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DNA FINGERPRINTING OF SUNFLOWER GENOTYPES (*Helianthus annuus* L.)

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SUMMARY

Sunflower (*Helianthus annuus* L.) being a cross-pollinated crop, genetic adulteration is a vested problem and varietal description is cumbersome. An investigation was carried out using sunflower hybrids, their parental lines and open pollinated varieties to identify and establish phylogenetic relationships among genotypes and characterize them based on both morphometric traits and PCR based RAPD markers. Data on twelve quantitative traits and thirty-two quantitative traits were analyzed. Data for 5 primers were used to establish genetic distance and construct dendrograms using statistical software package. Based on the analysis of clustering of sunflower genotypes it was found that morphometric traits were not in accordance with the molecular markers. At the molecular level, 25 scorable bands were produced with the 5 primers with the number of bands ranging from 3 for OPH-15 to 9 for OPG-10. Of the 25 bands, 14 were polymorphic (58.11%). A male specific third fragment amplified by the primer OPI-16 was inherited by the hybrid KBSH-1 and the primer OPH-15 failed to amplify DNA from all the varieties except Morden.

Key words: sunflower, DNA fingerprinting, PCR and RAPD

INTRODUCTION

Sunflower (*Helianthus annuus* L.) ranking fourth in acreage and production in India (2.4 million ha and 1.8 mt) is grown in the States of Karnataka, Maharashtra, Andhra Pradesh, Punjab, Haryana and Uttar Pradesh (Mangala Rai, 1999). The introduction of this crop to India has helped considerably to increase the country's oil seed production. The area under cultivation keeps increasing due to the crop's day neutrality, wide adaptability, short duration, high yielding potential, remunerative market price and good quality oil. The gene diversity estimated by the available variability for cultivated sunflower is one of the lowest described in crop plants (Tanksley and Orton, 1983). In order to broaden the genetic variability of cultivated sunflower more efforts are required. It would thus be relevant for sunflower breed-

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ing how new methods of DNA fingerprinting can contribute to overcome problems of narrow genetic diversity. Since sunflower is a cross-pollinated crop genetic adulteration has been the vested problem and varietal descriptions are cumbersome however, they are prerequisite for the granting of plant variety protection and utility patents and it is necessary to allow breeders to identify germplasm and monitor protection. National and international registration of the variety needs to pass the criteria of distinctiveness/uniformity/stability tests; varietal identity and purity are also critical elements in assessing the grain entering factories and seed production, etc., for checking of the sample identity to investigate the origin of off-types and distinguishing closely related species. As a result there is an immense need to control the quality of products of grains. In order to fulfil these requirements, variety descriptions must be discriminative, free from environmental effects, interpretable in genetic terms and reflective of pedigree and genetic constitution.

Although classical taxonomic approach has been the traditionally used method of varietal identification which can provide a unique identification of cultivated varieties, its ability to provide reliably discriminating identifications is at best cumbersome. Increased numbers of genetically related releases by plant breeders have made unique identification more difficult to achieve. Secondly, morphologies reflect not only the genetic constitution of the cultivars, but also the interaction of the genotype with the environment (G × E) within which it is expressed. Thirdly, for most morphological traits the genetic control is unknown, although it is known that multiple phenotypes have similar outward appearance. Therefore, it is impossible to determine how completely the genome is sampled by morphological description. Keeping in mind that descriptions based on morphological data are fundamentally flawed in their ability to provide reliable information for differentiating genotypes, we attempted to study phenotypic characters combined with DNA characterization which would be more accurate and easy.

MATERIAL AND METHODS

The genetic material consisted of 7 hybrids, 8 parental lines and 6 varieties. Table 1 depicts the genotypes used in the study.

Ten comparable plants from each genotype were used to study morphological characters and also subjected for RAPD analysis to identify markers differentiating the genotypes. The following were the observations recorded for qualitative and quantitative parameters.

Qualitative parameters

Leaf size, leaf shape, leaf color, leaf anthocyanin coloration, leaf glossiness, leaf blistering, leaf fineness of serration, leaf regularity of serration, leaf wings, leaf angle of lateral veins, length of blade from base to tip, leaf angle between lower part of petiole and stem, stem hairiness at top, ray flower color, ray flower shape, disk

flower color, disc flower anthocyanin coloration of stigma, bract shape, bract coloration, plant nature: position of the closest lateral head to the central head, heat altitude at maturity, head size, head shape, plant branching, type of branching, seed size, seed shape, seed thickness, seed main color, seed mottling, seed stripes, seed color of stripes and position of stripes.

Table 1: Genetic material used

No.	Genotype description		
I	Hybrid	Female parent	Male parent
	KBSH-1	CMS-234A	RHA-6D-1
	PKVSH-27	CMS-2A	AK-1R
	DSH-1	DSF-15A	RHA-857
	APSH-11	CMS-7-1A	RHA-271
II	Experimental hybrid		
	KBSH-41	-	-
	KBSH-42	-	-
	KBSH-44	-	-
III	Varieties		
	CO-2, CO-3, CO-4, EC-68414, GAU-SUF-15 and Morden		

Quantitative parameters

Days to star bud appearance, days to 50 percent of flowering, number of leaves per plant, plant height, number of ray flowers, number of bracts, seed yield per capitulum, test weight, percent seed filling and husk percentage. A one-way analysis of variance along with principal component analysis to cluster the genotypes based on their morphology was carried out following the multivariate statistical package program.

For RAPD analysis dried leaves from each of the ten plants per genotype were finely ground and filtered. The powder obtained was used to isolate DNA following CTAB method (Dellaporta, 1983). Using spectrophotometry, the extracted DNA was further quantified and RAPD analysis performed following the method recommended by Iouras *et al*. (1999), with required modifications. A total of 10 random primers with the following sequences were used for the study.

Primer	Sequence (5^1 to 3^1)
OPC-1	TTCGAGCCAC
OPG-2	GGCACTGAGG
OPG-10	AGGGCCGTCT
OPH-8	GAAACACCCC
OPH-10	CCTACGTCAG
OPH-15	AATGGCGCAG
OPI-1	ACCTGGACAC
OPI-16	TCTCCGCCTT
OPJ-11	ACTCCTGCGA
OPK-14	CCCGCTACAC

Genomic DNA (10 ng) was amplified *in vitro* in a 25 ml reaction mixture containing 2 mM magnesium chloride, 20 mM Tris-HCl (pH 8.4), 100 mM each dNTP's, 15 mg 10 mer primer and 0.50 Taq DNA polymerase. Reaction mixture was overlaid with mineral oil and placed in a thermocycler programmed for the following set of conditions.

Profile 1: initial denaturation: 94°C for 2 minutes;

Profile 2: denaturation: 94°C for 1 minute;

Profile 3: annealing: 36°C for 1 minute;

Profile 4: extension: 72°C for 2 minutes;

Profile 5: final elongation: 72°C for 2 minutes;

Profiles 2, 3 and 4 were programmed to run for forty cycles.

Amplification profiles of 5 primers were compared with each other and bands of DNA fragments were scored as present (1) or absent (0). The data were used to estimate genetic distance on the basis of the number of polymorphic bands and dendrograms were constructed using a statistical software package.

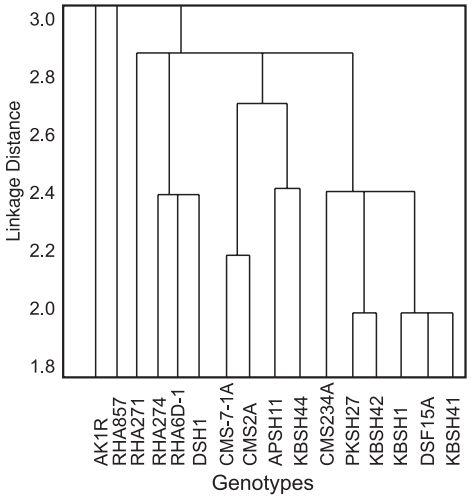

Figure 1: Dendrogram showing clustering of hybrids and their parental lines based on qualitative traits

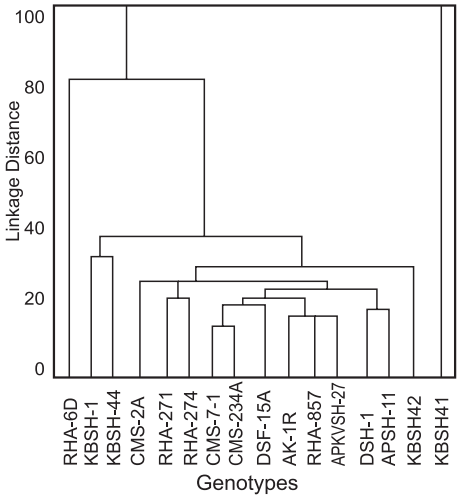

Figure 2: Dendrogram showing clustering of hybrids and their parental lines based on quantitative traits

RESULTS AND DISCUSSION

Figure 1 shows clustering of hybrids and their parental lines into 4 major clusters (based on qualitative traits). These clusters are:

1. CMS-234A, PKVSH-27, KBSH-42, KBSH-1, DSH-15A and KBSH-41;
2. CMS-7-1A, CMS-2A, APSH-11 and KBSH-44;
3. RHA-271, RHA-274, RHA-6D-1 and DSH-1;
4. AK-1R and RHA-857.

KBSH-1 and CMS-234A fell under the same cluster indicating a close relationship of the two. The same was noticed in the case of APSH-11 and CMS-7-1A, but Figure 2 shows all of them forming single genotype cluster. Clustering hybrids and their parental lines into eight groups based on quantitative traits is not in accordance with the qualitative groupings. Figures 3 and 4 show the grouping of varieties based on qualitative and quantitative parameters. While from Figure 3 we can see that GAU-SUF-15 formed a single genotype cluster it was Morden forming a single genotype cluster in Figure 4.

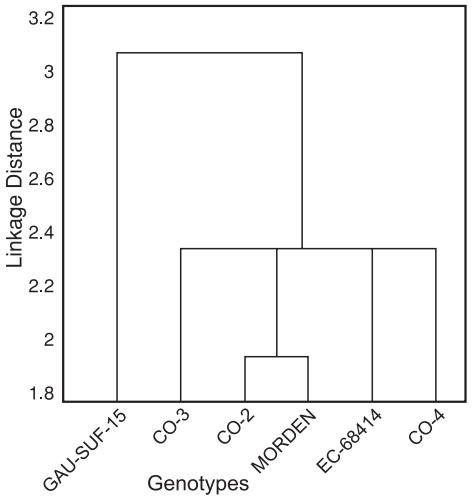

Figure 3: Dendrogram showing clustering of sunflower varieties based on qualitative traits

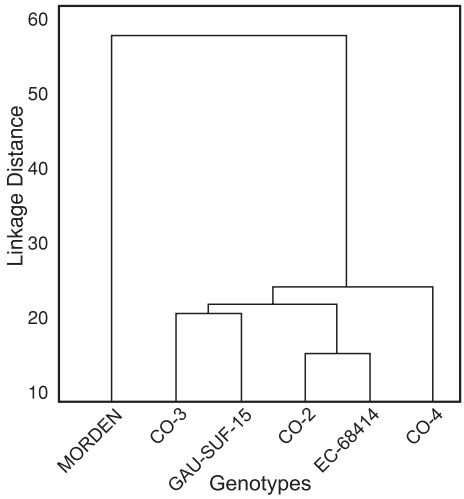

Figure 4: Dendrogram showing clustering of varieties based on quantitative traits

The twenty-two genotypes were initially screened with 10 random primers to detect primers showing amplification. Of the 10 primers used, DNA amplification was obtained with five primers. The RAPD profiles of parents and the hybrids were separately compared to find out whether a hybrid could be positively identified by the occurrence of male/female specific bands and RAPD profiles of six varieties were compared individually for each primer. In total, 25 scorable bands were produced in 22 genotypes with 5 primers (Table 2).

Table 2: Scorable DNA bands generated by different random decamer primers through PCR

Primer	Sequence no. of bands produced	No. of polymorphic bands	% polymorphism
OPC-01	4	2	50.00
OPG-02	4	1	25.00
OPG-10	9	5	55.55
OPH-15	3	3	100.00
OPI-16	5	3	60.00
Pooled	25	14	58.11
Average	5	2.8	

The number of bands produced per primer varied from three for OPH-15 to as many as 9 for OPG-10. Out of the 25 bands, 14 were polymorphic (58.11%). The average number of polymorphic bands was 2.8 per primer. Maximum number of bands was recorded by OPG-10 with 9 fragments while OPH-15 showed 100 percent polymorphism.

Detection of polymorphism between hybrids and their parents

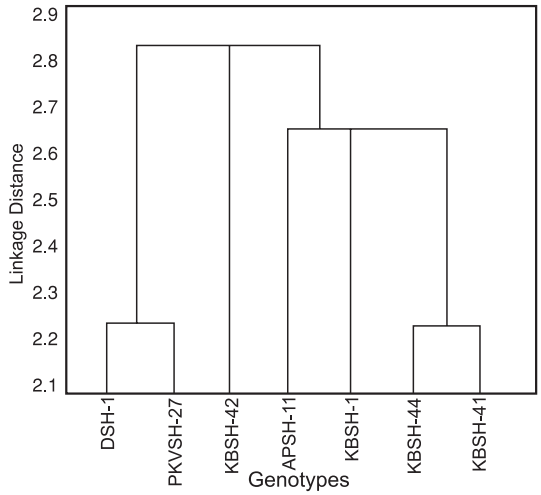

Figure 5: Dendrogram showing clustering of sunflower hybrids generated by RAPD primers

Considering the RAPD analysis of hybrids and their parental lines, it was possible to distinguish most of the hybrids based on the bands that were common to the F_1 and their parental lines. Of the five random primers used in this study, 4 were polymorphic and of the 110 PCR products generated 58.11 percent of the bands were polymorphic among the genotypes tested. This amounts to an average of 14 polymorphic bands per primer. Similar reports were reported by Besnard *et al.* (1997) in sunflower, Chevra *et al.* (1997) in *Brassica*, Heun and Helentjaris (1993) in maize and Noli *et al.* (1997) in barley. The primer OPI-16 (Figure 5) was particularly useful for discriminating most of the hybrids tested along with their parents. In the pair-wise comparison of marker bands, the primer OPI-16 generated 1 to 5 bands aiding in differentiating these genotypes.

The primer OPI-16 generated bands that were specific to male or female genotypes. A band of 2027 bp specific to RHA-6D-1, the restorer line for KBSH-1, was identified. A band of 2322 bp specific to CMS-7-1A, the female line of hybrid APSH-11, was identified while the same band specific to AK-1R, the restorer line of PKVSH-27, served as a marker for differentiating between hybrids and their parental lines. Among KBSH-41, KBSH-42 and KBSH-44, only the absence of the last two fragments (125 bp and 564 bp) in KBSH-42 helped in differentiating the KBSH series that were phenotypically very similar. Such paternal and maternal specific RAPDs are useful in phylogenetic studies. Similar study has been conducted on *Lycopersion esculentum* (Rom *et al.*, 1995). While estimating the genetic diversity as a function of DNA polymorphism, similarity was established between CO-2 and CO-3, Morden and GAU-SUF-15, EC-68414 and CO-4. The sixth and ninth frag-

ments amplified by the primer OPG-19 (Figure 6) were unique only to Morden and GAU-SUF-15.

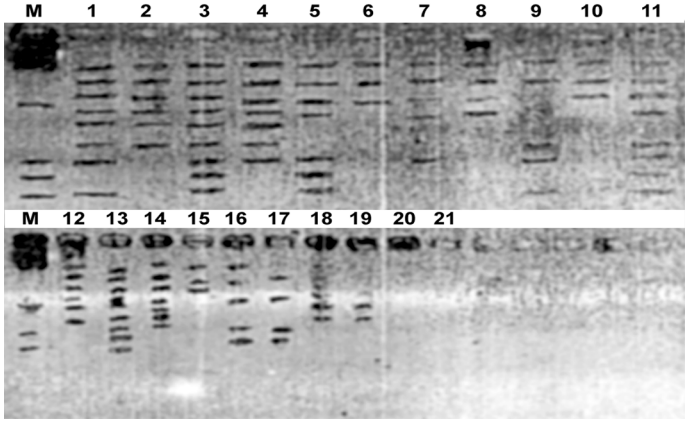

Figure 6: RAPD profile of sunflower genotypes using the primer OPG-10

1. KBSH-41	5. APSH-11	9. CMS-234A	13. RHA-271	17. EC68414	21. CO-2
2. KBSH-42	6. PKVSH-27	10. CMS-2A	14. RHA-6D-1	18. GAU-SUF-15	
3. KBSH-44	7. DSH-1	11. CMS-7-1A	15. AK-1R	19. Morden	
4. KBSH-1	8. DSH-15A	12. RHA-857	16. CO-4	20. CO-3	

Since the primer OPH-15 (Figure 7) failed to amplify DNA from all the varieties except Morden, this could very well serve as a marker for distinguishing Morden from other varieties.

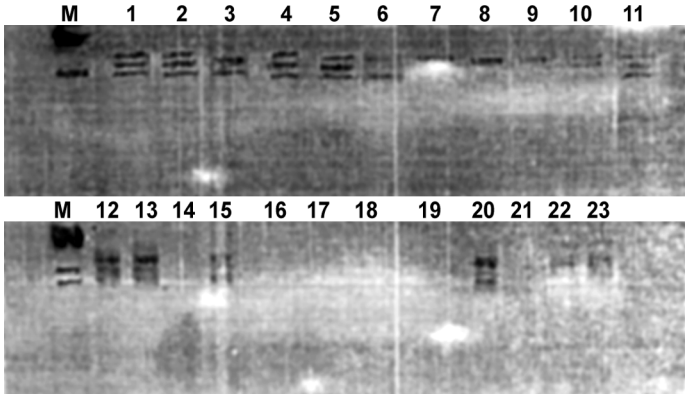

Figure 7: RAPD profile of sunflower genotypes using the primer OPH-15

1. CMS-234A	5. APSH-11	9. AK-1R	13. KBSH-41	17. CO-3	21. Morden
2. KBSH-1	6. RHA-271	10. DSH-15A	14. KBSH-42	18. CO-4	22. 89-B
3. RHA-6D-1	7. CMS-2A	11. DSH-1	15. KBSH-44	19. EC68414	23. 207B
4. CMS-7-1A	8. PKVSH-27	12. RHA-857	16. CO-2	20. GAU-SUF-15	

Figures 8, 9 and 10 show dendrograms based on RAPD analysis for hybrids, inbred lines and varieties, respectively. Similar results are reported by Concilio (1995), Araies and Reisberg (1995), Linder *et al*. (1998) and Reisberg *et al*. (1993).

An increasingly important and widespread use of RAPD is in quality control of seed material. Based on male specific fragments present in a given hybrid, it is possible to estimate the percent of outcrossing in a given amount of seed sample thereby checking the purity of the hybrid seeds.

CONCLUSIONS

It can be concluded from this study that morphologically very similar lines were found to be unrelated at the molecular level and such problems associated with taxonomical classification emphasize the need of complementary keys for identification and characterization of sunflower genotypes. Thus, usefulness of RAPD is demonstrated in this study. Our study also indicates that RAPD markers are readily detected and analyzed in *Helianthus annuus* and the technique lends itself to genotype characterization. Given the results obtained so far, it is possible to establish a standard set of primers that can be used to distinguish and characterize most of the common genotypes which thereby serve as a useful supplement to traditional morphological and agronomic data for plant variety protection.

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FINGERPRINT DNA DE GENOTIPOS DE GIRASOL (*Helianthus annuus* L.)

RESUMEN

Como el girasol (*Helianthus annuus* L.) es una planta de fecundación cruzada, la contaminación genética representaba un problema constante, y la descripción de variedades era algo difícil. Para identificar los genotipos y establecer las relaciones fitogenéticas, se ha realizado una investigación en los híbridos de girasol, sus líneas progenitoras y las variedades de fecundación cruzada, para efectuar la caracterización de genotipos sobre la base de las características morfométricas y los marcadores RAPD por la técnica PCR. Fueron analizados los datos obtenidos para 12 características cuantitativas y 32 características cualitativas. Los datos para 5 primers fueron utilizados con el fin de establecer la distancia genética y construir dendrogramas, mediante el programa estadístico. Sobre la base de la representación dendrográmica de agrupamiento de los genotipos de girasol, las características morfométricas no se encontraban en armonía con los marcadores moleculares. En el nivel molecular, mediante 5 primers se obtuvieron 25 cintas medibles, en las cuales el número de cintas varía desde 3, en OPH-15, hasta 9, para OPG-10. De 25 cintas, 14 eran polimorfas (58,11%). El tercer fragmento, masculinamente específico, obtenido por el primer OPI-16, fue heredado del híbrido KBSH-1, y el primer OPH-15 no multiplicaba DNA en ninguna variedad, excepto en Morden.

EMPREINTE ADN DES GÉNOTYPES DE TOURNESOL (*Helianthus annuus* L.)

RÉSUMÉ

Comme le tournesol (*Helianthus annuus* L.) est une culture fécondée par croisement, la modification génétique a toujours été un problème et la description des variétés s'en trouve compliquée. Nous avons fait cette recherche dans le but d'identifier les génotypes et d'établir les liens phylogénétiques. Nous avons utilisé des hybrides de tournesol, leurs souches parentales et les variétés fécondées par croisement pour caractériser les génotypes d'après les traits morphométriques et les marqueurs RAPD par la technique PCR. Les données obtenues pour 5 caractéristiques quantitatives et trente-deux caractéristiques qualitatives ont été analysées. Les données obtenues pour 5 amorces ont été utilisées pour établir la distance génétique et construire des dendrogrammes à l'aide de logiciels de statistiques. D'après le dendrogramme montrant le regroupement de génotypes de tournesol, les caractéristiques morphométriques ne correspondaient pas aux marqueurs moléculaires. Au niveau moléculaire, au moyen de 5 amorces, 25 bandes mesurables pour lesquelles le nombre de bandes variait de 3 pour OPH-15 à 9 pour OPG-10 ont été obtenues. Quatorze des 25 bandes étaient polymorphes (58,11%). Un troisième fragment spécifique mâle obtenu par l'amorce OPI-16 avait été hérité de l'hybride KBSH-1 et l'amorce OPH-15 n'avait réussi à multiplier l'ADN d'aucune variété sauf le Morden.