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A New Approach for Tracing Adulteration of Saffron with Safflower by Universal Barcoding Primers

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Abstract

Saffron adulteration dates back to antiquity a fraudulent act continued to modern day. In recent years the wide use of saffron in medicine and food has been associated with wider use of counterfeit to saffron, nevertheless this has been associated with development of methods to detect such adulteration.

The advancement of molecular biology methods has been one of key area used for detection of adulterants. The concept by its own is diverse and different approaches for better detection of adulterant has been developed to support detection of fraudulent activities in particular in food and agriculture industry.

To develop primers for detection of saffron adulteration with safflower we designed primer pairs based on universal reference sequence for chloroplast trnl(UUA) Intron that are different in length and size for saffron and sawfflower.

The approach was to save tedious work using otherwise RAPD /SCAR (sequenced characterized amplified region)markers to have accurate reproducible results with least interference and higher level of polymorphism of particular and specific conserved region in plants.

Key words: Saffron, the *Crocus sativus,* adulteration, safflower.

Introduction

Saffron exact origin is a matter of controversy, but most certain has originated from Iran, Greece and later spread over to Mediterian, India, and china. (1, 2). Saffron is a triploid,male-sterile species not known to be wild or spontaneous (3). It enjoys certain, agronomic, eco-physiological features including a relatively low water use, growth and development during fall and winter. It has very low harvest index as its cultivation, collection and handling practices, calls for careful and special attention, making it a direct and high labor and most precious agriculture commodity (4, 5). Saffron precious value should be sought in its limited production and wide spread application. Saffron is mainly used in food and pharmaceutical industries, two life-dependent and ever expanding markets although,textile industries too, does exploits saffron as a dye nevertheless it is used even in perfumery(6).

Saffron limited resources, and increasing demand, commands its high value and does justify the inexcusable alternative to compensate its short supply with deliberate substitution or adulteration with other materials. Saffron is known for its color, aroma and flavor providing grounds for easiest fraudulent activity which is use of synthetic dyes to increase the coloring strength of its aqueous extract, an act in violation of rules and regulations in most of the countries .The other fraudulent activities include substitution of other

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plants with saffron, such as *Carthamustinctorious* or safflower, *Calendula officinalis* or marigold, arnica and tinted grasses,besides mixing of beet and pomegranate fibers,are other frequent adulterants. The other practice in saffron fraudulent activities is to increase product mass by mixing yellow stamens of saffron with saffron stigma or powder, nevertheless amongst all these, most common fraudulent activity is substituting safflower petals with saffron stigmas (7).

There are various methods used for detection of adulterants in saffron, ranging from physical and microscopic examinations to spectroscopy method, in addition to high performance liquid chromatography which enjoy high precision and accuracy.The coloring property of saffron resulted from its various pigments has been applied in chromatography and spectroscopy methods for detection of some adulterants in saffron. Although the detection and sensitivity of HPLC method makes it the most accurate and acceptable method for detection of adulterants, the cost of methods compared to other methods are quite high.(6)

The international ISO-standard 3632-2 and its technical specification ISO/TS 3632 covers procedures for all the above methods which specify and define saffron taste, fragrance,color, moisture, minerals, exogenous dyes and foreign materials for authenticity and grading of saffron. Recent publications and comments over this ISO standard,highlights weak reliability of some of the mentioned methods and high cost of reliable methods to an extent that some of the researchers have suggested use of different methods for same sample to increase the reliability of results(8).

Advancement of molecular biology and DNA based techniques have initiated new approaches for detection of adulterants specifically for agriculture commodities.The advantages associated with DNA based techniques are important because of their application to all living organism(9).Unique genetic composition of each organism and their consistency and robustness in various physiological and environmental conditions makes them the most reliable elements for identification of organisms. Methods based on genetic materials have solved the problems made by age, degradation, and storage conditions of samples (9, 10).

The DNA basedtechniques could be classified into three types, namely polymerase chain reaction (PCR), sequencing and hybridization based, while the most robust, simple, sensitive, specific, rapid of all them is polymerase chain reaction (PCR), Which requires inexpensive markers, making it most cost-effective among DNA based methods compared to instrumented based methods, it is quite accurate,and accepted as an economical approach in food authentication (9).

The polymerase chain reaction amplifies specific DNA regions directed by oligonucleotide primers into easily detectable level, specifically in case of agricultural products.The main question in PCR reaction is how well a DNA region is characterized to serve the purpose of reaction and how accurate, specific and sensitive that region is reproduced along with oligonucleotide primers. Various PCR-based methods used for adulterants detection and authentication have been developed to increase the quality of detectionas well as the specificity and sensitivity of method reaction including PCR with arbitory primers (AP-PCR), PCR-RFLP (restriction fragment length polymorphism), RAPD and its modified improved version, SCAR marker, but the core issue has yet remained that how best and accuratein shortest time period particular region amplification is achieved. (1, 11, 12).

To initiate a DNA based method for identification of species, DNA based barcoding concept was developedfar beyond morphological classification in taxonomy.The application of barcodingis quite wide in different fields, forensic science, biotechnology, food industry, animal diet and many other areas.This concept uses standardized DNA region in specific loci as a tagfor species identification.DNA barcoding as an identification technique enjoys several advantages,

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including feasibility of performances even with degraded material, low DNA requirements, simplicity, rapidness, time and cost effective protocols, in addition to reproducible results between laboratories(13).

In wake of economic globalization and new trade approaches, authentication of food and its related products for food safety has been one of the most debated issues in recent years. Increasing the usage of agricultural products with dual application both as food and herbal medicine has strengthened concerns over food authenticity and safety. This has been one of the prime reason for exponential research in food analysis, an indispensable tool for authentication of food and food products, particularly in case of food which have dual or numerous biological applications like saffron (14, 15).

To develop an accurate, specific, primer sequence for authentication of saffron free of safflower in a time and cost efficient way, we used an international recommended standard sequence of chloroplast trnL (UAA) intron as template. This standard sequence has been recommended in ISO standard 21569 for plant authentication from other biological samples. The difference of nucleotide sequence in trnL (UAA) intron region of saffron and safflower implicated in their length was used to design primer sequence for each. These primer sequences were used to trace safflower if mixed with saffron by their difference in length sequence.

Material and Methods:

Plant materials: To ensure pure saffron sample for genomic DNA extraction ,we collected sample directly from harvesting site in Qanat city of Khorsan province with certificate of analysis for approval of its authenticity by HPLC. For safflower we collected the flower from Agriculture research center in Karaj of Alborz Province.

DNA extraction: Total genomic DNA was isolated using CTAB protocol of ISO standard 21571.

Designing of Specific Primers: For differentiation of saffron with safflower Multiplication of chloroplast trnl gene (UAA) intron.

To design specific primers for tracing adulteration of saffron with safflower samples of both plants were amplified with specific region of chloroplast trnl gene (UAA) intron referred in Gene Bank with accession No. Z00044, X1590 and referred in ISO standard 21589 whose amplification protocols has been specified in Table-1.The resultant PCR reaction was a 500 bp product as shown in Fig. 1 and expected by the said protocol of ISO 21589.

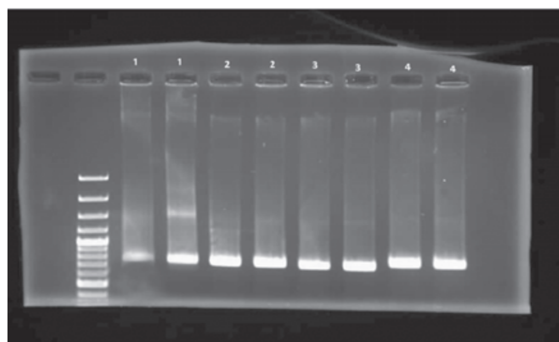

Fig. 1. The result of PCR products of universal trnl gene (UAA) Intron in four different Plants namely Pistachio, Favabean, safflower, Saffron having 500 bp nucleotide sequence.

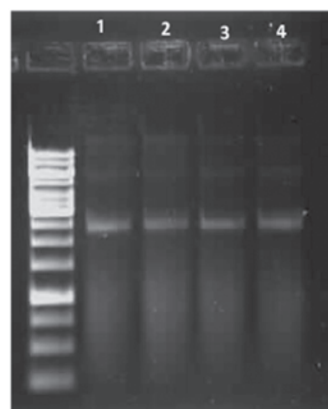

Fig. 2. Plasmid extraction.

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DNA Sequencing : Amplified PCR products were separated electrophoretically in 2% agarose gel, excised from the gel,and purified with IBRC extraction kit (MBK0061) and cloned with PGEM-T easy Vector (promega Crop, Madison, WI, USA). The transformed bacterial colonies were screened. Two colonies were cultured in LB medium and thereafter plasmid extraction was done with the help of IBRC kit seen in the Fig. 2 and extracted colonies were sent for sequence analysis to GATC company in Germany.

Primer design and optimization of PCR conditions: Specific primers for identification of saffron and safflower were designed according to nucleotide based difference between the two amplified trnl PCR products using programs, bio-Edit and prime blast. The primer sequence and their amplicon length' (Fragment size) are given in table 2.

Results

To evaluate the application of designed primers their validation was necessary. The validation of primers designed had to be sought in its properties and ability to trace safflower in saffron packing.

Validation of designed primers: A multiplex PCR program whose protocol is given in table-3 was set up to evaluate species specificity performance of primers and to rule out cross reaction with most common plants.

The target sequences are found in the plants chloroplasts and no sequence similarity should be seen in non-plants organisms,so amplification of both primer pairs were done with DNA extracted from human blood.

The primers were designed to amplify Crocus Sativus. (saffron) and C. Officinalis (safflower). To rule out cross reaction of the designed primer pairs with other plants DNA these primer pair were amplified with pistachio,corn, walnut and pepper. As seen in the Fig. 3 there were no cross reaction between primer pairs with DNA of the selected plants.

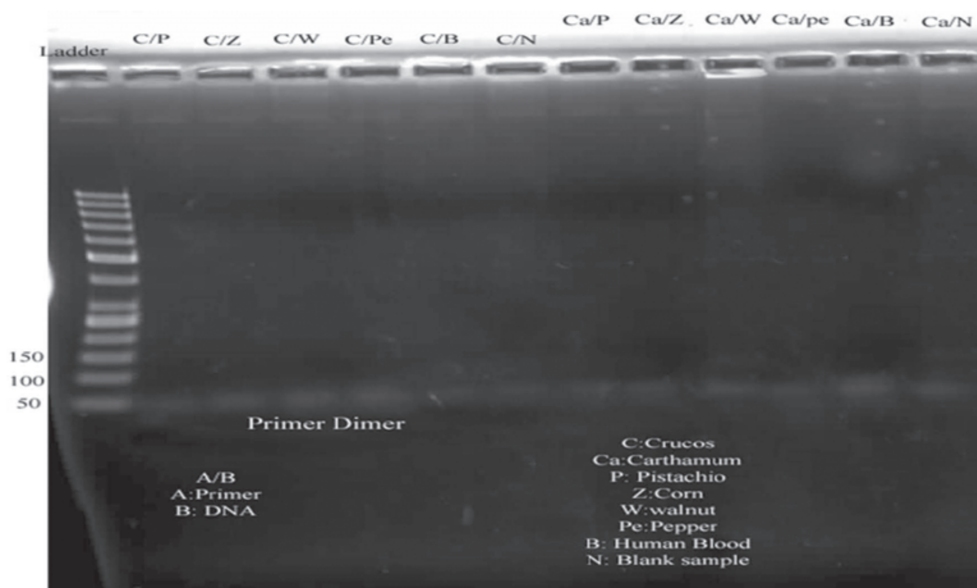

Fig. 3 : Evaluation of cross reaction of both of the designed primers with other plants DNA and non related Species DNA.

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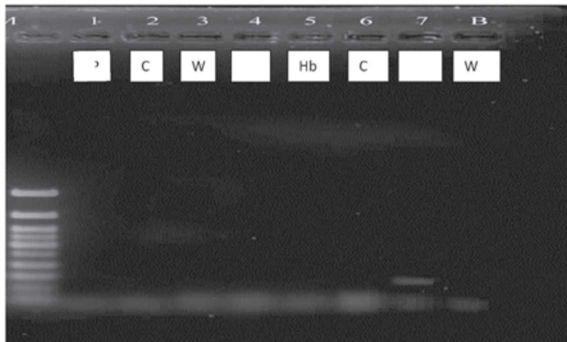
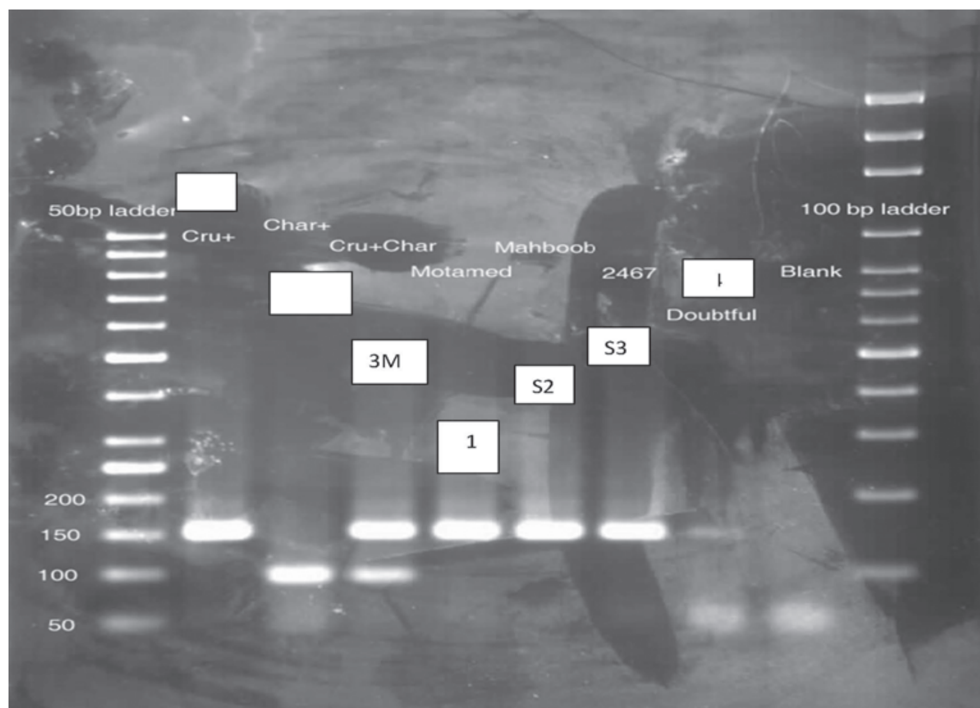


Fig. 4. Validation of primers specifity to rule out common adulterant interference of saffron along with other plants DNA. P- Pistacho C- Corn W-Walnut PE-Peper Hb-Human Blood C- Calendula officinalis T-Tumeric Powder W-water

As the difference of amplicon length was a discriminatory factor in different plants, the DNAs extracted from, pistachio, corn, marigold, pepper, human blood, and turmeric powder were amplified by the designed primers in order to evaluate the specificity of primers by the expected length of PCR product as shown in image 4. As shown in image 4 other than turmeric powder non of the primers gave band with the sample and the band for turmeric powder was in the vicinity of that of saffron but not in the same location.

To validate application of primer pair for tracing adulteration of saffron with safflower. We analyzed the designed primer reaction with commercial packed samples from the market. The image-5 shows the results. As Shown in



Fgi. 5. A market survey results for primer and PCR protocol confirmatory ability to trace adulteration of saffron. 1S- Pure Saffron sample 2F-Pure Safflower 3M-Mix sample of saffron and safflower .-S1sample one with trade name Motamed S2 sample with trade name Mahboob.S3-Unknown trade name sample with laboratory identification code.S4-Dyed inferior Saffron Sample.

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image samples have been designated with S and differentiated with numbers. The most important sample was 4th sample as it was saffron which had been dyed with edible colors to compensate for inferior quality of saffron packed where its PCR product was faint but enough to trace the sample as saffron.

Discussion

In this research we developed two pairs of primers from a conserved well recognized region in chloroplast (trnl),in order to detect safflower as an adulterant in saffron samples. The trnl region in chloroplast which is unique gene, in the plants, has been introduced in ISO standard 21569 as a reference sequence for confirmation of DNAs extracted from plants which has been validated and documented the oretically and experimentally by testing in various samples of plants and non-plants. However, we did trace and found a difference in the length of the mentioned sequence between safflower and saffron, which was used for detection of safflower in saffron as an adulterant.

The international standards for saffron (ISO/ TS 3632) consist of two parts.The standard has been drafted and approved by ISO technical committee ISO/TC 34 SC7 spices and condiments. The second part (ISO/TS 3632-2) covers test methods, for evaluation of quality and authenticity of saffron.The standard covers macroscopic and microscopic examinations as well as physical and chemical testused to specify the quality of saffron.

As number of reviews and research papers have indicated the chromatographic and spectroscopy methods have limitation in detection of adulterants since phytochemical in plants species varises with growing conditions harvesting periods, post harvest process and storage conditions a reason which could be misleading results if the sample has been adulterated with same adulterant compound (15, 16).

The UV-visible spectro photometric test methods such as TLC and HPLC,are used to characterize the phytochemical properties of saffron similar to many other standard test methods for authentication of medicinal plants. Sabestinia and etals have indicated that UV-Visible spectrophotometric method could not specify nature and type of adulterants,and it has some limitations to distinguishing blended adulterants with saffron below a certain w/w percentage.The adulterants could not be specified clearly by the TLC procedure stated in ISO/TS 3632-2 which has been developed based on the phytochemical specifications, so false results may be obtained by this method affected by age or storage conditions of samples. HPLC method has been designated by researchers as the most accurate method due to its ability to trace most colors used as adulterants of saffron due to their appearance and coloring properties. Sabestinia and etals have pointed out that HPLC/PDA/MS technique allows the unequivocal identification of adulterant characteristic marker molecules based on the values of absorbance and mass. Even though HPLC/PDA/MS method has been praised for accuracy and precision for detection of adulterants, it has been realized that this method is not easily available because of to its equipment basis and expensive instrumentation not preferable for field activities.

Although chemical methods based on phytochemical properties of saffron and spectro photometric absorption properties of additive colors provide useful clues for tracing adulterants, they can not provide sufficient and strong evidences to identify the type of the adulterants. (Anna Torelli and etals). Moreover, chemical finger printing could be influenced by various factors including age of sample, physiological conditions, environmental factors, cultivation area, harvesting period, drying and storage conditions (9).

The advancement of molecular biology and its rapid expansion and improvements has revolutionized many scientific fields including food safety and authenticity.The DNA-based techniques have been extensively used in food authentication for their specificity, time and cost efficiency (9) (6)..

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As DNA is extremely resistant, stable and long lived macromolecule that even its slightest amount can be recovered from any fresh, dried, and even processed material hence the techniques based on DNA are found ideal for molecular species identification with wider application even in food authentication as food are agro or animal based. Required properties of ideal DNA markers includes easy availability, highly polymorphic and reproducible, codominant inheritance and recurrent occurence in genome,selectively neutral to environmental conditions and easily applicable between laboratories (16). The DNA based techniques when compared to each other do differ for their analytical ability such as discriminatory power, sensitivity, reproducibility, cost and time efficiency besides their user friendliness. (5, 9, 11, 17).

Filipe Pereira reported that DNA-based hybridization techniques other than its high cost is not suitable for mixture detection due to cross-hybridization of closely related species nevertheless it is not even a time efficient method and requires good quality of DNA.The PCR based molecular techniques has been regarded as convenient for molecular studies as it facilitates any genomic region amplification providing genetic information of many individuals without requirements of cloning and isolating large amounts of ultra pure genomic DNA (18). This does not make PCR based method free of defects. Reports and reviews on PCR-based molecular DNA techniques like RAPD, ALFP and their improved versions like SCAR have high lighted the main difference revolving each PCR based techniques, including ,the requirement over quantity and quality of DNA,level of polymorphism, technical and instrumental demand and necessity for prior sequence information for producing reproducible results with affirmative decisive resolution free of interferences due to homology of similar size or limited size of amplified fragements for specific genetic loci(10, 12, 15, 18).These are implicit of two important issues lack of standardization and universtality(5).

With advancement of molecular biology and Herbert proposal, DNA barcoding has been used as a tool for species identification beyond single species. There is long way to reach a consensus on its application as a unique and most reliable method but it could be called a novel molecular and bioinformatical tool designed to provide rapid, accurate, automatable, cost effective method using a standardized DNA region as tag.The tag or standardized region is the solution of main issues associated with single species specificity of DNA-based techniques.The DNA based authentication assays require polymorphic and high copied, analytical target regions which also should be less variable within,than between species calling for conserved priming sites to make it extremely robust,and highly reliable DNA sequence and amplicon (5, 19).

Chloroplast DNA with its unique circular, small genomic size and conserved structure is one of the best candidates for plant barcoding. The chloroplast nucleotide substitution rate is far greater when compared to plant mitochondria DNA even though their genome size and arrangement vary enormously (20, 21). Moreover the chloroplast DNA circular structure could be divided into number of small and large repeat regions with number of loci which are used in barcoding studies.The most common loci which have been used for barcoding include rbcL, trnL-trnF, atpB-rbcL, trnLintron, matK, trnT-trnL (9, 13, 21).The plastid region of trnl (UAA) intron have been used in several studies discriminatings several plant genera and species. The trnL (UAA) intron region has unique evolutionary characteristic which gives it conserved secondary structure with alteration of conserved and variable regions (22).

The primer pair used in current study has been referred in the ISO 21589 and also one of the four primers referred by Taberlet.P in his studies, which encompasses the entire trnL (UAA) gene plus a few base pairs on each side belonging to trnL (UAA) itself. Taberlet. P has indicated the length of sequence amplified with c and d to vary from 254 to 767 (22). These primer sequence are

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extremely conserved in particular among angiosperms, and is most universality known sequence among plants which explains its robustness of amplification process that is a necessity for standardization of tag region (23).

As mentioned earlier in developing RAPD primers, the primer sequence is arbitary and no prior sequence information is required,the primers are cheap and process needs no blotting and hybridization, resulting in quick and simple matched stretches of DNA. The draw back of technique involves over results of amplified stretches of nucleotide which are mixture of various size and does not specify a specific loci. In addition these amplified nucleotide sequence are totally dependent over purity,quality, and molecular weight of DNA nevertheless PCR cycling conditions also does influence final product amplification resulting in total absence or decreased amount of banding profile of unspecific loci. This makes the RAPD results quite unreliable for decisive interpretation in authentication between two different biological product (11, 12, 24).

The modified version of RAPD is SCAR (sequenced characterized amplified region) the marker prepared through knowledge provided by RAPD or other alternative is to reduce repeatability problem and tedious procedure by means of primers designed from nucleotide sequence established in cloned RAPF fragments linked to a particular loci converting dominant markers into co-dominant markers. SCAR markers benefit from longer primer sequence which impart specifity, and higher level of polymorphism because of higher annealing temperature but as specified known standardized loci information is not used you cannot have information about trait of interest (10, 12, 16).

We exploited a short standard primer sequence known to amplify a conserved loci of

Tabel-1: PCR Temperature-time program for amplification of chloroplast trnl gene

The amplification Temperature-time protocol	
Activation/initial denaturation	4 min/94 °C
Amplification	30 s/95 °C 30 s/55 °C 120 s/72°C
Number of cycles	35
Final extension	5 min/72 °C

Table-2. The primer sequence of trnl PCR products of saffron and safflower used for differentiation

Marker	Sequence	No. of Nucleotide	Fragement Size
Carthamus	F: CAAAGGTTCAGAAAGCGAAAATCA R: TCTACCAACGTAAGACAATCAAC	24 22	94
Crocus	F: TTGACTACGTTGTGTTGGTAGCC R: CCACAATAACTCCCCCTTTTG	23 21	147

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Table -3 Multiplex PCR protocol for amplification of designed modified trnL primers.

Activation/initial denaturation	4min/94°C
Amplification	30s/95°C 30s/55°C 120s/72°C
Number of Cycles	35
Final Extension	5min/72°C

chloroplast DNA used primarily for plant barcoding to facilitate DNA extraction and amplification and establish a stable assay protocol to trace a single adulterant resulting in less labors, less tedious, and cost effective procedure to design a specific primer as compared to RAPD and its SCAR version.

We have used a sequence of universal primer pair for amplification of trnL (UAA) intron a known conserved chloroplast region of plants with significant sequence information available at database and known to be extremely well conserved among angiosperms. These are fundamental characteristic for a universal and robust primer. Robustness, of a primer signifies amplification of conserved and well documented region which could define a standardized region for a standardized protocol for identification and tracing different species and genus(22).

The trnL (UAA) intron region have been used in simple PCR approach to trace and identify specific food crops and food allergens, more advance approach for identification and authentication has been in combination with lab-on-based chip capillary electrophoresis system for tracing olive oil, coffee, and wines adulteration. The difference in trnL amlpicon target length/ (PCR fragements lenght) and or in combination,if recognized selected SNP position due to allelic variants, were used as discriminatory measures (25, 26).

The molecular methods for tracing safflower adulteration (6) in saffron or for phylogenic studies usually have focused on using RAPD/SCAR markers including Javanmardi.N and etal, Beiki H. A andetal, Marieschi.M, and etal, Gaiol.G and etalneverthless methods like ISSP primers has been use of by Han-jieZheng and et al (1, 6, 7, 27-29).

We designed set of primers from already well documented, conserved, standardized, known, polymorphic and high copy analytical targets, whereas other studies did have to trace and find such region even though then also the position of loci could have not been proved to be standardized neither its robustness nor its replication rate. We did avoid time consuming procedure of RAPD/SCAR and other molecular markers identification procedures.

The evaluation criteria for specificity of primers to distinguish between saffron and safflower did prove primers ability to detect safflower both in samples made in laboratory for evaluation of primers and also samples collected from market. Validation of designed primers showed no cross reaction with non-plant DNAs .

Comparing the length of PCR products amplified by saffron, sawflower, marigold, pistachio, corn and pepper showed that the designed primers are quite specific to saffron and sawflower according to their length. The PCR method was validated for possible cross reaction leading to conflicting results. There were no cross reaction other than with turmic powder, though band was not formed at the same location as that of saffron, it was in the vicinity of saffron band, but due to the aromatic characteristics of turmic powder, it would not be used as an adulterant in saffron packing restricting application of these primer for tracing saffron adulterants only for saffron packs.

In conclusion we could exploit successfully a universal conserved and standardized DNA trnL loci in chloroplast gene to design set of primers unique for plants and specific to saffron and sawflower differing in length of their PCR products

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to distinguish sawflower from saffron as an adulterant. The development of primers took a very short time with acceptable accuracy, robustness and cost effective approach. Considering wealth of various plastids DNA regions proposed and recommended to be exploited in DNA barcodes for identifying flowering plants this work only highlight the advantages of DNA barcode sequence in primer design and indeed there should be more accurate evaluations of all possible plastids DNA for better design of primers to avoid interferences.

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