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MICROWAVE ASSISTED EXTRACTION OF BIOLOGICAL COMPONENTS OF *CROCUS* SATIVUS WITH DNA BARCODING TECHNOLOGY

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ABSTRACT: Saffron is derived from the flower of Crocus sativus as a dried stigma. Kashmiri saffron is the most expensive herb and in skyscraping demand by herbal and pharmaceutical industries due to which the illegal trade is prevailing in the industry to earn instant Profit. Detection of adulteration and comparison of genuine saffron vs. fake saffron is the crucial aspect which needs a scientific solution. Samples were taken Mogra (A), Lacha (B), Iranian (C) and unknown grade of saffron (D) from Pampore and Anantnag region of Kashmir. The DNA from the samples was extracted with the help NucleoSpin® Plant II Kit and were amplified with the help of reported universal primers of matk, rbcL and MatK, and bidirectional Sanger sequencing was performed. Only ITS gene showed positive extraction and amplification results. ITS gene was capable of distinguishing the interspecific as well as intraspecific variations. The blind trial conducted on unknown grade sample revealed the presence of substituent, which turned out to be Safflower, *i.e. Carthamus tinctorius* as observed through nucleotide blast search. The site-specific variations were observed, and the nucleotide sequences of ITS obtained during the study were submitted to the NCBI gene bank with accession number MH984846, MH984847, and MH984848. The examination of saffron with DNA Barcoding methodologies may contribute to health care communities a new validated tool to authenticate the samples and improve the efficacy of herbal medicine used to treat various ailments.

INTRODUCTION: Saffron is the world's most expensive spice and is derived from the dried stigma of *Crocus sativus*. The Crocus genus belongs to family Iridaceae of order Asparagales and comprises of about 70 to 100 species². It is the main distributers are Mediterranean Europe and Western Asia.

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Iran accounts for about 90% of the world assembly of saffron. In India, Kashmir (J & K) is the largest producer of saffron, which is mainly grown in Pampore and Anantnag. The grades of saffron can be categorized into two major grades, *i.e.* 'Mongra' and "Laccha."

Mogra saffron contains stigma alone while Laccha saffron contains stigmas attached with parts of the style ³. The expensive nature of saffron is related to its production as 1 kilogram of saffron requires 110,000-170,000 flowers. The herbal plants with economic importance are often smuggled from one state to another or from one border to another to earn instant profit ³.

The illegal activities also include adulteration or substitution of expensive genuine herb with a cheaper similar looking herb which violates quality assurance and can be dangerous in consumption as well ⁴.

Saffron contains more than 150 volatile and many components which nonvolatile active are responsible for its medicinal properties and its higher demand in the herbal industry. The volatile components are terpenes, terpene alcohols, and their esters among which safranal $(C_{10}H_{14}O)$ is the main component. Non-volatile compounds comprise crocins C₄₄H₆₄O₂₄, crocetin C₂₀H₂₄O₄, picrocrocin C₁₆H₂₆O₇, and flavonoids. Crocin, glucosyl esters of crocetin are water-soluble carotenoids which are meant for saffron's characteristic color. Picrocrocin, a glycoside of safranal, is responsible for the bitter taste of the spice and Safranal, is responsible for its characteristic aroma. Saffron's taste and hay-like fragrance are due to picro-crosin and safranal. Its golden yellow-orange color is the result of crosin which is trans-crocetin di-ester, and its IUPAC name is 8,8-diapo-8,8-carotenoic acid ⁵. Saffron is of different quality and strength. The difference lies in the amount of style present and age of saffron. More style present in saffron means the saffron is less strong. Saffron is categorized into various grades.

Grades of Iranian Saffron:

- 1. Sargol- strongest grade, stigma only
- 2. Pushal- low grade, style present with the stigma
- **3.** Bunch- lowest grade, a large amount of style present with red stigma.

Grades of Kashmiri Saffron:

- **1.** Mogra saffron- strongest among all the saffron worldwide, Stigma alone
- **2.** Laccha saffron- Low-grade Kashmiri saffron, stigma attached with style

Kashmiri saffron is very expensive, and its demand is increasing day by day due to which it is very prone to adulteration ³. It is sold in the range of Rs.300-500 per gram in Kashmir. According to a study on saffron sold in Kashmir, only 52% is genuine, 30% is low grade and 17% adulterated. Adulteration also includes selling of mixes of different saffron grades. High-grade Kashmiri saffron is mostly mixed with low-grade cheaper Iranian saffron and is then sold as pure Kashmiri saffron.

DNA barcoding is one such technology which utilizes short sequences ranging from 300 to 1000 bp sequences to identify the biological specimens which aim and orients its applications in conservation biology, phylogenetic, ecology. taxonomic studies, Pharmaceutical studies as well as forensic studies to identify species in illegal trade ⁵. DNA barcoding is well established in animals as well as plants species. DNA barcoding could be utilized to identify and delineate the species in question ^{6, 7}. The present study is carried out to identify adulterant or any substituent among of saffron with DNA samples Barcoding Methodology.

MATERIALS AND METHODS: Dried commercial samples of *C. sativus* marketed as whole stigmas and from different geographic proveniences from Pampore, Anantnag and Srinagar district of Kashmir valley were also purchased and to conduct the blind trial the samples were also purchased from local herbal vendor of Chandigarh herbal market. Different varieties of samples claimed to be Mogra saffron, Kashmiri saffron, Iranian Saffron, and local sample (Chandigarh) were marked as Sample A to D.

Samples Collected:

- Sample A: (Mogra Saffron)
- Sample B: Lacha Saffron)
- Sample C: Iranian saffron.
- Sample D: (Unknown grade)

DNA Barcoding: 1 g of saffron stigmas were extracted by immersing in 5 mL of absolute ethanol at ambient temperature overnight, in the dark, followed by Microwave-assisted extraction. The extracts were sealed in 2 mL vials and stored at 4 °C. The genomic DNA from extracts was carried out with the help of NucleoSpin® Plant II Kit (Macherey Nagel). Plant DNA Kit was used for DNA extraction according to the manufactured protocol.

Quality of the extracted DNA was determined using gel electrophoresis PCR amplification reactions with PCR thermal cycler (GeneAmp PCR System 9700 with 1x 1X Phire PCR buffer (contains 1.5 mM MgCl₂), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers were carried out in a 20 µl reaction volume. Sequencing was performed using BigDye Terminator v3.1 the Cycle Sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The sequences analysis was carried out using Bioedit and Mega 7.0 editing software for by assembling both forward and reverse sequences for each gene amplified. The homology of sequences obtained during experimentation is confirmed by using NCBI blast tool. The Insilco analysis among crocus genus was carried out between obtained ITS sequences and Sequences obtained through NCBI for Crocus etruscus, Crocus biflorus, Crocus cancellatus, Crocus laevigatus.

RESULTS: The DNA was successfully extracted from samples types A, B, C and was further amplified for three markers matk, rbcL and ITS. The DNA extraction of dry samples are little cumbersome but all the samples were successfully extracted. The samples were further amplified with polymerase chain technology for three specific gene markers, *i.e.* matk, rbcL, and ITS. All the samples failed to show amplification for matk and rbcL except for ITS marker, which showed successful amplification in all the samples. The amplified DNA was sequenced, and sequences obtained were edited and assembled with the help of Mega 7.0 software. The sequences showed nucleotide composition, as shown in **Table 1**.

TABLE 1: SHOWING NUCLEOTIDE COMPOSITIONOF CROCUS GENUS

	T(U)	С	Α	G	Total
FIMPT-CS1-ITS	20.4	31.1	19.0	29.5	643.0
FIMPT-CS2-ITS	20.4	31.1	19.0	29.5	643.0
FIMPT-CS3-ITS	20.4	31.1	19.0	29.5	643.0
Crocus etruscus	20.2	29.3	20.2	30.3	634.0
Crocus biflorus	18.5	30.9	19.5	31.0	686.0
Crocus cancellatus	19.2	30.1	19.5	31.3	652.0
Crocus laevigatus	19.2	30.1	19.5	31.3	652.0
Avg.	19.7	30.5	19.4	30.4	650.4

The sequences obtained during the present study were further submitted to NCBI and accession numbers were obtained as MH984846, MH984847, and MH984848. The sequences obtained were analyzed using blast n of NCBI to compare the sequences with an existing database. The taijama neutrality test showed 595 segregating sites with 2.6 taijama test statistics. The barcoding gap was found to be much higher in case of ITS genes as the interspecific genetic distance was almost null as compared to interspecific genetic distance was calculated with P- distance to be 0.5537 as shown in **Table 2** and **Fig. 1** and **Fig. 4**.

Number of	S	egrega	-		ucleoti		Taijama tes	t	Interspecific	Intraspecific
sequences		sites]	Diversi	ty	statistics		Distance ITS	Distance
7		595			.5537		2.624352		0.5537	0.0
1. FIMPT-CS1-ITS										^{0.009} MPT-CS2-ITS
2. FIMPT-CS2-ITS	0.0000						0.017		1.118	0.001 PMPT-CS3-ITS
3. FIMPT-CS3-ITS	0.0000	0.0000						0.650	Crocus etruscus	
4. Crocus etruscus	0.6767	0.6767	0.6767						0.513 Cr	ocus biflorus
5. Crocus biflorus	0.7145	0.7145	0.7145	0.6530			0.389			0.000 Crocus cancellatu
6. Crocus cancellatus	0.7003	0.7003	0.7003	0.6893	0.6104				0.746	0.000 Crocus laevigatus
7. Crocus laevigatus	0.7003	0.7003	0.7003	0.6893	0.6104	0.0000	L	_		
FIG. 1: SHO	WING	PAIR	WISE	INTE	RSPEC	CIFIC	FIG. 2: SHO	WING	PHYLOGENETIC	C RELATIONSHI
	BETWE		ROCU			AND			M LIKELIHOOD	
~~	SPECIE	5 WI	TH I	TS B	ARCO	DING			METER WITH C	
MARKER							AND KELA	ED SP	ECIES WITH ITS	MAKKEK

TABLE 2: SHOWING TAIJAMA NEUTRALITY TEST AND GENETIC DISTANCE IN CROCUS GENUS

#FIMPT-CS1-ITS	gog	ace	aag	acq	gac	gat	cac	gac	ata	tta	cac	cat	act	tac	tta	ott	acq	act	000	tto	ogt	ecc	atc	act	oct	000
#FINPT-CS2-ITS			aag																							
\$FIMPT-CS3-ITS			880																							
#Crocus etruscus	TCG	AGA	CCC	GAA	CAA	ACG	GAC	GAT	COC	GAA	COT	GTT	ACA	TCC	TTA	CTT	ACT	ACT	CTG	TCT	COT	CCC	GCC	GCT	CCT	CGC
Crocus biflorus	TCG	AGA	CCC	GAA	CGA	ACG	GAC	GAT	CGC	GAA	COT	GTT	ACA	TAT	CTG	CTC	CCT	AAC	GAC	TCC	GTT	CGT	CCC	GCC	GCT	CCT
#Crocus cancellatus	TCG	AGA	CCC	GAA	CGA	ACG	GAC	GAT	CGC	GAA	CGT	GTT	ATA	TAT	TTG	CTA	ACC	AAA	ACA	ACT	TCG	TTT	CGT	CCC	GTC	GCC
#Crocus_laevigatus	TCG	AGA	CCC	GAA	CGA	ACG	GAC	GAT	CQC	GAA	COT	GIT	ATA	TAT	TTG	CTA	ACC	AAA	ACA	ACT	TCG	TTT	CGT	CCC	GTC	QCC
FIMPT-CS1-ITS	ggc	gtg	gog	aga	ttt	tgg	agg	888	cga	AAC	000	gge	gca	gtg	ggc	gec	aag	gaa	cac	tto	ttg	gaa	acg	cog	tog	cgg
\$FIMPT-C52-ITS	ggc	gtg	geg	aga	ttt	tgg	899	444	cga	aac	ccc	gge	gca	gtg	ggc	gee	aag	çaa	cac	tto	ttg	gaa	acg	cog	tog	cgg
FINFT-CS3-ITS	ggo	gtg	geg	aga	ttt	tgg	agg	444	cga	aac	000	gge	gca	gtg	gige	gec	aag	gaa	Cac	tto	ttg	gaa	acg	cog	tog	ogg
Crocus etruscus	GGC	GTG	GCG	TGG	CGA	GAC	GGA	GGA	AAC	GAA	ACC	CCG	GCG	CGG	TOG	GCG	CCA	AGG	AAC	ACT	TIT	TOG	AAG	CQC	COT	CGC
#Crocus biflorus	CGC	GGC	GCG	GCG	TGA	CGA	GAC	GGA	GGA	AAC	GAA	ACC	CCG	GCG	CGG	TGG	GOG	CCA	AGG	AAC	ACT	TTT	TAG	AAG	CQC	CGT
#Crocus cancellatus	CCT	COC	GQC	000	909	TGG	CGA	GAC	GGA	0GA	AAC	GAA	CCC	000	dea	C90	100	600	CCA	A00	AAC	ACT	TTT	TAG	AAG	CQC
#Crocus_laevigatus	CCT	CGC	GGC	GCG	GCG	TGG	CGA	GAC	GGA	GGA	AAC	GAA	CCC	CCG	GCG	CGG	TGG	GCG	OCA	AGG	AAC	ACT	TTT	TAG	AAG	CGC
FIMPT-CS1-ITS	ccc	tot	cca	CCL	occ	555	ctg	tet	gtc	cca	cga	tag	gga	aaa	agg	gag	gga	gag	gga	tat	ogt	att	ctg	tac	gac	tot
#FIMPT-CS2-ITS	ccc	tct	cca	oct	dee	ttt	ctg	tet	gtc	cca	cga	tag	gga	000	800	gag	gga	gag	gga	tat	cgt	att	ctg	tac	gac	tet
#FIMPT-CS3-ITS	ccc	tot	cca	CCL	ccc	ttt	ctg	tet	gtc	cca	cga	tag	gga	000	899	gag	gga	gag	QQ4	cat	cgt	att	ctg	tac	gac	tot
#Crocus_etruscus	GGC	CTC	CTC	COC	CIC	CCA	TCT	GTA	ACA	TGA	TAG	GGA	GGG	AGG	AGG	GGG	AAC	GCG	66C	GTG	GAT	ATA	GAT	ATC	GTA	TIC
#Crocus_biflorus	CQC	0QC	CIC	CIC	crc	CAC	CIC	CCC	ATT	900	ATC	ACA	ACA	GAT	AGC	000	990	200	ANG	GAG	00C	GAA	COC	995	COT	QGA
#Crocus_cancellatus	CGT	CGC	GGC	CTC	CIC	CTC	CTC	CCC	TAC	CIT	AGG	GAG	GGG	ATG	ACG	GAG	GGG	GAA	CGC	GGG	CGT	GGA	TAT	AGA	TAT	CGT
<pre>#Crocus_laevigatus</pre>	CGT	COC	GGC	CTC	CIC	CTC	CTC	CCC	TAC	CII	200	GAG	000	ATG	ACG	GAG	690	GAA	COC	000	CGT	66A	TAT	AGA	TAT	CGT
#FIMPT-CS1-ITS	000	caa	cgg	ata	tet	agg	ctc	tog	CAL	cça	tça	aga	acg	tag	cga	aat	gog	ata	ott	ggt	gtg	aat	tgo	aça	atc	cog
FIMPT-CS2-ITS	cgg	caa	cgg	ata	tet	400	CEC	tog	cat	oga	tga	aga	acq	tag	cga	aat	gog	ata	CEE	ggt	gtg	445	tgc	aça	ate	cog
\$FINPT-CS3-ITS	cgg	Caa	cgg	ata	tot	agg	oto	tog	cat	cga	tga	aga	acg	tag	cga	aat	gog	ata	ott	ggt	gtg	aat	cgo	aga	ato	ocg
#Crocus_etruscus	TGT	ACG	ACT	CTC	00C	AAC	00A	TAT	CTA	QQC.	TCT	CQC	ATC	GAT	GAA	GAA	CGT	AGC	GAA	ATG	CGA	TAC	TTG	GTG	TGA	ATT
#Crocus biflorus	TAT	AGA	TAT	CGT	ATT	CIG	TAC	GAC	TCT	CGG	CAA	ĊĠĠ	ATA	TCT	AGG	CTC	TOG	CAT	CGA	TGA	AGA	ACG	TAG	CGA	AAT	GCG
#Crocus_cancellatus	ATT	TIG	TAC	GAC	TCT	C99	CAA	C00	ATA	TOT	200	CIC	TCG	CAT	CGA	TGA	AGA	205	TAG	CGA	AAT	606	ATA	CTT	GOT	GIG
#Crocus_laevigatus	ATT	TTG	TAC	GAC	TCT	CGG	CAA	CGG	ATA	TCT	A96	CTC	TCG	CAT	CGA	TGA	AGA	ACG	TAG	CGA	AAT	GCG	ATA	CTT	GGT	GIG
FINPT-CS1-ITS	τga	acc	atc	gag	tot	ttg	aac	gca	agt	tgc	gcc	oga	ggc	cat	cog	gtc	gag	990	acg	oct	gcc	tgg	gog	tca	ogc	ctc
FIMPT-C52-ITS	tga	acc	ato	gag	tet	ttg	aac	gca	agt	tge	gee	oga	ggc	Cat	cog	gto	gag	00¢	acq	oct	gee	tgg	gog	tca	oge	ctc
#FIMPT-CS3-ITS	tga	acc	atc	gag	tet	ttg	aac	gca	agt	tgc	gee	cga	ggc	cat	cog	gtc	gag	ggc	acg	cct	gee	Egg	gog	tca	oge	ctc
Crocus_etruscus	GCA	GAA	TCC	CGT	GAA	CCA	TCG	AGT	CTT	TGA	ACG	CAA	GIT	GCG	CCC	GAG	GCC	ATC	CGG	TCG	AGG	GCA	CGC	CTG	CCT	GGG
#Crocus_biflorus	ATA	CTT	GGT	GTG	AAT	TOC	AGA	ATC	000	TGA	ACC	ATC	GAG	TOT	TTG	AAC	GCA	AGT	TOC	GCC.	CGA	GGC	CAT	005	GCC	GAG
Crocus_cancellatus	AAT	TGC	AGA	ATC	CCG	TGA	ACC	ATC	GAG	TCT	TTG	AAC	GCA	AGT	TOC	GCC	CGA	GGC	CAT	CCG	GIC	GAG	GGC	ACG	CCT	GCC
#Crocus laevigatus	AAT	TGC	AGA	ATC	CCG	TGA	ACC	ATC	GAG	TOT	TTG	AAC	GCA	AGT	TGC	GCC	CGA	GGC	CAT	CCG	GTC	GAG	GGC	ACG	COT	GCC

FIG. 4: MULTIPLE SEQUENCE ALIGNMENT OF CROCUS GENUS WITH THE HELP OF MEGA 7 SOFTWARE

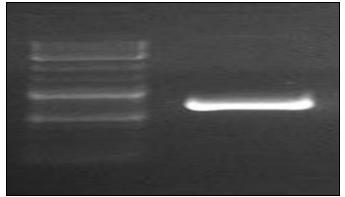


FIG. 3: SHOWING EXACTION AND AMPLIFICATION OF GENE IT'S IN 3kb LADDER

All the sequences obtained from sample A, B, C showed 100% homology with ITS, but sample D surprisingly showed 99.2% similarity with *Carthamus tinctorius, i.e.* Safflower. It was then observed that the *Carthamus tinctorius, i.e.* Safflower colored similar to the saffron plant is being sold on herbal outlets of Chandigarh with prize quite similar to the genuine herb.

Phylogenetic Tree: The phylogenetic relationships in the *Crocus sativus* with interspecific species of the same genus (*Crocus etruscus, Crocus biflorus, Crocus cancellatus, Crocus laevigatus*) was established by the combination of *ITS* **Fig. 2**, with the high average supporting values for nodes each tree showed a "fan" shape, with closely related species clustering together, whereas distantly related species relatively scattering. The analysis of the relative distribution of distances by K2P pairs using Taxon DNA showed that each loci have a global barcode gap. ITS gene marker shows the potential to distinguish the closely related species of crocus genus with the help of maximum likelihood phylogenetic tree method.

CONCLUSION: The present study was conducted to compare and evaluate the quality of saffron samples. Only ITS sample was successfully amplified and sequenced to produce DNA barcodes of the ITS gene. The gene was also tested for its resolution capability to distinguish the closely related species with the help of maximum like a hood phylogenetic tree. The study showed the successful identification of adulteration and substitution of the herbal product with similar looking cheaper Carthamus tinctorius. The preliminary examination of saffron with DNA Barcoding methodologies may contribute to the health care communities with validated PCR based methodology to authenticate the herbal samples and improve the efficacy of herbal

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