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ORIGINAL ARTICLE

Molecular Authentication of *Trichosanthes* Species Traded as "Patola:" An Ayurvedic Drug Resource

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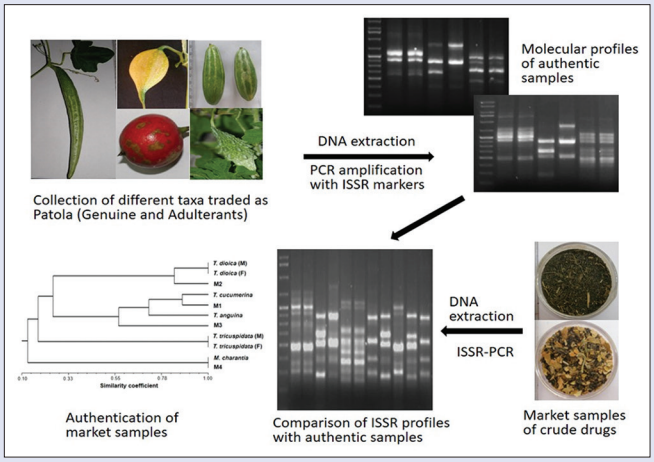
ABSTRACT

Background: Different interpretations of ayurvedic texts have recognized/recommended taxonomically distinct species/varieties of *Trichosanthes* as "Patola," which is used extensively in the Indian traditional system of medicine for curing a variety of disorders. Sources of "Patola" also vary depending on region-specific availability and diverse traditions in different parts of India, which creates ambiguity in correct identity of "Patola." Since "Patola" is one of the medicinal plant species traded in high volumes, its exact taxonomic identity and authentication is very essential. **Objectives:** Molecular profiling of different taxa recognized as "Patola" was carried out for correct identification and authentication of commercial crude drug samples. **Materials and Methods:** Molecular marker profiles of different taxa recognized as "Patola" were generated using inter-simple sequence repeat (ISSR) markers. Attempt was also made to authenticate commercial crude drug samples, using ISSR markers. **Results:** Selected 10 polymorphic ISSR primers generated distinct amplification profiles for the taxa traded as "Patola." Moreover, two varieties of *Trichosanthes cucumerina* (var. *cucumerina* and var. *anguina*) could be clearly distinguished based on the specific bands produced by primers UBC 812, 822, 887, 888, and 889. Molecular profiling of market samples along with the genuine plant samples confirmed the trade of different *Trichosanthes* species as well as *Momordica charantia* under the name of "Patola." **Conclusion:** To our knowledge, this is a first report on the application of ISSR markers for identification and authentication of "Patola." Developed DNA profiles should be useful in investigations of adulteration in market samples and establishment of correct identity.

Key words: "Patola," authentication, DNA profiling, inter-simple sequence repeat markers, *Trichosanthes* spp.

SUMMARY

- In the present work, ISSR markers are applied for characterization of different *Trichosanthes* taxa recognized under the name "Patola" as well as for authentication of commercial crude drug samples. Based on screening of ISSR primers for polymorphism, 10 primers were selected which could distinguish between the taxa traded under the name "Patola". ISSR primers UBC 812, 822, 887, 888, and 889 produced specific bands in two varieties of *Trichsanthes cucumerina* and were useful to differentiate between var. *cucumerina* (bitter) and var. *anguina* (non-bitter). Comparative molecular profiling of market samples along with the genuine plant samples confirmed the trade of different *Trichosanthes* species as well as *Momordica charantia* under the name of "Patola/Ran padval". The results confirm the utility of ISSR marker based DNA profiles in investigations of adulteration and establishment of correct identity of crude drug samples.



Abbreviations used: AFLP: Amplified fragment length polymorphism; AHMA: Agharkar Herbarium at Maharashtra Association; DNA: Deoxyribonucleic acid; ISSR: Inter-simple sequence repeat; MC: *Momordica charantia*; PCR: Polymerase chain reaction; RAPD: Random amplified polymorphic DNA; TAE: Tris acetate-EDTA buffer; TC: *Trichosanthes cucumerina*; TCA: *Trichosanthes cucumerina* var. *anguina*; TCC: *Trichosanthes cucumerina* var. *cucumerina*; TD: *Trichosanthes dioica*; TT: *Trichosanthes tricuspidata*; TE: Tris-EDTA buffer; UBC: University of british Columbia, Vancouver, Canada.

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INTRODUCTION

"Patola" is used extensively in the Indian traditional system of medicine for curing a variety of disorders. Its use can be traced back to the time of Charaka and Sushruta, around 2000 years ago.[1] It is one of the ingredients of ayurvedic preparations such as Gulguluthikthakam kashayam, Mahatiktaka ghritam, Mahatiktam kashayam, and Vajrakam kashayam.[2,3] The commercial herbal medicines such as Gulguluthikthakam ghee, Patoladi yoga, and Patolapatra capsules which contain Patola extract or powder are recommended for purifying blood, skin disorders, fever, and burning sensation of the body.[4]

Different interpretations of ayurvedic texts have recognized/recommended taxonomically distinct species/varieties of *Trichosanthes* under the name "Patola." Sources of "Patola" also vary depending on region-specific availability and diverse traditions

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within India, which creates ambiguity in correct identity of "Patola." For example, in North India, *Trichosanthes dioica* (TD) is used as "Patola," whereas in South India, *Trichosanthes cucumerina* (TC) (var. *cucumerina* and var. *anguina*) is used.[2,5] Occasionally, *Trichosanthes tricuspidata* (TT) is also used as a source.[6]

Among the taxa traded under the name "Patola," TD and TC var. *anguina* are non-bitter and edible while TC var. *cucumerina* and TT are bitter and nonedible. In the crude drug market, these bitter species/varieties are sold under the same trade name with suffix "bitter."[7] It is difficult to identify the sources when crude drug material is in dried, crumpled, and powdered form. The comparative macroscopic and organoleptic characters are not sufficient to establish exact identity of the species.[1] Since "Patola" is one of the medicinal plant resources traded in high volumes (estimated annual trade 500–1000 MT/year), its exact taxonomic identity and authentication is very essential.[5]

The quality of any medicinal plant product can be affected by many factors. Chemical analysis is reliable way to determine the alteration or adulteration in evaluating the quality of medicinal plant/herbal products.[8] Whereas molecular authentication using DNA sequences can successfully identify interspecies variation as well as adulterants.[9] A number of polymerase chain reaction (PCR)-based markers such as amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and inter-simple sequence repeat (ISSR) have been used to characterize medicinal plants.[10,11]

Among various markers, ISSR-based amplification is a quick and reliable technique which has been used extensively for phylogenetic studies, genetic diversity, and cultivar identification in a number of plant species.[12] They are also proven to be versatile molecular markers for assessing genetic relatedness as well as identification and authentication of medicinal plants.[13] In the present work, ISSR markers are applied for characterization of different taxa recognized under the name "Patola" as well as for authentication of commercial crude drug samples.

MATERIALS AND METHODS

Plant material

Young and healthy leaves from the taxa considered as "Patola" were collected at flowering/fruiting stage from different localities in Maharashtra, India. After identification, voucher specimens were deposited in Agharkar Herbarium at Maharashtra Association (AHMA), Agharkar Research Institute, Pune [Table 1]. For DNA extractions, leaf samples were frozen in liquid nitrogen and stored at −70°C till the use.

Market samples sold under the name of Patolah/Patol/Potol/Padval/Ran padval/Jangli padval were procured from eight different crude drug markets, namely, Akola, Dhule, Kolhapur, Mumbai, Pune, Ratnagiri (all from Maharashtra), Anand (Gujarat), and Lucknow (Uttar Pradesh). The market samples were checked for moisture and contamination and stored at room temperature in airtight containers till further analysis. Bitter gourd, *Momordica charantia* (MC) which is commonly admixtured with Patola, was also included in the analysis of market samples.

DNA extractions and inter-simple sequence repeat analysis

The total genomic DNA was extracted by modified cetyltrimethylammonium bromide method.[14] The DNA pellet was washed with 70% ethanol, air dried, dissolved in TE buffer, treated with RNase, and reprecipitated. The concentration of DNA samples was determined by comparison of band intensity with different concentrations of standard lambda DNA (Bangalore Genei, India) on 1% agarose gel stained with ethidium bromide. After quantification, the DNA samples were diluted with TE buffer to a working concentration of 5–10 ng/µl for PCR analysis. The same protocol was also used for DNA extractions from market samples.

Inter-simple sequence repeat–polymerase chain reaction amplification and agarose gel electrophoresis

For ISSR analysis, 94 anchored microsatellite primers from set #9 procured from University of British Columbia (UBC), Vancouver, Canada, were used. PCR amplifications were performed in 25 µl reaction volume in Veriti 96-well thermal cycler (Applied Biosystems, Foster City, USA). The composition of PCR reactions and thermal cycling conditions were as described in Tamhankar *et al.*[12] To ensure reproducibility of the amplifications, PCR reactions were repeated at least three times for each primer. Amplified products were separated on 1.5% agarose gels in 0.5X TAE buffer using GeNei™ Low Range DNA Ruler Plus (Merck Specialities Private Ltd., India) as the molecular weight marker and visualized on ultraviolet transilluminator (Fotodyne Incorporated, USA) after ethidium bromide staining. The gels were photographed and documented for further analysis.

Band scoring and data analysis

Only well-separated, reproducible, and intense bands that were observed in all the three independent amplifications were selected for scoring. Since ISSR markers behave as dominant markers, each ISSR band was considered as a character, and the presence or absence of the band was scored in binary code (present = 1, absent = 0). Pairwise comparisons between the taxa were made using the Dice coefficient.[15] Based on the similarity matrix, a dendrogram was constructed using unweighted pair group method with arithmetic averages.[16] All these analyses were performed with SAHN module in NTSYS pc 2.1 package (Exeter Software, Setauket, New York, United States of America).[17]

Table 1: Collection details of the taxa in "Patola"

Species	Short name	Locality	Voucher number AHMA
T. dioica Roxb. (male)	TD (male)	Nashik	25,493
T. dioica Roxb. (female)	TD (female)	Nashik	25,495
T. cucumerina var. *anguina* (L.) Haines	TCA	Kankavali	27,477
T. cucumerina var. *cucumerina*	TCC	Kankavali	27,006
T. tricuspidata Lour. (male)	TT (male)	Kolhapur	27,410
T. tricuspidata Lour. (female)	TT (female)	Kolhapur	27,414

AHMA: Agharkar Herbarium at Maharashtra Association; *T. dioica*: *Trichosanthes dioica*; *T. tricuspidata*: *Trichosanthes tricuspidata*; *T. cucumerina*: *Trichosanthes cucumerina*

Table 2: Inter-simple sequence repeat primers selected for profiling of "Patola"

Primer	Sequence	Number of bands
808	5'-$(AG)_n$C-3'	10
809	5'-$(AG)_n$G-3'	12
812	5'-$(GA)_n$A-3'	08
813	5'-$(CT)_n$T-3'	08
818	5'-$(CA)_n$G-3'	10
836	5'-$(AG)_n$YA-3'	11
842	5'-$(GA)_n$YG-3'	07
887	5'-DVD $(TC)_n$-3'	10
888	5'-BDB $(CA)_n$-3'	20
889	5'-DBD $(AC)_n$-3'	08
	Total	104

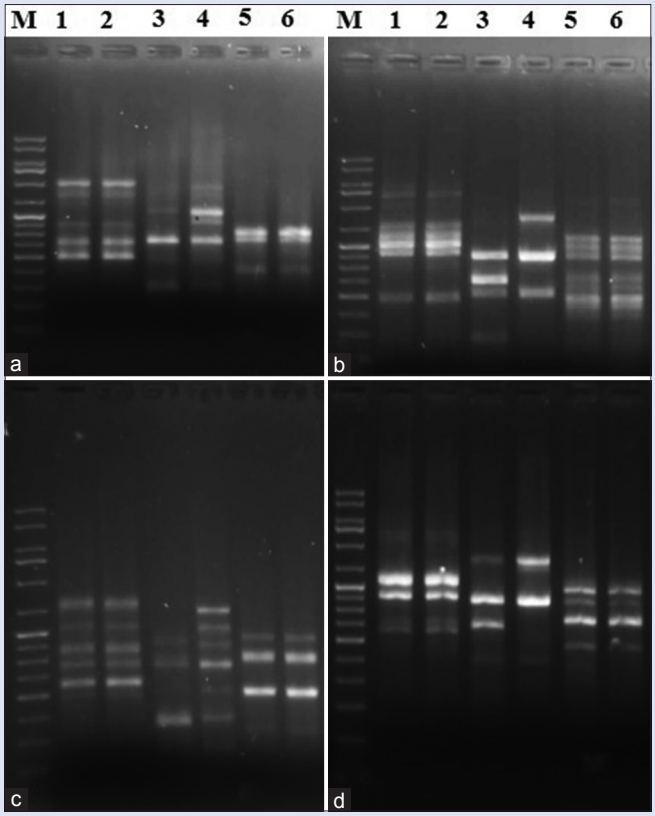

Figure 1: Inter-simple sequence repeat amplification profiles for *Trichosanthes* spp. considered as "Patola" using primers 808 (a); 887 (b); 888 (c), and 889 (d). M: Low Range DNA Ruler Plus; 1: TD (male); 2: TD (female); 3: TCC; 4: TCA; 5: TT (male) and 6: TT (female). TD: *Trichosanthes dioica*; TCC: *Trichosanthes cucumerina* var. *cucumerina*; TCA: *Trichosanthes cucumerina var. anguina*; TT: *Trichosanthes tricuspidata*

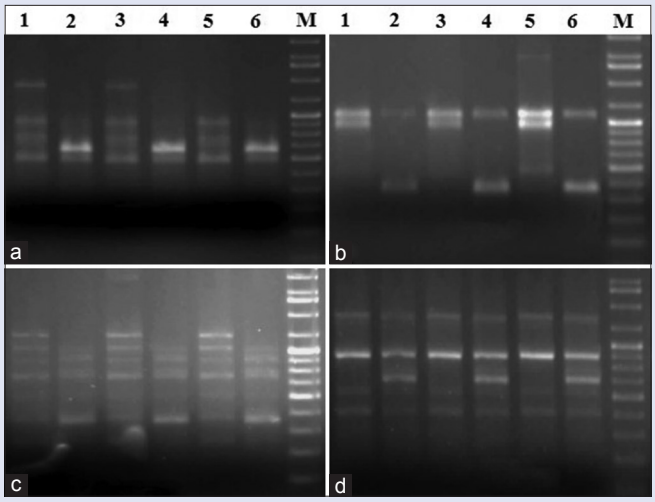

Figure 2: Inter-simple sequence repeat amplification profiles showing specific bands in TCC and TCA primer UBC 812 (a); 822 (b); 887 (c), and 889 (d); M: Low Range DNA Ruler Plus; 1, 3, 5 TCA samples collected from 1: Amboli, 3: Kankavali, and 5: Junnar; 2, 4, 6 TCC samples collected from 2: Akola; 4: Kankavali, and 6: Kolhapur. TCA: *Trichosanthes cucumerina var. anguina*; TCC: *Trichosanthes cucumerina* var. *cucumerina*

Table 3: Diagnostic markers for identification of two varieties of *Trichosanthes cucumerina*

Variety	Markers showing specific bands*
T. cucumerina var. *anguina*	UBC $812_{718;\ 883,1474}$; UBC 822_{960}; UBC 887_{950}
T. cucumerina var. *cucumerina*	UBC 812_{624}; UBC 822_{456};UBC 888_{632}; UBC 889_{685}

*Primer number followed by molecular weight in base pairs (bp) in subscript.
T. cucumerina: *Trichosanthes cucumerina*

RESULTS

DNA profiling of the taxa

The samples of the taxa considered as "Patola" were collected from different locations of Maharashtra. Identified plant samples were used to develop comparative molecular profiles using ISSR markers. Initially, total 94 primers (UBC 807–900) were screened for their ability to amplify DNA fragments using six samples. Out of the 94 primers tested, 68 did not amplify any fragments; 10 produced either smear or faint bands on a highly smeared background; and remaining 16 generated band patterns. Based on the clear, scoreable, and reproducible band patterns, 10 primers were selected for further profiling work [Table 2].

A total of 104 bands were scored from the selected 10 primers. The profiles generated by selected markers did not show any differences in male and female plants of TD as well as in TT. All the selected polymorphic primers contained dinucleotide repeats and generated distinct band profiles for selected *Trichosanthes* spp. It was possible to distinguish between TD, TC (TC var. *cucumerina* [TCC] and TC var. *anguina* [TCA]), and TT based on the profiles. The amplification profiles generated by primers UBC 808, 887, 888, and 889 are shown in Figure 1. The sizes of the amplified products ranged from 400 to 1815 bp. The number of bands varied from 7 (UBC 842) to 20 (UBC 888).

Primers UBC 812, 822, 887, 888, and 889 produced specific bands which clearly distinguished the closely related varieties of *Trichosanthes cucumerina* (TC), i. e. TC var. *anguina* (TCA) and TC var. *cucumerina* (TCC) from each other. To validate the specificity of the bands, DNA samples of three sets of well-identified TCC and TCA samples collected from different locations from Maharashtra (Akola, Amboli, Junnar, Kankavali, and Kolhapur), India, were amplified using primers UBC 812, 822, 888, and 889 [Figure 2]. The results confirmed the presence of diagnostic bands, thus indicating that primers UBC 812, 822, 888, and 889 could be used as for correct identification and distinguishing these two varieties. Specific bands used for identification of these varieties are listed in Table 3.

Molecular marker analysis of market samples

Market samples collected from eight different markets were processed/checked for moisture and foreign matter percentage as per pharmacopoeial norms. Samples from Akola and Anand contained a higher amount of moisture while samples from Dhule, Mumbai, Ratnagiri, and Anand exceeded the limit of % foreign matter. The higher percentage of these parameters indicated the inferior quality of crude drug. However, in an attempt to use DNA profiles for authentication of Patola market samples, the amplification profiles of DNAs extracted from the market samples were compared with the profiles of selected three authentic species of *Trichosanthes* along with MC which is very commonly admixtured and sold as "Patola." The quality of DNA extracted from market samples was not good and visible on the agarose gel as smear. Out of eight samples, only four samples (from Akola, Lucknow, Pune, and Mumbai) could be consistently amplified, showed clear and reproducible band pattern, and hence were used for further analysis. The representative amplification profiles are presented in Figure 3A, and the dendrogram based on the amplification profiles is

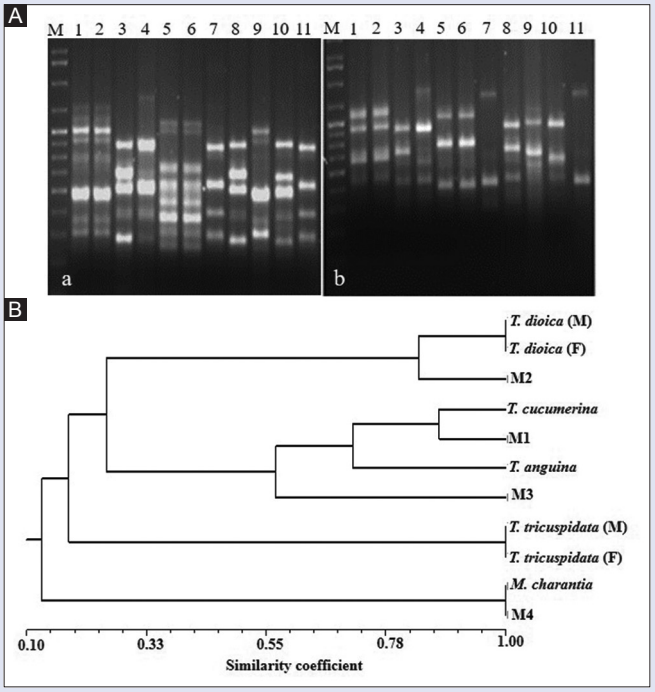

Figure 3: Analysis of market samples (A) comparative inter-simple sequence repeat profiles for identification and authentication of market samples. M: Low Range DNA Ruler Plus; 1–7 authentic samples of 1: TD (male); 2: TD (female); 3: TCC; 4: TCA; 5: TT (male); 6: TT (female); 7: MC; Samples 8–11 market samples from 8: Akola; 9: Lucknow; 10: Pune; 11: Mumbai (B) dendrogram of genuine and market samples of "Patola." TD: *Trichosanthes dioica*; TCC: *Trichosanthes cucumerina* var. *cucumerina*; TCA: *Trichosanthes cucumerina anguina*; TT: *Trichosanthes tricuspidata*; MC: *Momordica charantia*

shown in Figure 3B. Market sample from Lucknow (M2) showed more than 70% similarity with genuine TD, and sample from Akola (M1) sample showed 76% similarity with genuine TCC. The market sample from Pune (M3) was distinct but grouped in the same clade along with TCA and TCC with 40% similarity. However, market sample from Mumbai (M4) showed 100% similarity with MC.

DISCUSSION

Utility of inter-simple sequence repeat markers to distinguish between different taxa considered as "Patola"

In the present study, 94 ISSR primers were screened and 10 were selected to generate amplification profiles for all the *Trichosanthes* species considered as "Patola." All the selected primers generated distinct amplification profiles which could distinguish between the taxa traded under the name "Patola." ISSR markers have been successfully used earlier for DNA profiling of many crude drugs.[12,18-20] Moreover, primers UBC 812, 822, 887, 888, and 889 produced specific bands in TCC and TCA and were useful to differentiate between both the varieties of TC (TCC and TCA). The observation was further validated using samples collected from multiple locations confirming that these bands would be useful as diagnostic markers to identify and differentiate these two varieties. In the recent revision of family Cucurbitaceae, these taxa are not recognized as separate varieties and are merged under single species TC. However, wild variety *cucumerina* bears bitter fruits and is used in traditional systems of medicine while var. *anguina* is cultivated and used mainly as vegetable. The use of these diagnostic markers will be useful for authentication of bitter and non-bitter forms. To our knowledge, this is a first report on the application of ISSR markers for identification and authentication of these plant species.

Authentication of market samples using marker profiles

The molecular profiling of market samples and their comparison with the genuine plant samples confirmed the trade of different *Trichosanthes* species as well as MC under the name of "Patola/Ran padval" in Maharashtra as well as some other parts of country. The sample procured from Lucknow was closest to genuine TD confirming the use of TD as Patola in the northern parts of the country. Two out of the three samples procured from Maharashtra showed 40%–80% similarity with TC var. *anguina* and *cucumerina* and were grouped in the same cluster while the market sample from Mumbai was 100% similar to *M. charantia* based on the DNA profiles. The study thus demonstrates the utility of ISSR markers for authentication of market samples and also substantiates the need for standardization of medicinal plant resources.

CONCLUSION

To our knowledge, this is a first report on the application of ISSR markers for identification and authentication of *Trichosanthes* species considered as "Patola." The present study confirms that different *Trichosanthes* species are traded under common name "Patola/Kadoo patolam/Ran Padval" and supports previous reports. Developed DNA profiles should be useful in investigations of adulteration in market samples and establishment of correct identity.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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