Theor Appl Genet (1994) 89: 435–441

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Molecular marker analysis of *Helianthus annuus* L. 1. Restriction fragment length polymorphism between inbred lines of cultivated sunflower

Received: 20 September 1993 / Accepted: 11 February 1994

Abstract cDNA and *Pst*I genomic clones have been used to assess levels of restriction fragment length polymorphism (RFLP) in *Helianthus annuus* and to determine the inter-relationships between a diverse set of 24 inbred lines. Of the cDNA clones screened 45% were useful as RFLP probes, compared to less than 20% from the *Pst*I library, which showed high levels of redundancy for high copy sequences. Fifty-seven low-copy DNA probes (23 *Pst*I and 34 cDNA clones) were used to fingerprint 12 maintainer (B) lines and 12 restorer (R) lines. The average number of RFLP variants per probe was found to be 3.2, with a mean polymorphic index of 0.49, indicating that high levels of nuclear DNA polymorphism are to be found in cultivated sunflower. Cluster and principal coordinate analysis of the fingerprinting data clearly separated the maintainer and restorer lines, but there was a degree of association between 2 unbranched R-lines and the B-line germ plasm pool.

Key words Restriction fragment length polymorphism (RFLP) · *Helianthus annuus* · Cytoplasmic male sterility (CMS) · Restorer · Maintainer

Introduction

The genus *Helianthus* contains both annual and perennial species that exhibit various ploidy levels where x=17 (Heiser et al. 1969). Cultivated sunflower (*Helianthus annuus* L.) is an allogamous, diploid annual grown mainly for its seed oil, which is high in unsaturated fatty acids. Early commercial cultivars were open pollinated; however the discovery of a source of cytoplasmic male sterility (CMS) from *Helianthus petiolaris* (Leclerq 1969) led to the rapid introduction of hybrids during the 1970s. Commercial sunflower germ plasm can now be functionally divided into two broad groupings: the maintainer or B-lines and the CMS restorer or R-lines. In addition to having the dominant CMS restorer gene Rf_1 (Kinman 1970), R-lines are usually mildew resistant and possess the recessive branching genes b_1 and b_2 (Kovacik and Skaloud 1990), which produce multicephalous plants; the advantage of which is prolonged pollen production. It is the male sterile A-lines (which are alloplasmic to the B-lines) that are crossed to the R-lines for hybrid seed production.

Although sunflower hybrids are now one of the world's most important sources of vegetable oil, the application of biotechnology to this crop has been extremely limited (Friedt et al. 1991). For example, there is no genetic linkage map for sunflower, in contrast to other oil seed crops such as *Brassica napus* (Landry et al. 1991) and *Glycine max* (Shoemaker et al. 1992). To date the use of nuclear restriction fragment length polymorphisms (RFLPs) on sunflower germ plasm has been limited to taxonomic studies of the genus *Helianthus* (Choumane and Heizmann 1988, Gentzbittel et al. 1992) and to the identification of interspecific hybrids (Krauter et al. 1991). The study presented here was therefore undertaken to assess the level of nuclear DNA polymorphism in proprietary and publicly available breeding material, with the ultimate aim to determine the feasibility of constructing a genetic linkage map for sunflower.

Communicated by J. W. Snape

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Materials and methods

Plant material

In order to assess the level of RFLP within cultivated sunflower inbred lines, 12 maintainer (B) lines and 12 restorer (R) lines were chosen to represent geographically diverse germ plasm pools (Table 1).

Table 1 The origin and source of the sunflower inbred lines fingerprinted in this study. Lines which have the branching phenotype are marked with an asterisk (*)

Inbred	Version	Origin	Source
BFR2603	Maintainer	Moroccan population	INRA
BSA52	Maintainer	South African material	INRA
BHA89	Maintainer	Russian population VNIIMK 8931	USDA
ZENB1	Maintainer	Argentinian material	ZENECA
ZENB2	Maintainer	Argentinian material	ZENECA
ZENB3	Maintainer	Argentinian material	ZENECA
ZENB4	Maintainer	Australian material	ZENECA
ZENB5	Maintainer	Australian material	ZENECA
ZENB6	Maintainer	Australian material	ZENECA
ZENB7	Maintainer	Australian material	ZENECA
ZENB8	Maintainer	Argentinian material	ZENECA
ANSBCPL	Maintainer	Bulgarian material	NOVI SAD
ZENR1*	Restorer	Argentinian material	ZENECA
ZENR2*	Restorer	Australian material	ZENECA
ZENR3*	Restorer	Australian material	ZENECA
ZENR4*	Restorer	Australian material	ZENECA
ZENR5*	Restorer	Australian material	ZENECA
ZENR6	Restorer	Australian material	ZENECA
RFRPR56*	Restorer	American, Yugoslave material	INRA
RFRPAT4*	Restorer	Armvir 9345 × *H. tuberosus*	INRA
RFRPW3	Restorer	American, Yugoslave material	INRA
RHA297*	Restorer	American material	USDA
RFRPAC2*	Restorer	HA61 × *H. petiolaris*	INRA
RHA801*	Restorer	American inbreds	USDA

For a more detailed description of the pedigrees of the public lines refer to Korell et al. (1992). This material was grown under standard glasshouse conditions until several fully expanded leaves could be harvested. The tissue was then frozen in liquid nitrogen, lyophilised and ground to fine powder in a mill.

DNA isolation

DNA was extracted from the freeze-dried leaf powder using the method described by Saghai-Maroof et al. (1984) with the modification that the DNA was not recovered by isopropanol precipitation, but via CsCl density gradient centrifugation.

Genomic library construction

Twenty microgrammes of DNA were digested to completion with *Pst*I, and the resulting fragments run out on a 1.0% TBE-agarose gel. Two size fractions, 1.0–2.0 kb (fraction 1) and 0.5–1.0 kb (fraction 2), were collected onto DE81 paper (Whatman) according to Dretzen et al. (1981), and the eluted DNA ligated into *Pst*I-digested, dephosphorylated pUC13. Following transformation into *E. coli* strain TG2, recombinants were selected on ampicillin, IPTG and X-Gal, and plasmid isolated according to Birnboim and Doly (1979). Recombinant clones from the fraction 1 were designated H1001, H1002 etc., and those from fraction 2 as H2001, H2002 etc.

mRNA isolation and cDNA cloning

Total RNA was extracted from etiolated seedlings using the method described by Brandt and Ingversen (1978). mRNA was purified via oligo-dT cellulose column chromatography and the cDNA synthesised using an Amersham cDNA Synthesis System Plus kit. The sunflower cDNA was then cloned into lambda ZAPII (Stratagene) after the addition of *Eco*RI linkers and size-fractionation on a Sephacryl S300 column (Pharmacia). The ligation was packaged using Giga Pack Gold II extracts (Amersham) and plated onto *E. coli* strain XL-1 Blue; 2.3×10^5 clones/µg of vector were obtained and individual recombinant plaques picked into SM buffer. cDNA clones were coded with the prefix C.

Preparation of DNA probes

DNA probes were produced by polymerase chain reaction (PCR) amplification (Saiki et al. 1988) from plasmid in the case of genomic clones, or from phage in SM buffer for the cDNA clones. Inserts were amplified using 25 base primers, designed to include the universal and reverse-sequencing priming sites. PCR products were purified through 0.6% low-melting-point agarose (Seakem) gels, and the excised DNA labelled with [α-^{32}P]dCTP (Amersham) via random priming (Feinberg and Vogelstein 1983).

DNA restriction and Southern analysis

Sunflower DNA was digested using 4 units of enzyme/µg according to the manufacturers instructions (Northumbria Biologicals). Ten micrograms of DNA per lane was loaded onto 0.8% TBE-agarose (Seakem) slab gels, and electrophoresis was carried out overnight at 3 V/cm. The DNA was blotted onto Hybond-N membrane (Amersham), and hybridisations were carried out overnight using standard conditions. Unbound probe was removed by two 30-min washes in 0.5× SSC, 0.1% SDS at 60 °C. Filters were exposed to X-omat ar X-ray film (Kodak) with two intensifying screens at –80 °C.

Statistical analyses

All of the following analyses were carried out using programmes written in SAS (1985).

Genetic distances were calculated for all possible pairs of lines as: the number of probes for which the lines were identical plus half the number of probes for which the lines shared one variant, divided by the number of probes for which data was present for both lines. A dissimilarity matrix was then constructed from these measures of genetic distances and used to produce an average linkage cluster analysis and a principal coordinate analysis (PCOA) (Gower 1966).

A canonical discriminant analysis was also carried out to determine whether the RFLP genotype could be used to predict the phenotype of the inbred lines (i.e. maintainer or restorer and branched or unbranched). This procedure included: an analysis of variance for each probe to test that the class means were equal, a resubstitution to check whether the discriminant function would classify the observations correctly and a cross-validation to see whether the discriminant function, calculated without an observation, would still produce the same classification.

Results

Screening of libraries for low-copy clones

Individual clones from the two source libraries were screened against Southern blots of genomic DNA isolated from 6 sunflower inbred lines and digested with four restriction enzymes; *Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII. In general, the hybridisation patterns obtained could be classified into four groups on the basis of signal strength and the number of restriction fragments detected by the probe:

1) simple/polymorphic – fewer than four variable hybridising restriction fragments/bands;

2) complex/polymorphic – more than four hybridising bands, some of which are variable;

3) monomorphic – one or a number of hybridising bands, all of which are constant;

Table 2 *Pst*I and cDNA library screening results

	*Pst*I (Fraction 1)	*Pst*I (Fraction 2)	cDNA	Total
Simple/polymorphic	8 (15%)	18 (19%)	43 (45%)	70
Monomorphic	1	14	24	39
Complex	5	6	24	35
Repetitive	2	0	5	7
Redundant	38	55	0	93
Total	54	93	96	244

4) repetitive – hybridisation to many tens or hundreds of fragments to produce a smear.

The distribution of clones between these classes for both source libraries is shown in Table 2. Only clones in the simple/polymorphic class were considered as putative RFLP probes, and the abundance of such clones was found to vary by a factor of 2.5 between the *Pst*I and cDNA libraries. However, the *Pst*I library contained a high level of redundancy for several families of either repetitive sequence, based on the very short exposure times or organellar DNA (Table 2), which significantly reduced the efficacy of RFLP probe production.

Inbred line fingerprinting

From the 70 clones identified as potential RFLP probes, 23 *Pst*I clones and 34 cDNA clones were used to fingerprint 12 maintainer (B) lines and 12 CMS restorer (R) lines of diverse pedigree and origin (Table 1). Only single probe/enzyme combinations were used to generate the fingerprinting data set, and these were selected on the basis of the highest level of polymorphism seen on the screening autoradiographs. In total, 14 probes were used in conjunction with the restriction enzyme *Dra*I, 19 with *Eco*RI, 18 with *Eco*RV and 6 with *Hin*dIII. The majority of probes hybridised to single restriction fragments, and these were considered, in the absence of segregation data, as allelic variants at a single locus. These variants were scored by designating the largest molecular weight variant as AA, the next largest BB and so on. However, in some inbreds a small number of loci appeared to be heterozygous, and these were scored, for example, as AD or CE (Fig. 1). The four probes which hybridised to multiple restriction fragments were scored according to the overall patterns shared by different inbred lines.

The number of RFLP variants detected by the 57 probes ranged from 2 up to 7, with the average value being 3.2 variants per probe. In total, 185 RFLP variants were observed in this set of 24 sunflower inbreds, with the B-lines (149 variants) and the R-lines (145 variants) showing similar levels of polymorphism; however some variants were found to be specific to either B- or R-line germ plasm. Of all the variants 24% had a frequency of greater than 0.5, whilst the mean polymorphic index [obtained by subtracting from unity the sum of the squared RFLP variant frequencies (Marshall and Allard 1970)] was found to be 0.49. There was no significant difference between the mean polymorphic indices calculated for cDNA and for *Pst*I clones.

Multivariate analyses of the RFLP data

Table 3 shows the dissimilarity matrix derived from the RFLP fingerprinting data. The majority of distances lie in the range 40–70%, with the most dissimilar lines being ZENB8 and RFRPAC2 and the most similar being ZENB4 and ZENB7. Tables of genetic distances are unable to reveal the complex genetic relationships within the germ plasm being studied. However, these multidimensional relationships may be partially visualised using multivariate analyses such as cluster and principal coordinate analysis. Figure 2 shows an average linkage cluster analysis of the 24 sunflower lines, with inbreds generally being grouped into a B-line cluster, BSA52 to ZENB8 (including RFRPW3 and ZENR6), and two R-line clusters, RFRPAC2 to RFRPAT4 (including ANSBCPL) and ZENR5 to

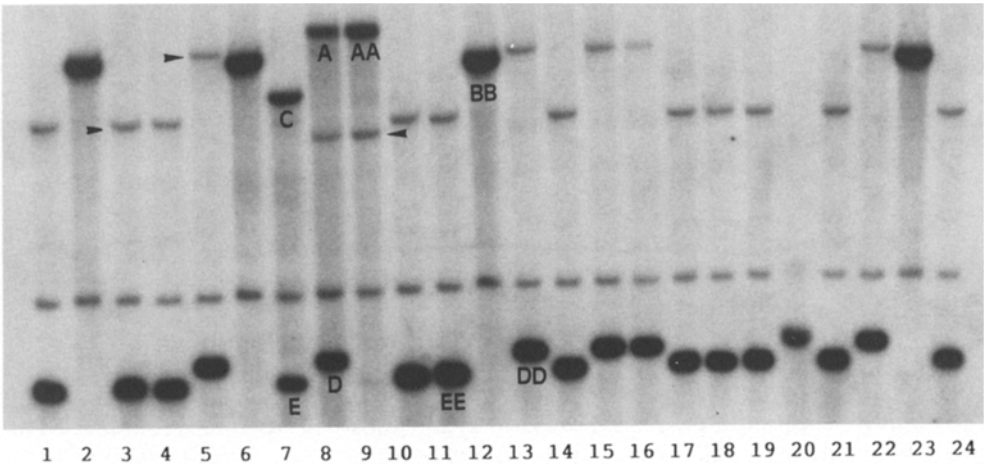

Fig. 1 An autoradiograph of the cDNA probe C0151 hybridised to the 24 inbred lines (*lanes 1–12* B lines, *lanes 13–24* R lines) digested with *Eco*RI. The RFLP variants scored in the analysis are labelled *A–E*. The *arrowheads* indicate the presence of a putative duplicated, polymorphic locus based on the lower signal intensity

Table 3 Genetic distances between all possible pairs of lines calculated as dissimilarities

	BFR2603	BSA52	BHA89	ZENB1	ZENB2	ZENB3	ZENB4	ZENB5	ZENB6	ZENB7	ZENB8	ANSBCPL	ZENR1	ZENR2	ZENR3	ZENR4	ZENR5	ZENR6	RFRPR56	RFRPAT4	RFRPW3	RHA297	RFRPAC2	RHA801
BFR2603	0	55	55	54	51	49	59	59	62	58	51	53	48	57	53	66	51	55	54	54	39	56	55	58
BSA52	55	0	40	54	36	47	42	50	51	44	68	54	61	52	58	45	62	54	54	45	44	58	64	46
BHA89	55	40	0	25	38	33	17	41	34	15	54	48	51	50	58	52	53	45	56	52	27	54	67	54
ZENB1	54	54	25	0	47	39	39	46	41	36	29	56	53	44	55	56	66	49	61	53	37	54	69	59
ZENB2	51	36	38	47	0	37	42	57	54	42	67	45	47	48	46	47	60	43	58	54	48	46	61	46
ZENB3	49	47	33	39	37	0	41	57	48	41	59	42	45	49	52	41	46	56	55	46	40	54	55	57
ZENB4	59	42	17	39	42	41	0	47	40	4	58	47	51	57	66	56	49	51	61	53	36	55	67	48
ZENB5	59	50	41	46	57	57	47	0	22	44	62	58	56	60	54	63	54	57	52	57	54	54	61	52
ZENB6	62	51	34	41	54	48	40	22	0	37	54	53	53	54	60	60	61	59	54	53	46	56	68	57
ZENB7	58	44	15	36	42	41	4	44	37	0	57	47	49	56	64	47	49	59	54	35	54	66	66	50
ZENB8	51	68	54	29	67	59	58	62	54	57	0	59	60	51	59	59	66	56	65	64	42	65	70	67
ANSBCPL	53	54	48	56	45	42	47	58	53	47	59	0	39	57	61	57	53	53	64	53	45	45	45	59
ZENR1	48	61	51	53	47	45	51	56	53	49	60	39	0	42	41	47	60	51	61	47	42	25	39	53
ZENR2	57	52	50	44	48	49	57	60	54	56	51	57	42	0	41	39	67	56	65	53	38	34	65	49
ZENR3	53	58	58	55	46	52	66	54	60	64	59	61	41	41	0	53	59	57	46	58	54	32	51	53
ZENR4	66	45	52	56	47	41	56	63	60	56	59	57	47	39	53	0	59	58	52	46	45	47	56	59
ZENR5	51	62	53	66	60	46	49	54	61	47	66	53	60	67	59	59	0	60	29	55	61	67	47	47
ZENR6	55	54	45	49	43	56	51	57	59	49	56	53	51	56	57	58	60	0	62	59	52	59	58	56
RFRPR56	54	54	56	61	58	55	61	52	54	59	65	64	61	55	46	52	29	62	0	54	54	60	53	32
RFRPAT4	54	45	52	53	54	46	53	57	53	54	64	53	47	53	58	46	55	59	54	0	45	52	50	47
RFRPW3	39	44	27	37	48	40	36	54	46	35	42	45	42	38	54	45	61	52	54	45	0	46	59	51
RHA297	56	58	54	54	46	54	55	54	56	54	65	45	25	34	32	47	67	59	60	52	46	0	45	51
RFRPAC2	55	64	67	69	61	55	67	61	68	66	70	45	39	65	51	56	47	58	53	50	59	45	0	49
RHA801	58	46	54	59	46	57	48	52	57	50	67	59	53	49	53	59	47	56	32	47	51	51	49	0

RHA801 (including BFR2603). The majority of lines clustered below a dissimilarity of 45%; however some inbreds such as BFR2603, RFRPAT4 and ZENR6 failed to do so, indicating their genetic distinctness from the other germ plasm in this study.

A clearer representation of the genetic relationships can be seen in the two-dimensional (2-D) PCOA (Fig. 3), which explains 25% of the total variation in the RFLP data. The first principal coordinate alone (which only explains 14% of the total variation) is sufficient to split the majority of the lines into the two main germ plasm pools (i.e. maintainer and restorer lines). The validity of these groupings was checked by canonical discriminant analysis, which removes each line in turn and then attempts to reclassify it on the basis of the imposed group structure (i.e. maintainer versus restorer and branched versus unbranched). By this method the two restorer lines RFRPW3 and ZENR6 were always classified as unbranched, maintainer lines.

Discussion

From the data presented here it appears that sunflower cDNAs are a richer source of RFLP probes than genomic *Pst*I clones, as is also the case in tomato (Miller and Tanksley 1990), lettuce (Landry et al. 1987) and lentil (Havey and Muehlbauer 1989). Traditionally, *Pst*I libraries have been used as source of plant RFLP probes, owing to their ease of construction and potential enrichment for hypomethylated genic (low-copy) regions (Antequera and Bird 1988). However, the literature shows a wide variation in the content of low-copy DNA sequences in *Pst*I libraries [e.g. from 22% in lentil (Havey and Muehlbauer 1989) up to 95% in *Phaseolus vulgaris* (Vallejos et al. 1992)], which is probably a reflection of differences in the frequency of C-methylation between plant genomes (McCouch et al. 1988). However, sunflower has a 5-methyl cytosine content of 37% (Ergle and Katterman 1961), which is the highest level found in any crop plant species studied to date (Messeguer et al. 1991). Therefore the low success rate in isolating RFLP probes from the sunflower *Pst*I library was probably due to the high levels of redundancy for a number of high-copy, monomorphic sequences. These clones may have been organellar in origin, as the preferential cloning of chloroplast DNA has been reported in the *Pst*I libraries of bamboo (Friar and Kochert 1991), barley (Jahoor et al. 1991) and *Brassica* (Figdore et al. 1988). Low-copy, polymorphic clones were successfully isolated from both *Pst*I size fractions (Table 2); however no correlation was found between the insert size and the level of polymorphism detected. Similar results have also been reported in tomato (Miller and Tanksley 1990).

In total 185 RFLP variants were detected by 57 probes, which gives an average value of 3.2 RFLP variants per

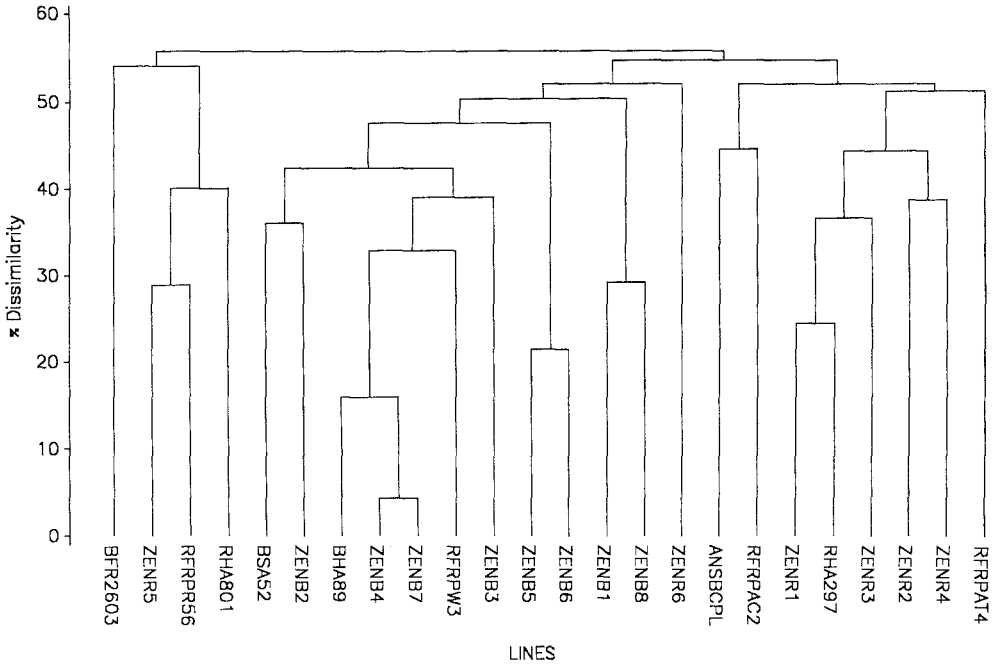

Fig. 2 An average linkage cluster analysis of the 24 sunflower inbred lines based on 57 unmapped probes

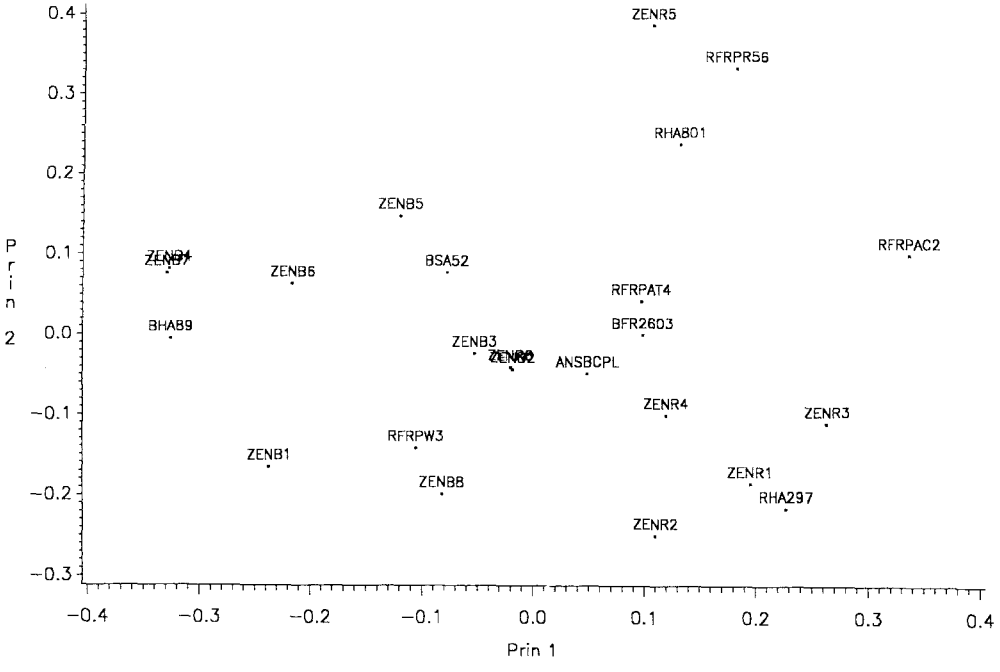

Fig. 3 A two-dimensional PCOA which explains 25% of the variation in the RFLP data. Two pairs of lines are not clearly separated in this 2-D analysis, and these are ZENB2 and ZENR6 at coordinates (−0.02,−0.04) and ZENB4 and ZENB7 at (−0.33, 0.08)

probe. This maybe an underestimate of the variation in the genome as a whole because the majority of probes were selected on the basis of hybridisation to single restriction fragments. Livini et al. (1992) have shown that higher levels of polymorphism can be detected in maize using probes that produce complex hybridisation patterns, thereby increasing the average number of RFLP variants per probe from 3.8 to 7.7. However, the utility of such probes in segregating populations may be reduced due to difficulties in assigning the variant bands to the multiple loci being detected.

The mean polymorphic index for a sunflower RFLP probe was found to be 0.49, which is considerably higher than the value of 0.3 calculated for soybean probes (Keim et al. 1992). In general, allogamous crops (e.g. maize and potato) have higher levels of intraspecific variation than autogamous plants (e.g. tomato and soybean), which has been reported as being due to higher frequencies of insertion/deletion events in the nuclear genome (Gebhardt and Salamini 1992). Therefore, it is not unexpected to find a relatively high level of restriction fragment length polymorphism in sunflower, and this fact should facilitate the rapid construction of a RFLP linkage map for this crop.

The statistical analyses of the RFLP fingerprinting data clearly show that some lines are very closely related. For example, ZENB4 and ZENB7 have a genetic similarity of

96% (Table 3) and are in fact sib-selections out of the same pedigree cross involving HA89. These 3 lines are grouped together in both the cluster analysis and the two-dimensional PCOA (Figs. 2 and 3). Other related lines include ZENB1, which was selected out of a cross between ZENB8 and HA89. This line is intermediate between its progenitors in the 2-D PCOA; however the three-dimensional PCOA, which explains a further 9% of the variation (data not shown), clearly shows it to be more closely related to ZENB8 (the recurrent parent) than to HA89. In general, however, the majority of dissimilarities were in the range of 40–70% (Table 3), reflecting the diverse origin of the germ plasm selected for study (Table 1). The pairs of lines which showed the highest dissimilarities (e.g. ZENB8/RFRPAC2) were used to construct F_2 populations for RFLP linkage analysis.

The ability of RFLPs to distinguish major phenotypic and heterotic groupings has been shown in melon (Neuhausen 1992), maize (Godshalk et al. 1990) and now in this study of sunflower inbred lines. Although an unmapped set of RFLP probes were used, the 2-D PCOA (Fig. 3) illustrates the genetic distinctness of maintainer and restorer lines. Similar results have also been obtained by Gentzbittel et al. (1994) using an independent set of probes to fingerprint 17 sunflower inbreds. This clear separation of the sunflower germ plasm is the result of breeders generally confining their inbred line development to crosses made within one or other of these groups (i.e. B × B or R × R). An added advantage to the breeder, only in making R × R populations, is that screening for the inheritance of mildew resistance and a CMS restorer gene can be avoided.

The validity of the B- and R-line groupings revealed in the multivariate statistical analyses was tested by canonical discriminant analysis. When the RFLP data was used to classify each line as either maintainer or restorer, 2 R-lines (i.e. RFRPW3 and ZENR6) were consistently classed as B-lines. These inbreds are clearly associated with the B-line germ plasm in both the cluster analysis (Fig. 2) and the 2-D PCOA (Fig. 3). RFRPW3 and ZENR6 each possess a CMS restorer gene; however they are unbranched, like the maintainer lines (Table 1). It is therefore possible to speculate that either these two R-lines were derived from B × R crosses or that a number of probes linked to the branching gene(s) were used in this study. The development of a mapped set of RFLP probes for fingerprinting, including ones known to be linked to the branching and restorer genes, will enable a more accurate study of the genetic relationships within cultivated sunflower germ plasm.

Acknowledgements The authors would like to thank Alberto Leon and Chiara Livini for their constructive criticism of the draft manuscript, Marie-Laure Ribet for supplying the seed of the inbred lines and Angela Woods and Penny Hill for their technical assistance.

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