235. <https://doi.org/10.1186/1741-7015-11-222>
- Osathanunkul M, Suwannapoom C, Osathanunkul K, Madesise P, Boerf HD (2016) Evaluation of DNA barcoding coupled high resolution melting for discrimination of closely related species in phytopharmaceuticals. *Phytomedicine* 23:156–165. <https://doi.org/10.1016/j.phymed.2015.11.018>
- Quandt D, Muller K, Stech M, Frahm JP, Frey W, Hilu KW, Borsch T (2004) Molecular evolution of the chloroplast *trnL-F* region in land plants. *Monogr Syst Bot Missouri Bot Gard* 98:13–37
- Ridgway K, Duck J, Young J (2003) Identification of roots from grass swards using PCR-RFLP and FFLP of the plastid *trnL* (UAA) intron. *BMC Ecol* 3:8. <https://doi.org/10.1186/1472-6785-3-8>
- Rohlf FJ (1997) NTSYS-pc Version. 2.02i Numerical taxonomy and multivariate analysis system. Applied Biostatistics Inc., Exeter Software, Setauket
- Seth BP, Thaker VS (2015) Identification of a herbal powder by deoxyribonucleic acid barcoding and structural analyses. *Pharmacogn Mag* 11:S570–S574. <https://doi.org/10.4103/0973-1296.172963>
- Shaw J, Lickey EB, Schilling EE, Small RL (2007) Comparison of whole Chloroplast genome sequence to choose non-coding region for phylogenetic studies in angiosperm: the tortoise and the hare III. *Am J Bot* 94:275–288. <https://doi.org/10.3732/ajb.94.3.275>
- Sikdar S, Tiwari S, Sapre S, Thakur VV (2018) Simple approach for species discrimination of fabaceae family on the basis of length variation in pcr amplified products using barcode primers. *Int J Curr Microbiol App Sci* 7:921–928. <https://doi.org/10.20546/ijcmas.2018.712.115>
- Soni HB, Dabhi M, Thomas S (2013) Perspective on phytochemical and biochemical compounds of selected Indian medicinal plants. *Int Res J Chem* 1:37–45
- Spaniolas S, Bazakos C, Spano T, Zoghby C, Kalaitzis P (2010) The potential of plastid *trnL* (UAA) intron polymorphisms for the identification of the botanical origin of plant oils. *Food Chem* 122:850–856. <https://doi.org/10.1016/j.foodchem.2010.02.039>
- Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol Biol* 17:1105–1109. <https://doi.org/10.1007/BF00037152>
- Taberlet P, Coissac E, Pompanon F, Gielly L, Miquel C, Valentini A, Vermat T, Corthier G, Brochmann C, Willerslev E (2007) Power and limitations of the chloroplast *trnL* (UAA) intron for plant DNA barcoding. *Nucleic Acids Res* 35:e14. <https://doi.org/10.1093/nar/gkl938>
- Taberlet P, Gielly L, Pautou G, Bouvet J (2009) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol Biol* 17:1105–1109. <https://doi.org/10.1007/BF00037152>
- Techen N, Parveen I, Pan Z, Khan IA (2014) DNA barcoding of medicinal plant material for identification. *Curr Opin Biotechnol* 103–110. <https://doi.org/10.1016/j.copbio.2013.09.010>
- Tsai L, Yu Y, Hsieh H, Wang J, Linacre A, Lee JC (2006) Species identification using sequences of the *trnL* intron and the *trnL-trnF* IGS of chloroplast genome among popular plants in Taiwan. *Forensic Sci Int* 164:193–200. <https://doi.org/10.1016/j.forsciint.2006.01.007>
- Vijayan K, Tsou CH (2010) DNA barcoding in plants: taxonomy in a new perspective. *Curr Sci* 99:1530–1541
- Wang W, Wu Y, Yan Y, Ermakova M, Kerstetter R, Messing J (2010) DNA barcoding of the Lemnaceae, a family of aquatic monocots. *BMC Plant Biol* 10:205. <https://doi.org/10.1186/1471-2229-10-205>
- Yadav A, Ahmad J, Chaudhary AA, Ahmad A (2012) Development of sequence characterized amplified region (SCAR) marker for the authentication of *Bacopa monnieri* (L.) Wettst. *Eur J Med Plants* 2:186–98. <https://doi.org/10.9734/EJMP/2012/1192>