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Genetic diversity in European and Mediterranean faba bean germ plasm revealed by RAPD markers

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Abstract Broadening of the genetic base and systematic exploitation of heterosis in faba bean requires reliable information on the genetic diversity in the germ plasm. Three groups of faba bean inbred lines were examined by means of RAPDs (random amplified polymorphic DNAs) assays: 13 European small-seeded lines, 6 European large-seeded lines, and 9 Mediterranean lines. Out of 59 primers, 35 were informative and yielded 365 bands, 289 of which were polymorphic with a mean of 8.3 bands per primer. Monomorphic bands were omitted from the analyses and genetic distances (*GD*) were estimated via the coefficient of Jaccard. The mean *GD* among the European small-seeded lines was significantly greater than those among the lines of the other two groups. Repeatability of *GD* estimates was high. Cluster (UPGMA) and principal coordinate analyses identified European small-seeded lines and Mediterranean lines as distinct groups with European large-seeded lines located in between. The results are in harmony with published archaeobotanical findings. We conclude that RAPDs are useful for classification of germ plasm and identification of divergent heterotic groups in faba bean.

Key words RAPD · Genetic diversity · *Vicia faba* L. · Germ plasm · Gene pools

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Introduction

Faba bean (*Vicia faba* L.) is a grain legume grown for its high seed-protein content (about 30%). Although its potential for high yield and its low input requirements have long been recognized, its acceptance in European agriculture is rather low owing to insufficient yield and yield stability. A broadening of the genetic variability and systematic exploitation of heterosis have been suggested as means to overcome these problems (Kittlitz et al. 1993; Bond 1993). Thus, information about the structure of the genetic diversity and the identification of germ plasm groups are mandatory if crossing plans and selection procedures are to be optimized.

Faba beans have attracted the interest of taxonomists and evolutionists for a long time. Traditionally, the species is divided according to seed size into small-seeded minor beans (seed weight of 0.2–0.5 g), large-seeded major beans (seed weight of 0.8 to more than 2.0 g), and beans of intermediate seed size. Large-seeded beans are mainly used for human consumption, whereas beans of smaller seed size are grown for feed.

There is substantial variation in seed size in all regions of cultivation, indicating that this criterion is of questionable validity for taxonomic classification of subspecies. Alternative approaches for intra- and interspecific taxonomy have been reported based on geographic origin, morphology, karyotype, or isozymes (Cubero 1974; Hammer et al. 1986; Hanelt 1972; Käser and Steiner 1983; Ladizinsky 1975; Mancini et al. 1989; Yamamoto 1973). These criteria are either influenced by environmental factors and stage of plant development or they reveal only limited variation. Recently, the advent of molecular markers [e.g., restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNAs (RAPDs)] has opened a new vista to study genetic diversity. These markers have the potential to reveal a large amount of variation with good coverage of the entire genome (for review, see Melchinger 1994). RAPDs circumvent many technical limita-

tions of RFLP assays. Both RAPDs and RFLPs have been used to characterize genetic diversity in several species (Tingey et al. 1992).

Genetic diversity determined with molecular markers has been explored in the *Vicia* family mainly at the interspecific level (Van de Ven et al. 1993). Information about the genetic diversity within elite germ plasm of faba bean is far from being adequate for breeding purposes. Moreover, breeders can only capitalize on the genetic variation within the species because *Vicia faba* cannot successfully be crossed with any related species.

The objectives of the study presented here were to (1) examine the degree of RAPD polymorphism and the repeatability of RAPD analyses in *Vicia faba* and (2) study the genetic diversity within and between the European minor and major germ plasm and the Mediterranean germ plasm.

Materials and methods

Plant materials

Twenty-eight *Vicia faba* inbred lines, comprising 13 European minor lines, 6 European major lines, and 9 Mediterranean lines, were selected to represent the corresponding faba bean germ plasm groups (Kittlitz et al. 1993; Table 1). The lines were generated by the single-seed descent method from their progenitor varieties. The minor lines are tall (up to 180 cm), non-tillering spring beans. The major lines are less tall, tillering spring beans. Both develop large leaflets and a high number (five to nine) of flowers per inflorescence. The Mediterranean, winter-sown lines are shorter (about 100 cm), tillering, and have marked genotypic variation for leaflet size and a low number (one to four) of flowers per inflorescence.

Ten lines (nos. 2, 3, 4, 9, 14, 18, 20, 23, 24, 25) were assayed in duplicate as controls, generated by using DNA from the respective standard lines. Four lines (nos. 2, 3, 10, 11) were further replicated as independent controls. These were generated by using a second plant within the line, grown in a different environment, and DNA was extracted in a different laboratory using a different method.

DNA isolation and PCR amplification

Plants were grown in isolation cages in the open field. DNA was isolated according to the procedure described by Rogers and Bendich (1985) with 5–10 g young leaf tissue from 1 plant per line at anthesis. Isolated DNA was frozen at $-80\,°C$. For the independent replicates of lines 2, 3, 10, and 11, plants were grown in the greenhouse, and DNA was extracted according to the procedure described by Saghai-Maroof et al. (1984).

For the polymerase chain reaction (PCR) reactions, the number of cycles (35, 40, and 45), Mg^{2+} concentration (1.5, 2.0, 2.5, and 3 m*M*), *Taq* polymerase concentration (0.25, 0.5, and 1.0 U), and content of genomic DNA (20, 60, 120, and 240 ng DNA in a final volume of 25 µl) were optimized in pilot assays. A Hybaid thermocycler was used. As a result, the assays were run with 120 ng genomic DNA, 100 µ*M* each of

Table 1 Coding and pedigree information of 28 faba bean inbred lines representing three germ plasm groups

Number	Code	Single seed weight (*g*)	Progenitor population	Provenience	Germ plasm group	Generation
1	D03	0.33	Diana	Germany	Minor	F_9
2	D07	0.32	Diana	Germany	Minor	F_9
3	H20	0.39	Herz Freya	Germany	Minor	F_9
4	H20s[a]	0.45	Herz Freya	Germany	Minor	F_6
5	H23	0.36	Herz Freya	Germany	Minor	F_9
6	K25	0.38	Kristall	Germany	Minor	F_9
7	K31	0.39	Kristall	Germany	Minor	F_9
8	N36	0.54	Nixe	Germany	Minor	F_9
9	KT42	0.43	K. Thüringer	Germany	Minor	F_9
10	KT43	0.35	K. Thüringer	Germany	Minor	F_9
11	KT47	0.38	K. Thüringer	Germany	Minor	F_9
12	C14	0.52	Cagnote	France	Minor	F_8
13	SCI[b]	0.37	Cantalupe	England	Minor	$>F_5$
14	CAm	1.60	Con Amore[f]	Netherlands	Mijor	F_9
15	CEx	1.56	Canner Express[g]	England	Major	F_9
16	Fel	0.95	Felicia[f]	Netherlands	Major	F_9
17	Hed	0.80	Hedosa[f]	Netherlands	Major	F_9
18	Min	1.20	Minica[f]	Netherlands	Major	F_{11}
19	Row	0.76	Rowena[h]	Netherlands	Major	F_9
20	Aqu[c]	1.05	Aquadulce	Spain	Mediterranean	F_4
21	Pel[d]	0.87	Peleponnes	Greece	Mediterranean	F_{11}
22	34M[e]	0.68	BPL35/ILB141	Marocco	Mediterranean	$>F_8$
23	98T[e]	0.49	BPL649/ILB388	Tunesia	Mediterranean	$>F_8$
24	129T[e]	0.47	BPL1164/ILB923	Tunesia	Mediterranean	$>F_8$
25	Giz[d]	0.61	Giza 4	Egypt	Mediterranean	F_8
26	Reb[c]	0.65	Rebaya	Egypt	Mediterranean	F_4
27	13S[e]	1.40	BPL35/ILB19	Syria	Mediterranean	$>F_8$
28	135E[e]	0.43	BPL1815/ILB1243	Ethiopia	Mediterranean	$>F_8$

[a] Line from the cross 'H20' × ('Diana' × 'Russian'), 'H20S' is black-seeded
[b] Obtained from D.A. Bond, Cambridge, UK
[c] Bred by D. Stelling, Göttingen, Germany
[d] Bred by E. von Kittlitz, Hohenheim, Germany
[e] Obtained from ICARDA (Robertson and El-Sherbeeny 1988)
[f] Bred by Nickerson Zwaan, The Netherlands
[g] Bred by Nunhems Zaden, The Netherlands
[h] Available from Nunhems Zaden, The Netherlands

dATP, dCTP, dGTP, and dTTP, 15 ng primer (random 10-mers, OPM-01 to OPP-07, Operon Technologies, Alameda, Calif.), 0.5 units *Taq* polymerase and *Taq* polymerase buffer (Supertaq, P.H. Stehlin & Ciag). The reaction mix was overlaid with mineral oil.

DNA amplification was performed as follows: 50 s at 93 °C, followed by 40 cycles with (1) 10 s at 93 °C, (2) 10 s at 36 °C, and (3) 1 min at 72 °C. After the final cycle, samples were held for 2 min at 72 °C. Amplification products were analyzed by electrophoresis in 2.5% agarose gels. Permanent records were obtained by photographing ethidium bromide-stained gels under UV light (312 nm).

Recording of observations

Visual scoring for the presence or absence of amplification products at identical positions on the gel was performed as described by Vierling and Nguyen (1992). In the following, the term "band" will be used for a visible amplification product at a given gel position (*GP*). Five classes of bands were defined according to intensity (very strong, strong, medium, light, very light). Very light bands were omitted from the analyses. These and other questionable results at a *GP* were designated by 9.

Statistical analyses

Pairwise comparisons of lines for both unique and common bands were used to calculate a coefficient of genetic distance (*GD*). *GD* was calculated as the complement of Jaccards' (1908) coefficient of community according to the following equation:

$$GD_{ij} = (N_i^* + N_j^*)/(N_{ij} + N_i^* + N_j^*).$$

Here, N_i^* is the number of *GP*s with bands in line *i* and not in line *j*, N_j^* is the number of *GP*s with bands in line *j* and not in line *i*, and N_{ij} is the number of *GP*s with bands in lines *i* and *j*. Thus, GD_{ij} reflects the proportion of *GP*s with bands in only one of both lines relative to the total number of *GP*s with bands occuring in this very pair. *GD* values may range from 0 to 1. *GP*s designated by 9 in at least one of the two lines were omitted from the analyses. The same applied to *GP*s being monomorphic across the entire set of genotypes.

Two types of error variances for *GD* values were calculated. First, we compared the *GD* values of a standard line to a given other line with the corresponding *GD* value of its control line. Calculating the differences between these two *GD*s for all 270 combinations of the 10 pairs (standard line and control line) with all other lines yielded the error mean square of error type A. Second, perfoming the same calculations for the 4 lines assayed as independent replicates yielded an estimate of error type B.

Graphic representations of the associations among the 28 faba bean lines were obtained by standard procedures of numerical taxonomy. Principal coordinate analysis (PCoA) (Gower 1972) was performed with the matrix of RAPD-based *GD* estimates by using appropriate procedures of program NTSYS-pc (Rolf 1989).

Results

Variation for RAPDs

Fifty-nine random primers were used for DNA amplification. Eight primers (OPM-09, 17, 18, 19; OPN-06, 10; OPO-02, 03) yielded no amplification products, 11 primers (OPM-11, 14, 15; OPN-04, 14, 17; OPO-12, 13; OPP-01, 06, 07) yielded no clear or scorable bands, and 5 primers (OPN-02, 05; OPO-08, 10, 14) yielded only monomorphic *GP*s. Hence, 35 primers were found to be informative. Only *GP*s referring to fragment lengths of 500–2100 bp were scored. They yielded a total of 365 *GP*s across the set of 28 *Vicia faba* inbred lines. Among these, 289 *GP*s (79.2%) were polymorphic and provided the basis for calculating *GD* estimates. The number of polymorphic *GP*s per primer ranged from 2 to 14 with an average of 8.3. The proportion of polymorphic *GP*s per primer was not significantly correlated with the total number of bands per primer ($r = 0.30$).

Genetic distances within and between germ plasm groups

Line combinations of type minor × minor had a significantly ($P < 0.05$) greater mean genetic distance and also a wider range of individual *GD* estimates than line combinations of type major × major and Mediterranean × Mediterranean (Table 2). Mean genetic distances of line combinations including major lines were small compared to those of the other groups of line combinations. The largest *GD* value was observed for line combination KT43 × Aqu ($GD = 0.646$). The second largest *GD* value as well as the smallest *GD* value was found between two minor lines (H20S × SCI, $GD = 0.622$; KT42 × KT43, $GD = 0.302$).

Estimation of error variances

Estimates of error variances referring to individual *GD* values were rather low for error A ($2.57 \cdot 10^{-4}$) and error B ($4.05 \cdot 10^{-4}$). Using an *F* test, the latter was found to be significantly ($P < 0.01$) greater than the former. The *GD* estimates between the 10 standard lines and their control lines and between the 4 standard lines and their independent control lines averaged both 0.03.

Principal coordinate analysis

Figure 1 shows associations among the 28 lines revealed by PCoA, based on *GD* estimates of all 378 line combinations. The first (PC1) and second (PC2) principal coordinate explains 7.44 and 6.53% of the total vari-

Table 2 Mean, minimum, maximum, and standard deviation of genetic distance estimates from RAPD data (of 35 primers yielding 289 polymorphic gel positions) for various groups of faba bean lines

Group of line combinations	*N*[a]	Mean[b]	Minimum	Maximum	SD
Minor × Minor	78	0.528a	0.306	0.622	0.052
Minor × Major	78	0.513b	0.428	0.596	0.033
Minor × Medit.	117	0.556c	0.447	0.646	0.034
Major × Major	15	0.442d	0.370	0.500	0.034
Major × Medit.	54	0.496e	0.418	0.532	0.030
Medit. × Medit.	36	0.506bef	0.440	0.567	0.028

[a] Number of line combinations in respective group
[b] Numbers followed by different letters are significantly different at the 0.05 probability level based on *t*-test

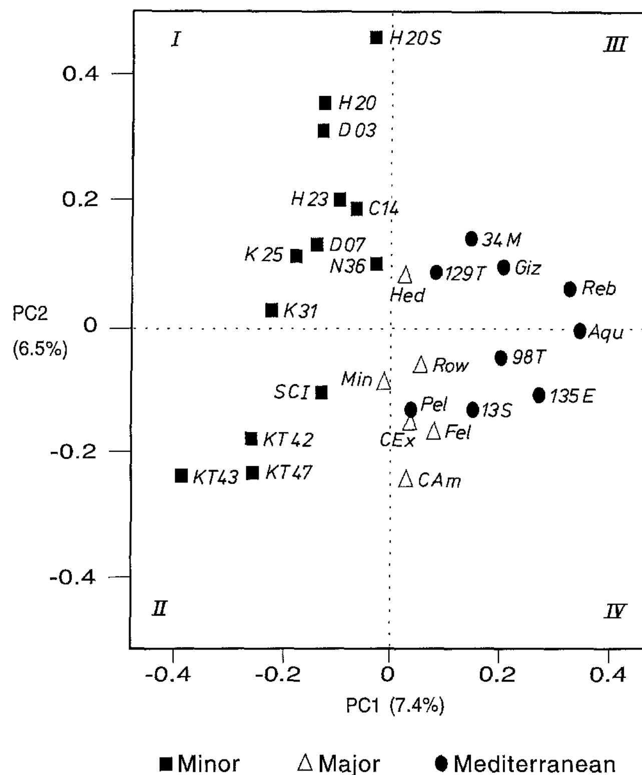

Fig. 1 Association among 28 faba bean inbred lines revealed by principal coordinate analysis based on genetic distance (*GD*) estimates, calculated from RAPD data (of 35 primers yielding 289 polymorphic gel positions). *PC1* and *PC2* denote the first and second principal coordinate, respectively

ation in RAPD data, respectively. The minor lines were clearly separated from the major and Mediterranean lines with respect to PC1. They had a markedly wider spread across PC2 than the major and Mediterranean lines. Lines tracing back to the same cultivars were placed adjacent to each other (KT42, KT43, KT47; K25, K31; D03, D07; H20, H23). The same was true for related lines H20 and H20S.

Lines from cultivars 'Herz Freya' (H) and 'Kleine Thüringer' (KT) were positioned quite distantly within the minor group. The major lines were located as a distinct group in between the group of minor and Mediterranean lines, adjacent to the latter ones. Mediterranean line Pel was located in the center of the major beans. The minor lines also displayed a wider range than the major and Mediterranean lines with regard to the third principal coordinate (not shown), which accounted for 5.44% of the variation.

Discussion

RAPDs as basis of *GD* estimates

The same statistical procedures are often applied to analyze RFLP and RAPD data (Skroch et al. 1992). Crucial characteristics of RAPDs are not adequately taken into consideration. Important items in this respects are (1) the choice of the coefficient for measuring genetic distances, (2) the treatment of monomorphic *GP*s, and (3) the repeatability of *GD* estimates based on RAPDs.

Inheritance of RAPDs

Unlike RFLPs that display mostly a codominant inheritance, RAPDs have been found to be dominant markers with rare exceptions (presence vs. absence of one band per homozygous marker locus; Chalmers et al. 1992; Kesseli et al. 1992; Wilde et al. 1992; Williams et al. 1992; Tinker et al. 1993). Within a species, visually identical bands among accessions appear to be homologous (Thormann and Osborn 1992). For a given *GP*, the simultaneous absence of bands in two genotypes provides little information about their genetic similarity, particularly if the genotypes are not closely related. *GP*s with the simultaneous absence of bands are generally ignored when calculating coefficients of community for RAPDs.

Choice of genetic distance measure

As has been done in previous RFLP studies, the coefficient of community of Nei and Li (1979) has repeatedly been applied for evaluating genetic diversity based on RAPDs (e. g., Chalmers et al. 1992, Van de Ven et al. 1990). *GD* estimates of two genotypes according to Nei and Li quantify the proportion of bands occurring only in one of the two genotypes relative to the total number of bands occuring in both genotypes. *GD* estimates according to Nei and Li (GD_{NL}) versus *GD* estimates according to Jaccard (GD_J) differ in the weighting of polymorphic marker loci with dominant and codominant inheritance. While both measures lead to identical rankings of *GD* estimates among pairs of inbred lines, they differ in their relationship to the percentage of heterozygous marker loci in hybrids between these lines. Melchinger (1993) pointed out that for codominant markers, the expected GD_{NL} of related pairs of lines is a linear function of their coancestry coefficient. By way of contrast, it can be demonstrated that for dominant markers, this property applies to GD_J but not to GD_{NL}. Hence, we recommend the use of GD_{NL} for RFLP data but GD_J for RAPD data.

When RAPD data are used, the denominator of GD_J estimates ($N_{ij} + N_i^* + N_j^*$) varies among pairs of lines. In particular, it increases with increasing *GD*. In our data set, the mean value of the nominator ($N_i^* + N_j^*$) was 73.2. The denominator had a mean of 140.6 and a standard deviation of 11.3. In order to eliminate the variation in the demonimator, Tinker et al. (1993) used the same nominator as the one used in our study, but the total number of *GP*s as the constant denominator for all

line combinations. With this treatment, pairs of lines each with a small number of bands tend to show smaller *GD* values than those with a large number of bands, thus introducing a systematic bias.

Monomorphic fragments

The ranking of *GD* values for a greater set of lines depends upon whether monomorphic *GP*s (*MGP*s) are included in the analyses or not. The coefficient of determination (R^2) between *GD* values estimated with and without *MGP*s in our data set was 0.904. In the materials examined, 76 *MGP*s occurred. When this constant number is included in calculating *GD* values, it receives greater weight in pairs of lines with a small number of *GP*s than in pairs of lines with a great number of *GP*s. We recommend excluding *MGP*s from the analyses. The opposite point of view is put forward by Skroch et al. (1992). By omitting *MGP*s prior to the analyses, the range of *GD* values was shifted and increased (to 0.306–0.646 as given in Table 2 compared to 0.199 to 0.449 with *MGP*s included).

Repeatability of *GD* values based on RAPDs

The error mean squares based on *GD* values of the 10 pairs and the 4 pairs of replicated genotypes were both rather small. This indicates that *GD* estimates based on RAPDs are highly repeatable. Error A refers to differences that evolve after DNA extraction. Error B additionally includes genotypic variation among individual plants within inbred lines and differences among laboratories and methods of DNA extraction. This is reflected by a moderately greater variance for error B.

As shown by Skroch et al. (1992) for the simple matching coefficient, a *GD* estimate of 0.5 has a standard error of about 0.05–0.04 with the usual sample size of 100–200 bands per individual line comparison. Hence, the error caused by the sampling of marker loci seems to be larger than errors A and B. The sampling error refers to the difference between *GD* estimates obtained from different sets of marker loci, whereas error A and B refer to the repeatability of laboratory assays with a given set of genotypes and marker loci.

Degree of polymorphisms revealed with RAPDs

In our materials and with the laboratory procedures applied, the average number of bands per primer was greater than in barley, soybean, and snap bean (Skroch et al. 1992; Tinker et al. 1993; Williams et al. 1992). This may partly be caused by the inclusion of light bands in our analyses. The degree of polymorphism with RAPDs detected in the present study was also substantially greater compared with a previous RFLP study in faba bean (Van de Ven et al. 1990). Likewise, a preliminary investigation (A. Schilling, unpublished data) of faba bean lines D07, H20s, Hed, and T129 with RFLPs revealed a much lower degree of polymorphisms than with RAPDs. Looking at repeatability, degree of polymorphism, and simplicity of the laboratory assay, we conclude that the RAPD assay is a very effective method of obtaining reliable *GD* estimates for inbreds of faba beans.

Implications for faba bean breeding

Genetic variation within and among European and Mediterranean faba bean germ plasm

Data on heterosis for yield of crosses among the lines K25, K31, KT43, and KT47 (Link 1988) revealed a significant mid-parent heterosis for yield in the crosses among lines from the same progenitor cultivar. However, heterosis of crosses among unrelated lines was nearly twice as large. Our results with RAPDs are in good agreement with these field data.

Marked progress has been reported for breeding hybrid cultivars in faba beans (Duc et al. 1992). At present, minor × major hybrids are regarded as being most promising in Central Europe (Duc et al. 1992; Melchinger et al. 1994). However, in view of the fact that certain minor × minor combinations displayed greater *GD* values than the minor × major combinations, it might also be promising to establish genetically divergent heterotic groups within the minor germ plasm. To answer this question, research is warranted whether large RAPD-based *GD* estimates are indeed indicative of increased heterosis for yield (Melchinger 1993). From the Mediterranean breeders' point of view, Mediterranean × minor seems to be an attractive hybrid combination. However, the extremely poor adaptation of minor genotypes to Mediterranean environments will hamper their direct utilization (Kittlitz et al. 1993). Major beans are much better adapted to Mediterranean environments. In order to assist breeders in the establishment of divergent heterotic groups, we are presently evaluating a subset of these genotypes in intra- and intergroup crosses at Central European and Mediterranean environments for hybrid performance and heterosis.

Evolutionary aspects

Region and time of origin of the *Vicia faba* is supposed to be the Near East or West Asia and the late neolithic period (Hanelt 1972). As early as 2000 B.C., faba beans had been introduced to Portugal and only few centuries later to the Jersey Islands off the French Coast. During the Roman time, cultivation spread throughout Europe up to Scandinavia. Earliest findings of major faba beans in Eastern Iraq date after 1000 A.D. The presence of major faba beans in Central Europe is proven only as late as the Middle Ages (Hanelt 1972). Thus, minor

beans had already undergone a long history of cultivation and evolution in West and Central Europe before a large-seeded type evolved near to the original center of domestication. This type should have been extremely attractive for human consumption and might have spread rapidly around the Mediterranean Sea and north into Europe. In conclusion, archeaobotanical findings agree with our main results from RAPD assays in that (1) they suggest a close relationship between European major beans and Mediterranean beans and (2) they explain the large amount of genetic variation present in the European minor bean germ plasm.

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