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Genetic Diversity analysis in five accessions of *Trigonella* based on Seed protein Electrophoresis and RAPD

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ABSTRACT

Biochemical and molecular characterization of five *Trigonella* accessions were carried out based on seed storage protein electrophoresis and RAPD marker. The electrophoretic pattern of proteins produced 3 monomorphic bands and 23 polymorphic bands and 7 species specific bands. RAPD results revealed high level of polymorphism among the studied genotypes. The primer OPP-02 produced reproducible polymorphic bands. A total of 19 amplified bands were generated across the studied accessions. 16 bands out of total number were polymorphic and 7 were unique. UPGMA dendrogram based Jacquard's similarity matrix grouped all the accessions into two clusters. Based upon similarity matrix accessions A4 and A2 were close. On the other hand UPGMA dendrogram generated on the basis of jaccard's similarity matrix revealed that accessions A1 and A2 Were very close to each other. No significant correlation was observed among the two marker systems utilized. It is concluded that Seed protein profiles could be useful, but RAPD profiles are reliable tools in the genetic diversity studies and genotype classification which can be used to improve the efficiency of *Trigonella* breeding programme.

Introduction

The genus *Trigonella* is one of the largest genera of the tribe Trifoliatae in the family Fabaceae and sub-family Papilionaceae (Balodi and Rao, 1991) represented by about 110 species in the world and 50 species in Turkey (Sechmen *et al.*, 1998). Among *Trigonella* species, *Trigonella foenum graecum* (commonly known as fenugreek) is a flowering annual, with autogamous white flowers occasionally visited by insects indigenous to countries on the eastern shores of Mediterranean, Irano-Turanian and Europe-Siberian phytogeographical regions. Fenugreek is widely cultivated in India, Egypt, Ethiopia, and Morocco and occasionally in England (Polhil and Raven, 1981). *Trigonella foenum-graecum* is extensively grown in the tropical and subtropical regions of India during winter season for its seeds, tender shoots and fresh leaves. Different parts of the plant such as leaves and seeds are consumed in India. The current productivity of fenugreek is 1,245 kg/ha. The value added products of fenugreek such as fenugreek seeds, fenugreek powder and oleoresins are exported to Europe North America, South Africa and other Asian countries (Malhotra and Vahishtha, 2008). Fenugreek is gaining importance due to its rare medicinal properties (Sharma et *al.*, 1990). Fenugreek is reported to have anti-diabetic, anti-fertility, anticancer, anti-microbial, anti-parasitic and hypocholesterolaemic effects (Al-Habori and Raman, 2002). In India, fenugreek is used as a lactation stimulant (Tiran, 2003). Fenugreek seed in powder or germinated form exhibits anti-diabetic properties (Broca *et al.*, 2004; Devi *et al.*, 2003; Hannan *et al.*, 2003; Tahiliani and Kar, 2003 a ; Thakaran, 2003 and Vats *et al.*, 2003), Across the world only known and well-defined cultivars are grown in specific areas. Gene banks also harbor scanty germplasm collection of *Trigonella* species (Hymowitz, 1990). The neglected and the underuse status of these locally important crops indicates a risk of disappearance of important plant material developed over thousands of years of cultivation. One of the important factors restricting their large-scale production and development of better varieties is that very little information is available about their genetic diversity, inter and intra-specific variability and genetic relationship among these species. Therefore, attempts to analyze possible untapped genetic diversity become extremely essential for breeding and crop improvement

Protein is a primary product of the genetic system, on the basis of this, a protein can serve as a marker of genome, and protein marker as protein phenotype indicate the genome structure and phenotype specificity as a whole. Availability of genetic information is important for genetic improvement of the crop; local and primitive germplasm can be used as a source of genetic variation. Total protein is not sensitive to environmental fluctuations, its banding pattern is very stable which advocates for cultivar identification purpose in crop plants (Duran *et al.*, 2005) Among biochemical techniques, sodium dodecyle sulphate polyacrylamide gel electrophoresis is the most common and widely used, due to its reliability and simplicity in describing the genetic structure of crop germplasm (Gepts, 1989; Murphy *et al.*, 1990). Over the last two decades, considerable interest has been focused on the use of electrophoretic methods for reliable discrimination and identification of plan varieties (Sammour, 1985; Sammour *et al*, 1994; Przybylska and Zimnlak- Przybylska, 1995). Electrophoresis adds information to taxonomy and should not be dissociated from morphological,, anatomical and cytological observations. (Boulter *et al.*, 1966; Ghafoor *et al.*, 2002). Seed protein profiles have been successfully used to study taxonomical and evolutionary relation ships of several crop plants (Gepts and Bliss, 1988; Rao *et al.*, 1992; Das and Mukarjee, 1995).

Phenotypic characters have limitations since they are influenced by environmental factors and the developmental

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stage of the plant. In contrast, molecular markers, based on DNA sequence polymorphisms, are independent of environmental conditions and show higher levels of polymorphism. The latter have proved their utility in fields like taxonomy, physiology, embryology, genetics, etc. Among the different types of molecular markers available, random amplified polymorphic DNA (RAPD) is useful for the assessment of genetic diversity among rare species (William *et al.*,1990), despite of the existing limitations (dominant mode of inheritance of RAPD loci), because of their simplicity, speed and relatively low costs as compared to other molecular markers. RAPD markers have been used extensively in analyzing genetic diversity (Garacia *et al.,* 1998; Gwanama *et al.,* 2000; Levi *et al.,* 2001; Artyukova *et al.,* 2004; Sureja *et al.*,2006; Guerra *et al.,* 2010).

Materials and Methods

Seeds of five accessions of *Trigonella* viz., IC 143851, IC 144225, IC 332236, IC 371755 and IC 433589, designated in the experiment as A1, A2, A3, A4 and A5, respectively, were collected from National Bureau of Plant Genetic Resource (NBPGR), New Delhi, to study the genetic variation based on protein profiling and RAPD.

Sds Page Analysis

Extraction of proteins

Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) was used to characterize the protein profiles of the accessions by using 12.25% (w/v) separating gel and 4.5% (w/v) staking gel as developed by Laemmli (1970). 1.0 g seeds from each accession were ground to fine powder with mortar and pestle for the extraction of proteins. 400µl of protein extraction buffer was added to 0.01g seed flour and vortexed thoroughly to homogenize. For purification the homogenated samples were centrifuged at 15000 rpm for 10 minutes at room temperature. The extracted crude proteins were recovered as clear supernatant, which were transferred into1.5 ml centrifuge tubes and stored at 2^0C until electrophoresis.

Electrophoresis (SDS- PAGE)

The elecrophoretic procedure was carried out using slab type SDS-PAGE (AE 6530 M, Japan) with12.25% polyacrylamide gel. A resolving gel (3.0 M Tris HCl, pH 8.0, 0.4% SDS) and a 4.5% stacking gel (0.4 M Tris HCl, pH 7.0, 0.4% SDS) was prepared and polymerized chemically by the addition of 17 ml of N, N, N, N tetra methylene diamine and 10% ammonium persulphate. Electrode buffer solution was poured into the bottom pool of the apparatus. Gel plates were placed in the apparatus carefully so as to prevent bubble formation at the bottom of gel plates .Then electrode buffer (0.025 M Tris, 0.12 M Glycine, 0.12% SDS) was added to the top pool of the apparatus. 15µl of the supernatant was loaded with the help of a micropipette into each well of the gel. The apparatus was connected with uninterrupted electric supply (100 V) until the bromophenol blue reached the bottom of the gel. Gels were then stained for one hour with the staining solution containing 0.2% (w/v) Comassie Brilliant Blue R 250, dissolved in 10% (v/v) acetic acid, 40 % (v/v) methanol and water in the ratio of 10:40:50. Gels were destained in a solution of methanol: acetic acid: water in the ratio of 30:10:60 respectively. After destaining, gels were photographed using gel documentation systems.

Gel analysis

The gels were analyzed directly and data recorded on the basis of presence and absence of protein bands. Similarity index was calculated for all possible pairs of proteins. Presence and absence of bands were entered in a binary data matrix. Based on result of electrophoresis band spectra Jaccard' s (1979) a dendrogram was constructed by the unweighed pair group method with airthmatic mean (UPGMA).All calculations were carried out using NTSYS-pc software version 2.2 (Rohlf, 2004).

RAPD analysis

Genomic DNA was isolated from the mature seeds of five accessions using the procedure developed by Kang et al., 1998 with slight modification. After quantification DNA samples were diluted to working concentration of 10ng/ul. Amplification was carried out in 25µl reaction mixture containing about 25 ng genomic DNA,0.2 mM of each dNTP, 0.2 µM primer, 2 mM of $MgCL_2$ and I U of Taq DNA polymerase (Fermentas, life sciences) .DNA amplification was performed in a PTC 200 thermal cycler (MJ Research) according to the following thermal profile: initial denaturation at 94^0C for 3 minutes, followed by 40 cycles of denaturation at 94^0C for 45 seconds, annealing at 35^0C for 1.5 minutes and extension at 72^0C for 1.5 minutes, followed by final extension at 72^0C for 3 minutes. Amplification products were separated in 1.5-2% agarose gel and detected by staining with ethidium bromide (Sambrook *et al.,* 1989). The gels were photographed under UV light.

Data analysis

The amplified fragment profiles were visually scored for the presence (1) or absence (0) of bands and entered into binary matrix. The variation in intensity was not taken into consideration to avoid confusion in scoring. Jaccard's similarity coefficients were calculated and used to construct dendrogram based on unweighed pair group method with arithmetic averages (UPGMA).The computer package NTSYS-pc version 2.0 (Applied Biostatistics,Inc.) was used for carrying out cluster analysis.

Results and Discussion

Electrophoresis of proteins is a powerful tool for population genetics Parker *et al.,* (1998). The most commonly used proteins are seed storage proteins, which are known to be polymorphic with respect to size, charge, or both these parameters (Cooke, 1984; Martinez *et al.,* 1997). Germplasm characterization based on morphological traits is not upto the mark and requires confirmation at molecular or at least at protein level. Electrophoresis of proteins is a powerful tool for detection of the genetic diversity and the SDS-PAGE of seed protein is particularly considered as a reliable technology because seed storage proteins are highly independent of environmental fluctuations (Iqbal et al., 2005 and Javid et al., 2004). Genetic diversity of *Trigonella* germplasm elucidated through SDS-PAGE of proteins from seeds revealed distinct electrophoretic patters. Twenty seven bands ranging from 50.0 to 97.0 kDa were recognized among five accessions, three were monomorphic. (Table 2, Figure 1). The genotypes showed considerable variation in protein band number ranged from 12 to 17. Out of 27 peptide bands 24 bands were polymorphic with 88.88% polymorphism. Our finding reveals that considerable intra – specific variation was available in the analyzed accessions. The variation in the major bands was present in case of A1 (IC-143851) and A5 (IC-433589), where as the accessions showed variations for the minor bands. Band number 5 (93 kDa), 7 (89 kDa) and 27 (50 KDa) were common in all the accessions. Polypeptide band number 8a(87akDa, A1), 12 (78 kDa, A1) 18(69 kDa, A2), 21(65 kD, A4), 23 (59 KDa, A4), 24 (58 kDa, A3) and 26 (56 kDa, A5) were accession specific. Band number 20 (67 kDa) was absent only in case of A1.Species specific

bands may be exploited for hybrid identification in breeding experiments (Maity et al., 2009).

Table 2: Molecular weight of polypeptide bands 0f *Trigonella* accessions

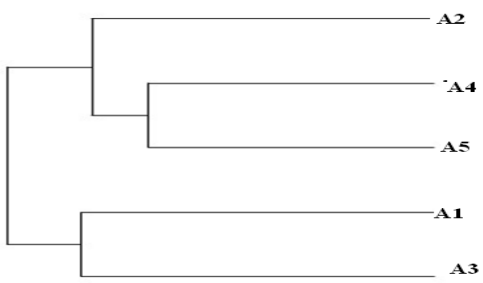
Band No.	Rf value	Molecular Weight (KDa)	*Trigonella* accessions				
			A1	A2	A3	A4	A5
1	0.03	97	—	+	—	+	+
2	0.04	96	+	—	+	—	—
3	0.05	95	+	—	—	+	+
4	0.06	94	+	+	+	—	—
5	0.07	93	+	+	+	+	+
6	0.10	90	+	+	+	—	+
7	0.11	89	+	+	+	+	+
8	0.13	87	(+)	—	—	—	—
9	0.14	86	—	+	—	—	+
10	0.15	85	—	+	—	+	+
11	0.16	84	+	—	—	—	+
12	0.22	78	(+)	—	—	—	—
13	0.25	75	—	+	—	+	+
14	0.26	74	—	+	+	+	—
15	0.27	73	+	—	+	+	+
16	0.28	72	+	—	+	—	—
17	0.30	70	—	—	+	+	+
18	0.31	69	—	(+)	—	—	—
19	0.32	68	—	—	+	+	+
20	0.33	67	—	+	+	+	+
21	0.35	65	—	—	—	(+)	—
22	0.38	62	—	—	+	—	+
23	0.41	59	—	—	—	(+)	—
24	0.42	58	—	—	(+)	—	—
25	0.43	57	—	+	—	—	+
26	0.44	56	—	—	—	—	(+)
27	0.50	50	+	+	+	+	+
Total no. of bands			12	13	14	14	17

(+) ;Species specific.

Where , A1 =IC-143851, A2=IC- 144225, A3= IC- 332236, A4=IC- 371755 and A4= IC- 433589

The cluster analysis performed using UPGMA revealed two distinct clusters as evident from dendrogram (figure 2) constructed from jaccards similarity matrix (Table- 3). Cluster 1 consisted of two accessions (A3 and A1) while the cluster 2 grouped rest of the accessions (A2, A4 and A5). Clustering based on seed storage protein profiles provides information about the phylogenetic relationship of genotypes as all the genotypes have at least one or more unique seed storage protein marker that can separate them from one another and also from other *Trigonella* genotypes. The results are in consistence with Hameed et al., 2009.

Figure 2. UPGMA dendrogram showing relationship among five *Trigonella* accessions based on genetic distance of SDS page.



The SDS-PAGE results revealed that the total amount of polymorphism accounted for principal component was 88.88% which revealed a considerable genetic diversity among the studied accessions. Our results are in consistence with that of Landizinsky, (1979) who found genetic diversity among three species of *Trigonella foenium-graecum* based on seed protein profiles. The variability within the investigated accessions agrees with previous biochemical studies (Chowdhury and Slinkard (2000); Tadesse and Bekele (2001, 2004).Various reports on the same line are present from the previous investigations. Sammour *et al*., (2007) used SDS-PAGE technique in *Latharus sativa* and found 72.72% polymorphism in case of seed proteins .The electrophoretic analysis of seed proteins in the *Trigonella* accessions revealed a considerable intraspecific variation. This observation is consistence with the electrophoretic data of *Latharus sativa* (Przybylska *et al*., 1998). Marked protein polymorphism may be explained by the presence of out crossing in this self-pollinated species as have been augmented by Chowdhury and Slinkard, (1997) in case of *Latharus sativa.* Kaumar and Tata, (2010) found 85.57 % polymorphism in case of chilli peppers. Present protein profiles of the selected experimental accessions of *Trigonella* revealed that accession A4 and A2 are very close to each other at molecular level. These accessions almost possess same similarity index of about (0.50000), (Table 2).

Table 3. Jaccard binary similarity coefficients

Accession No.	TI	A2	A3	A4	A5
TI	1.000				
A2	0.25000	1.000			
A3	0.44444	0.35000	1.000		
A4	0.23810	0.42105	0.40000	1.000	
A5	0.31818	0.50000	0.40909	0.55000	1.000

Seed protein patterns can be used as a promising tool for distinguishing cultivars of particular crop species (Jha & Ohri, 1996; Mennella *et al*., 1999). However, only few studies indicated that cultivar identification was not possible with the SDS-PAGE method (Ahmad & Slinkard, 1992; De Vries, 1996). The SDS-PAGE is considered to be a practical and reliable method for species identification (Gepts, 1989)

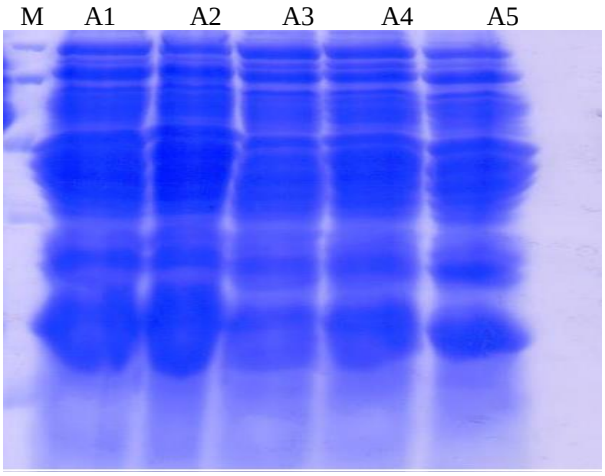


Figure-1: Gel illustrating variation in Electrophoretic seed protein profiles in the investigated Trigonella accessions.

RAPD Analysis:

The amplification profiles were in the form of 19 bands out of these 15 were polymorphic with a polymorphism of about 78.79% .The amplified DNA fragments varied in size from 200-3830bp.(Table-4,Fig.4) The number of bands amplified from different accessions varied from 8-11 bands. Pairwise

comparisons between the tested genotypes were used to calculate the genetic similarity (Table.5).

Band No.	Rf value	(Kbp)	A1	A2	A3	A4	A5
1	0.03	3830	0	1	1	0	0
2	0.08	3680	1	1	0	1	1
3	0.10	3600	0	0	1	0	0
4	0.15	3400	1	0	0	0	0
5	0.18	3280	0	1	1	0	1
6	0.21	3160	1	1	1	1	1
7	0.22	3120	0	0	0	1	0
8	0.23	3080	0	0	0	0	1
9	0.25	3000	0	0	0	1	0
10	0.28	2880	0	0	0	1	1
11	0.31	2760	1	0	0	0	0
12	0.33	2680	0	0	1	0	0
13	0.38	2480	1	1	1	1	0
14	0.50	2000	1	1	0	1	1
15	0.56	1760	0	0	1	1	1
16	0.75	1000	1	0	0	0	0
17	0.77	920	0	0	1	1	1
18	0.86	560	1	1	1	1	1
19	0.95	200	1	1	1	1	1

Table 4 : Showing the presence and absence of amplified DNA bands.

The similarity index (Table-5) revealed the maximum similarity between accessions IC-144225 (A1) with IC-144225 (A2) and IC-371755 (A4) with IC-433589 (A5) while distantly related varieties were IC-332236 (A3) and IC-433589 (A5).

A1	A2	A3	A4	A5	A1	
A2	0.54545					
A3	0.26667		0.50000			
A4	0.42857		0.46154		0.40000	
A5	0.35714		0.50000	0.42857		0.61538

Table 5: Jaccard´s similarity coefficient

The Bivariate 1-0 data matrix generated dendrogram shows (Table:4) that the genotypes analysed on DNA basis belong to two clusters (Fig.3) Cluster -I includes two accessions IC-144225(A1) and IC-144225(A2).The major cluster-II included IC-371755(A4) and IC-433589 (A5). The accession IC-332236 (A3) was placed distantly from the studied accessions as revealed by the dendrogram constructed.

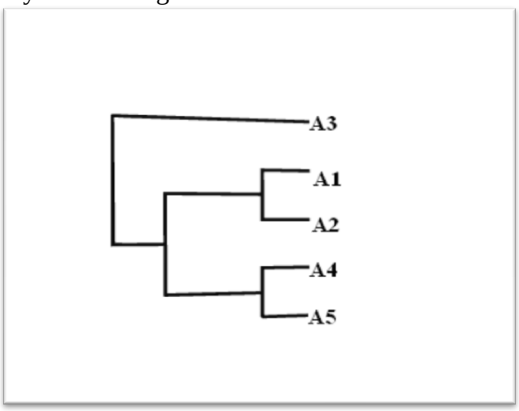


Fig.3: Dendrogram showing the result of clustering on five *Trigonella* accessions.

Where, A1= IC-143851, A2 = IC- 144225, A3 = IC- 332236, A4 = IC- 371755 and A5 = IC-433589

The results obtained during the present investigation seems to be consistant with the studies of Dangi *et al.,* (2004) who reported 70.12% polymorphism in case of *Trigonella foenum-greacum* and 94.83% polymorphism in *Trigonella caerulea* using 40 and 10 decamer primers , while 64 % polymorphism was reported by Kakani *et al* ., (2011) in case of *Trigonella* by using 10 decamer primers. Moreover different workers worked on different plant species and repoted almost similar pattern of polymorphism e.g. Rao *et al.,* (2006) reported 77.8% polymorphism in chick pea by using 10 decamer primers. On the other hand 78.8% polymorphism was reported by Thomas *et al.,* 2006 in wheat upon utilizing 50 random decamer primers, Patra and Chawla, (2010) also found 76.5% polymorphism in eighteen Basmati rice varieties by utilizing 12 random primers. Malviya and Yadav (2010) also observed the same extent of polymorphism 74.7% in pigeon pea by usind 17 random decamer primers. Reby et al., (2011) also observed 72.27% polymorphism in rice genotypes from Kerala by using twenty 10-mer random primers. The detection of high level genetic diversity observed in *Trigonella* genotypes confirms the findings of Welsh and McClelland, (1990) and Dos Santos *et al.,* (1994) who demonstrated that RAPD markers are effective for visualizing high level of polymorphism in plant species. The high level of polymorphic products generated by certain products might be attributed to the fact that in RAPD even small divergence between two cultivars can result in distinct pattern as polymorphism may be the result of any of the various reasons: a) Single nucleotide change within the primer binding site. b) Insertion or deletion with the amplified region so that part of the primer binding site in one of the strand is missing. 3) Complete absence of complimentary sites and 4) The region between the binding site on opposite strand is beyond the normal amplifiable length.

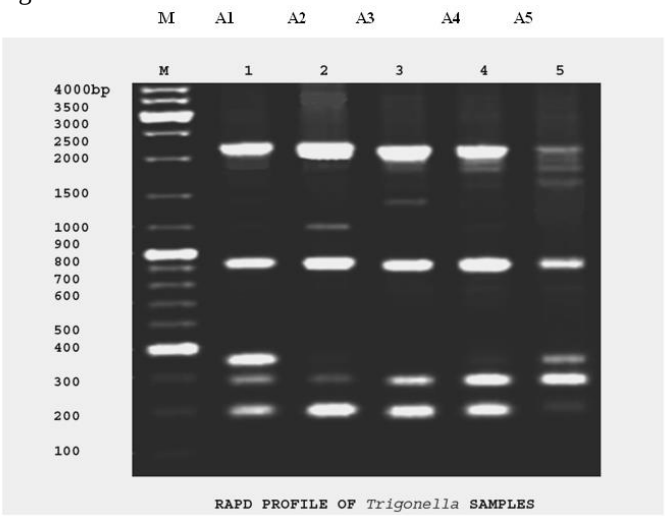


Figure-4 : RAPD profiles from genomic DNA of five *Trigonella* accessions using primer OPP-02 (5' TCG GCA CGC A 3') .Where, M = Marker (100-4000bp). 1 = IC 1438851, 2 = IC 144223, 3 = IC 332236, 4 = IC 371755 and 5 = IC 433589.

The two marker systems used in this study were compared using various parameters like percentage polymorphism, Similarity matrices and clustering of genotypes. A low level of correlation was observed between the two marker systems. RAPD similarity coefficients ranged from 0.26 to 0.61 with 78.79% polymorphism where as similarity coefficients showed a wide range (0.23-0.55) in case of SDS-PAGE analysis with 88.88% polymorphism. Almost similar results were obtained by Mohammad Farooq et al., (2010) in wheat. Fufa et al., (2005) reported that seed storage protein was the major determinant of

end use quality (a highly selected trait)and that genetic estimates based on seed storage proteins were therefore lowest. However, the present study showed relatively higher degree of divergence among *Trigonella* accessions based on seed storage protein and RAPD analysis. The possible reason for the high polymorphism obtained for SDS-PAGE could be due to genetic diversity based on total seed protein as well as DNA marker system used in the present study. As protein markers are selectively neutral, genetic diversity based on these is relatively higher than that of high molecular weight peptides. Also RAPD marker system may amplify similar size fragments from different genomic regions, resulting in underestimation of genetic diversity. Molecular markers such as RAPD and seed storage protein analysis and subsequent banding pattern should be included in the testing of advanced breeding lines. This will not only help in the development of varieties with wider genetic base but will also generate fingerprints of such varieties.

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