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Simple Approach for Species Discrimination of Fabaceae Family on the Basis of Length Variation in PCR Amplified Products Using Barcode Primers

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A B S T R A C T

Fabaceae is one of the most diversified and complex family of flowering plants. The important pulses and the medicinally important plant species under this family have a high market value. Now a day, adulteration in the food and herbal medicinal products has become a severe problem. Adulteration of therapeutic herbs and major pulses with related or conflicting species has proved to be hazardous to human health in several cases. We have projected here, a PCR-based method using some of the major universal DNA barcode primers from the plastid region to address this problem. The basic idea behind this study was to utilize the amplicon length polymorphisms exhibited by these primers to differentiate the plant species. PCR amplification success and species discrimination ability of five major DNA barcode primers (*trnH-psbA*, *trnL*, *atpF-atpH*, *matK* and *rbcL*) was studied among 24 representative plant species of Fabaceae family. The results showed that the primers *atpF-atpH*, *trnH-psbA* and *trnL* exhibited amplicon length polymorphism can simultaneously discriminate all the 24 species under study whereas the primers *rbcL* and *matK* produced monomorphic band and hence failed to do so. Differentiation of plant species on the basis of amplicon length polymorphism which can be clearly visualized on agarose gel and does not require sequencing hence it is a noble and cheap approach to discriminate plant species and to check adulteration. This technique can give the way to identify adulteration both in herbal drug formulations as well as in processed food materials.

Introduction

The family Fabaceae specified as pea, bean or legume family, is the third largest family of land plants which is traditionally being divided into three sub families: Caesalpinioideae, Mimosoideae, and Papilionoideae (Lewis *et al*., 2005). After cereals, it is the second most important family in both nutritional and economical aspects. Many plant species of this family are yet to be identified and many are at the point of extinction due to the drastic climatic change and rapid increase in habitat devastation. This

family includes almost all the major pulses like mung, urad, pigeonpea, moth bean, cow Pea, adzuki bean, etc. The plant species under this family with medicinal importance includes Cassia, Senna, Mimosa, Crotoleria etc. which are being extensively used by the therapeutic drug industries so has high demands on the market of herbal medicine. Due to this high demands, exceeding the availability of quality raw materials the major pulses and the herbal products in the market suffers adulteration problems. The major pulses are adulterated with less important and abundantly available weed seeds, oil seeds or damaged pulse seeds of other pulses or less important and non-consumable pulses like Khesari (*Lathyrus sativus*). There are severe hazard to public health, diseases like (Lathyrism) or spastic paralysis, food poisoning and some skin allergies reported several times due to the consumption of such adulterated dal (pulses) (Sudheer *et al*., 2015).

The trade of medicinal plants in India is about 5.5 billion US$. India is an exporter of crude drugs to various countries like USA, Germany, France, Switzerland, UK and Japan. The major crude therapeutic drugs exported by India to these countries are Aconite, Aloe, Belladonna, Acorus, Cinchona, *Cassia tora*, Dioscorea, Digitalis, Ephedra, Plantago and Senna. The presence of various adulterants and toxic components in the Indian herbal drugs which is maximum in wild plants grown in the lack of policy attention, adversely affects the quality as well as the demands of the Indian herbal medicine (Mohammed, 2009).

In today's situation, the identification based on the molecular markers is widely used for biological samples. One of such technique is DNA barcoding which is based on PCR followed by sequencing and analysis of sequenced products (El-Atroush *et al*., 2015). Seven major plastid and nuclear region (*psbK–psbI*, *atpF–atpH*, *trnH–psbA* spacers and *matK*, *rbcL*, *rpoB*, *rpoC1* genes) have been examined by Plant Working Group of Consortium for the Barcode of Life (CBOL) out of this seven barcode region CBOL has recommended *rbcL* and *matK* as the most promising regions along *with trnH-psbA* and ITS as subsidiary barcode region (CBOL Plant Working Group, 2009).

DNA barcoding has proved itself as an ideal technique for the identification of plant species. The technique of DNA barcoding is exclusively used for amplification of target sequence, so the success rate of PCR is the pre-requisite for barcoding success. Then after the successful amplification the sequencing of the PCR product is the next important step in barcoding technique which is quite expensive and time consuming then comes the part of bioinformatics which is required for the analysis of data generated after sequencing. Only to identify the variable species there is a need of an easy and less expensive technique. Vijayan and Tsou (2011) has described about the variability in the amplicon size of all the suggested barcode regions. This property of variability in amplicon length can be used to differentiate different plant genus prior to sequencing which will save the time and expenditure required for sequencing. So this feature of the variability in sequence length is used in the present investigation for identification of different plants of Fabaceae family at genus level by evaluating the polymorphism in the amplicon through agarose gel electrophoresis using five universal barcode primers (*trnH-psbA, trnL, atpF-atpH*, *matK* and *rbcL*) for differentiating 13 different genus of Fabaceae family.

Materials and Methods

Collection and storage of plant material

Seeds of 24 individual plants belonging to 14 different genus from the family Fabaceae were collected from several states of India and

placed individually in envelopes and stored at 4°C.

Extraction of DNA from seed samples

The DNA was extracted by crushing the seeds into fine powder using sterile pestle and mortar. This powder was further processed using the DNeasy® plant mini kit (Qiagen) to get high quality and pure DNA.

The quality and quantity of DNA on each sample was tested using Gel electrophoresis and Spectrophotometer. The isolated DNA samples were stored at -20°C to be used further.

Amplification using barcode primers

The polymerase chain reaction (PCR) was carried out for five universal barcodes. The of PCR reaction mixture of total volume 10 µL containing 1 µL of 10x PCR buffer (Genei), 200 µM each deoxynucleotide, 1.5 mM $MgCl_2$, 1U *Taq* polymerase, 5 pmol primers (Integrated DNA Technologies, USA, Table 1), 1 µL of genomic DNA and the rest was adjusted with deionized water. PCR amplification was carried out at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30s, 55 °C for 30s and 72 °C for 1 min, followed by final extension step at 72 °C for 5 min in a thermal cycler (Agilent). PCR conditions were similar for all the primer-pairs beside the annealing temperature (T) for *matK* (58 °C).

Agarose gel electrophoresis and data analysis

The PCR products were electrophoresed on agarose gel of 1.2% and (20 x 14 cm) in length using 1x TAE buffer containing 0.5 µl/mL ethidium bromide. Gel images were obtained using bio-imaging system (Vilber Lourmat, France). The amplicon size of PCR products resulting from the primer pairs of the specific barcoding gene was determined using a 100bp ladder (Fermentas).

Results and Discussion

Amplification efficiency of all five primers

The amplification efficiency of all the 5 primers were found to be 100% for all 24 individual samples it represents the effectiveness of these primers to successfully amplify and produce single, intact and clear bands within desired range of size for each individual sample. In terms of absolute discriminatory power on the basis of amplicons length polymorphism, *atpF-atpH*, *trnH–psbA* and *trnL* were found to be suitable for species discrimination while, *rbcL* and *matK* were failed to discriminate species under study.

Potential of *atpF-atpH* to discriminate legume species

In the present study the primer pair *atpF-atpH* produced five different types of amplicon based on their size. On the basis of this difference in amplicon size, the thirteen different genus involved in this study can be discriminated in 5 different groups. The different amplicon sizes produced were 300 bp in species *Crotolaria*, 500bp in *Atylosia*, 600bp amplicon size differentiated *Vicia*, *Vigna* and *Cajanus* from the other species and 650bp discriminated *Arachis*, *Mimosa*, *Trigonella*, *Senna*, *Casia*, *Clitoria*, *Tephrosia* and *Alysicarpus* species. This primer discriminated plants at genus level but failed to produce polymorphism at species level.

The *atpF-atpH* is a noncoding intergenic spacer region which can be used as potential DNA barcode region for plant as proposed in the second international barcode of life conference. Similar to our results, amplicon length polymorphism produced by this region

was also revealed in the plant samples of South African Kruger National park flora, in which variable band size ranging from 225-758bp and 218-847bp for different species under study (Lahaye *et al*., 2008) and same variation in the amplicon length was observed in the study conducted on 31 species of family Lamiacea in which the maximum length of the amplicon was 622bp and minimum length was 579bp (Wang *et al*., 2010).

Potential of *trnL* to discriminate legume species

This primer produced three unique amplicon lengths of 400, 600 and 650bp. Based on these variable amplicon lengths, the entire 24 specimens of 13 different genus under the study can be separated into four different groups. The maximum number of samples falls under the group with amplicon length of 600bp (*Vigna, Trigonella foenum-graecum, Crotalaria, Senna, Cassia, Clitoria, Tephrosia, Alysicarpus Mimosa and Vicia*). Another group of amplicon length *i.e.* 650bp contained two plant genus (*Arachis* and *Cicer*), the third group of 400bp amplicon length contained three genus (*Cajanus cajan*, *Trigonella corniculata* and *Atylosias carabaeoides*). This primer was not proved successful to discriminate the samples at species level but it produced polymorphic bands of 400 and 600bp for the two species of *Trigonella*. This primer differentiated all the other samples at genus level.

It is a noncoding spacer region of plastid tRNA intron which has the tendency to evolve more rapidly than other coding regions. This region is most unstable at between species level where as frequently conserved at within species level and abundant insertion and deletions and variable single nucleotide polymorphism make *trnL* (UAA) an potential candidate for identification of several plant species (Taberlet, 2007). James *et al.,* (2004) utilized intron region of *trnL* gene for identification of food crops and observed variation in amplicon length in different food crops ranging from 387 to 642bp in various crops like potato, wheat, soybean etc.

D'yachenko *et al.,* (2015) also observed variation in the amplicon size produced by this region within 16 legume species. They obtained amplicon size ranging from 442 to 555bp and segregating *Phasiolus vulgaris*, *Cicer arietinum* and *Triforium repens* from other legume species.

Potential of *trnH-PsbA* to discriminate legume species

This primer has showed the potential to produce polymorphism at the species level as in the present investigation. It showed slight variation in the amplicon length produced within species. It produced four unique amplicon of variable length of 300, 380, 400 and 500bp.

On the basis of this variation in band size, all the 24 samples can be discriminated into four groups. Maximum amplicon length of 500bp was produced for species (*Cicer arietinum, C. pinnatifidum, Cajanus cajan, Clitoria ternatea, Tephrosi apurpurea, Vicia hirsute)* which discriminated them from all the other species.

This primer showed slight polymorphism at species level and has produced different sized amplicons from 380bp for (*Vigna aconitifolia*, *V. radiate*, *V. unguiculata*, *V. mungo*, *V. trilobata*, *Mimosa pudica, Trigonella corniculata*, *T. foenum-graecum, Crotalaria pallida, Senna occidentalis* and *Cassia sericea*) *and* 400bp for *(Vigna umbellate*, *Crotalaria spectabilis, Sennatora, Atylosias carabaeoides, Alysicarpus vaginalis).* It discriminated the two species of *Arachis* (*A. hypogaea* and *A. monticola*) producing amplicon size of 300bp.

This primer region has proved itself as a promising option for barcoding of land plants due to its properties like high level of PCR success and easy amplification efficiency. It is region of the plastid region of flowering plants (Vijayan and Tsou, 2011).

In the present investigation it also has shown 100% PCR efficiency and produced intact and sharp bands of variable length. This primer has been reported to produce a variable length polymorphism ranging from 296-1120bp (CBOL 2009; Chase *et al.*, 2007). Monkheang *et al.*, (2011) has obtained different size amplicon among fourteen species of *Senna* (400-600bp) using this primer and has called it as species specific marker sequence.

Potential of *rbcL* and *matK* to discriminate legume species

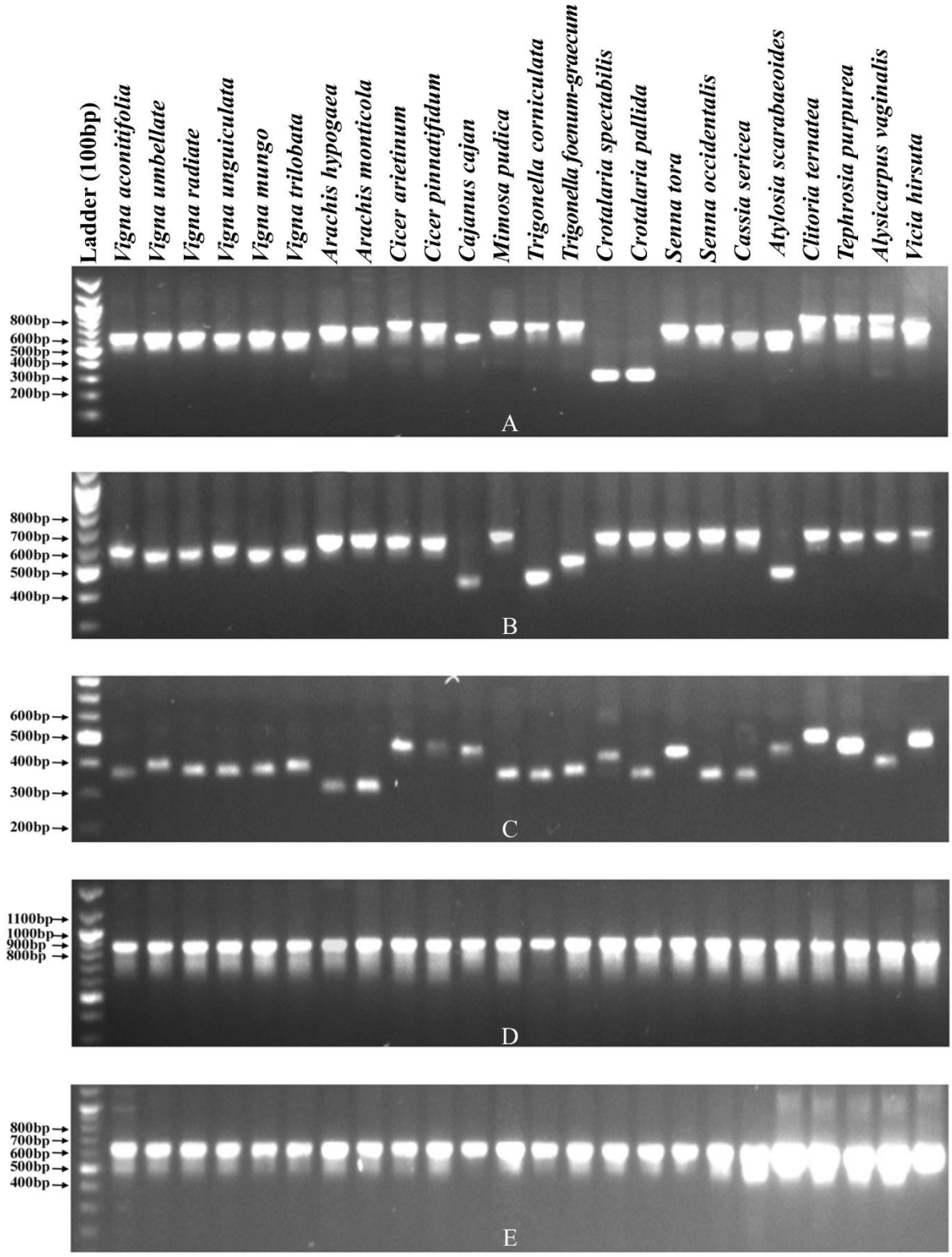
The primers *rbcL* and *matK* are the genes of choloroplast region which are recommended as the two standard DNA barcode region for plants (Bafeel *et al.*, 2001) due to its high rate of evolution. In present investigation, *rbcL* and *matK* produced monomorphic bands of 650bp and 850bp respectively.

In this way, these primers were unable to discriminate the samples at genus as well as species level. Zhang *et al.*, (2011) also obtained similar monomorphic bands of 648bp and 763bp using primer pairs of *rbcL* and *matK* respectively.

Table.1 List of plant species from Fabaceae family and their collection sites

Species	Collection site
Vigna aconitifolia	Jabalpur
Mimosa pudica	
Vigna umbellate	Samastipur, Bihar
Vigna unguiculata	Local Farm, Jagdalpur, Bastar (C.G.)
Arachis monticola (acc. ICG 8135)	International Crops Research Institute for the Semi – Arid Tropics, Hyderabad
Cicer pinnatifidum (acc. ICC 17153)	
Cajanus cajan (cv. Laltondi)	Jabalpur, Harda (M.P.)
Trigonella corniculata	Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur
Trigonella corniculata	
Vigna radiata	
Senna tora	ICAR- Directorate of Weed Research, Jabalpur
Senna occidentalis	
Vigna trilobata	
Cassia sericea	Shaheed Gundadhur College of Agriculture & Research Station, Jagdalpur (C.G.)
Atylosia scarabaeoides	
Clitoria ternatea	
Tephrosia purpurea	
Alysicarpus vaginalis	
Arachis hypogia (var. KH-4)	
Vigna mungo (var. Barkha) and (var. Kala urad)	
Cicer arietinum (var. C- 170)	
Crotalaria spectabilis	

Crotalaria pallida	



In conclusion, the present investigation demonstrated about the discrimination ability of five barcode primers namely *atpF-atpH*, *trnL*, *trnH–psbA*, *rbcL* and *matK* on the basis of variable lengths of amplicon. We found three primers *atpF-atpH*, *trnL*, *trnH–psbA* to show polymorphism at species and genus level. These three primers in combination or singly can be used successfully to differentiate important plant samples both in processed and fresh form on the basis of the difference in amplicon length. This method can prove very useful for accurate discrimination and validation of adulterated and processed medicinal therapeutic products and food products as well as fresh plant samples. This method is simple, cheap and time saving compared to several other existing molecular techniques.

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