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USEFULNESS OF AFLP MARKERS TO ESTIMATE HOMOGENEITY OF RAPESEED INBRED LINE VARIETIES

prepared by experts from France

USEFULNESS OF AFLP MARKERS TO ESTIMATE HOMOGENEITY OF RAPESEED INBRED LINE VARIETIES

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Summary

The ability of AFLP markers to assess the uniformity of rapeseed inbred line varieties was investigated in the context of plant registration. Three candidates varieties were chosen on a scale of morphological heterogeneity in the field. Thirty lines per variety were characterized with 4 primer combinations (173 markers). Principal component analysis and distribution of pairwise Rogers' distances between lines revealed exactly the same off types than those observed in the field. The Nei's genetic diversity computed from the binary data matrix revealed the same diversity level as the one observed with morphological traits: the Nei's diversity value for the most homogeneous variety, a microspore-derived doubled haploid line, was 0.0011, whereas the value for the most heterogeneous variety was 0.0706. The agreement between the results with AFLP markers and morphological traits showed that AFLP markers associated with adapted analysis tools are very useful to assess the uniformity of candidate varieties in the context of plant registration.

Introduction

The legal right to market a newly bred variety in the European countries depends on the results of a statutory testing. This statutory testing provides information to establish if a new variety is distinct (D) from all the other released varieties, uniform (U) and stable (S), for the traits used in the test. This testing guarantees the quality of the new variety for farmers and merchants.

The assessment of the uniformity of rapeseed inbred line varieties only relies on morphological traits evaluated in field experiments. The French protocol is very efficient to reveal the defaults of uniformity of the varieties. It is a very stringent protocol for the candidate varieties, but it was time and area consuming. As the number of varieties increases, a powerful, rapid and cost effective additional protocol is required to maintain the efficiency of the French DUS trial.

To complement field trials, analysis of isozymes has been successfully applied for variety distinctness and was included in DUS trial for some crops like maize, wheat and barley. However, only 7 enzyme systems could be easily used in rapeseed and the number of isozyme markers is limited (Lee et al., 1996 a, b). Then, the use of isozyme markers to assess the uniformity of the varieties seems to be unsuitable because the diversity within varieties may be lower than between varieties.

In this framework, uniformity testing would benefit from the use of molecular markers that are almost unlimited in number in comparison with isozymes. Molecular markers have been successfully applied in rapeseed for diversity analysis (Diers & Osborn, 1994), for variety identification (Mailer et al., 1994), and for controls of seed purity of F1-hybrids (Marshall et al., 1994). Among the available DNA molecular techniques, AFLP (Amplified Fragment Length Polymorphism) is a powerful technique for variety identification (Lombard et al., 2000, Powell et al., 1996). It provides a large number of markers on a single analysis without requiring sequence information for their development (Vos et al., 1995).

The aim of this study was to evaluate the ability of AFLP markers to identify the homogeneity defaults previously observed in the field for three varieties, in the context of plant registration.

Materials and Methods

Experimental design to assess the uniformity of varieties

In France, a specific experiment is used to assess the uniformity of candidate varieties. For each variety, breeders must provide 40 inbred plants of their varieties and a basic seed lot. Twenty inbred plants are planted in La Minière, near Paris and the remaining 20 inbred plants are planted in Le Magneraud, in the center-west. Each location is planted with two replications. For each candidate variety, a plot consists in five lines of basic seeds followed by twenty lines corresponding to twenty inbred plants. In addition, among the inbred plant lines, one line per five is planted with basic seeds. Each plot is grown with 75 individuals spaced in a row 2.5 m long with 35 cm between lines.

Morphological description

Morphological data for 10 qualitative traits and two quantitatives traits are collected at the two locations (Table 1). Qualitative traits are noted on an intensity scale that ranged from 1 to 9. According to the protocol, a line is declared off type if its morphological description clearly differs from the remainding lines of the variety for at least one trait. If more than three lines are off types, the variety can not be registered.

[Table 1]

Plant material

On the basis of the results of field experiments in 1997, three varieties (A, B and C) were chosen on a scale of homogeneity. Variety **B,** a microspore-derived doubled haploid line, was strictly homogeneous, all the 40 lines had the same morphological description. Varieties **A** and **C,** selected from a pedigree breeding program, were heterogeneous. Seven out of the 40 lines of variety A were off types. Variety **C** was very heterogeneous, probably due to an insufficient number of selfing, and one line was off type. For each variety, 30 out of the 40 lines were harvested, including the off type lines. For each line, pieces of leaves from 20 plants were pooled.

DNA extraction and AFLP assays

Each sample was frozen and ground in liquid nitrogen and 100 mg was used for total DNA extraction using the DNeasy Plant Mini Kit®(Qiagen). AFLP analysis was performed with the AFLP analysis system I® (Gibco BRL). After the pre-amplification, selective amplification were performed with E*co*RI primer labeled with IRD700 or IRD800 fluorescent dyes (LI-COR). PCR products were loaded on a 5% polyacrylamide gel containing 8 M urea and analyzed with a LI-COR model 4200 automated DNA sequencer. A 50-700 bp sizing marker (ScienceTec) was loaded in three lanes per gel to facilitate the sizing of the fragments revealed. Six primers combinations (173 markers) chosen on the basis of a previous study on a collection of 83 rapeseed varieties (Lombard et al., 2000) were used to characterize the thirty lines of each variety.

Statistical analysis

One hundred and seventy three markers were scored as biallelic loci of which alleles were the presence and the absence of the fragment. The variability within each variety was estimated using the Nei's diversity index (Nei, 1973) as, $H = \frac{1}{L} \sum_{j=1}^{L} \left(2 p_j (1 - p_j)\right)$, where L is the number of loci and p_j the frequency of marker j. Because of problems during experiments, two lines of variety A (a normal line and an off type line) did not enter the data set.

For each variety, the Rogers' distance (Rogers, 1972) was computed between each pair of lines. With the assumptions that each line is homozygous and each marker is a biallelic locus, the Rogers' distance reduce to the percentage of markers which differ between two lines. Relationships between lines were further analyzed with a principal component analysis (PCA). All the statistical analyses were performed with SAS (SAS institute Inc., 1996).

Results

Variability within each variety

The variability revealed within each variety is presented in table 2. Among the 173 polymorphic markers previously identified in Lombard et al. (2000) on a collection of 83 winter and spring rapeseed varieties, only one band was polymorphic within variety B, 29 within variety C and 54 within variety A. Based on the data matrix, the Nei's diversity index values was 0.0011 for variety B, 0.0251 for variety C and 0.0706 for variety A.

[Table 2]

Identification of the "morphological" off types

The Figure 1 shows the associations among lines within each variety. The first two principal components accounted for 55.9 % of the total variation, which provided a good representation of the relationships among lines on these axes. For variety A, the lines are located in two opposite directions. The first group (Var A, A10 and A12) included 22 lines that were very similar. Twenty lines had the same molecular pattern and were located at the

point named varA. The second group included the six morphological off type lines. Five off types clustered and one (A13) was located outside of this group. For variety B, 27 out of the 30 lines were located at the same point named var B which corresponded to the gravity center. The remaining three lines (B24, B25 and B40) had the same molecular pattern and were closely located near the gravity center of the variety. The lines of variety C were more scattered around the gravity center of the variety. Two lines were clearly separated from the remaining lines: a morphological off type line (C34) and a morphological normal line (C12).

[Figure 1]

Genetic distances between lines of each variety

Figure 2 shows the distribution of the pairwise distances between lines for each variety. For variety B, more than 80% of the distance values equaled zero and the remaining distance values ranged from 0 to 0.01 (Figure 2a). The 0.01 value corresponded to one marker which differed between lines B24, B25 and B40 and the remaining lines of the variety. For variety A, the shape of the distribution showed that variety A was a mixture between two types of genotypes; the morphological normal lines and morphological off type lines (Figure 2b). Distances between normal lines ranged from 0 to 0.02, and more than 50 % of distances equaled zero. Distances between normal lines and off types lines ranged from 0.15 to 0.21. The case of variety C is different because the shape of the distribution of the distances between morphological normal lines is more spread (Figure 2c). About 10% of distance values equaled 0 and the remaining distances ranged from 0 to 0.1. The distances between the off type line and the normal lines ranged from 0.09 to 0.13 and the distribution overlapped the distribution of distances between normal lines.

[Figure 2a, b, c]

The Nei's diversity values were 0.0011 forvariety B, 0.0706 for variety A and 0.0251 for variety C (Table 2). If off types lines were removed from the calculation, the Nei's diversity equaled 0.015 for variety A, which was close to the value for variety B. Without off type lines, the Nei's diversity equaled 0.0192 for variety C, which was more than 10 times higher than the value for variety A without off type lines.

[Table 2]

Discussion

Agreement between "morphological" off types and "molecular" off types

AFLP markers identified all the morphological off types. PCA and distribution of pairwise genetic distances were useful tools to reveal lines that have original molecular pattern. For variety A, the six morphological off types were clearly distinguished from the remaining normal lines. These lines were also clearly identified in the field, because they were very different from the remainder of the variety for some traits such as the number of leaf lobes, the dentation of leaf margin and the color of leaf. The distance value between the

normal lines and an off type lines was the same as distances between different registered varieties (Lombard et al., 2000). This result showed that for variety A, the off type lines may not belong to the variety but resulted from an error of production. Among the off types of variety A, AFLPs differentiated the A13 line from the remaining off type lines, which was also previously observed in the field. A13 had a more erected habit. Then, AFLPs were powerful to discriminate between the off types. The case of variety C was different because of its large heterozygosity. C34, a morphological off type line, was identified with AFLP markers but C12 had also an original molecular pattern while it was a morphological normal line. This is only an seeming discrepancy between morphological observations and molecular patterns. For this variety, there was a morphological variation between lines but also within lines. It was difficult to reveal off type lines with morphological traits among lines with a global heterogeneity. The line C12 was a molecular off type and it may be also a morphological off type but variety C was rejected for a global heterogeneity and not because the number of off types exceeded the norm. For variety C, AFLP markers were more efficient to reveal off type lines than morphological traits in a case of a globally heterogeneous variety.

Estimation of the diversity within variety

The diversity found within the varieties studied was very low. Even if the heterogeneity of variety C without the off types (C12 and C34) was 0.0147, this value was very lower than diversity values for population varieties (0.200 for ryegrass, P. Dubreuil, personal communication). For variety B, only one marker was polymorphic between lines. This result was expected because variety B was a microspore-derived doubled haploid line variety. Theoretically, all its loci are homozygous and all the lines are identical. The results confirmed that this variety could be considered as a reference of uniformity. But all the rapeseed varieties are not selected from microspore derived lines. The general case is pedigree breeding programs as for variety A. When only the normal lines are taken into account (the off type lines do not belonged to the variety) the diversity become comparable with the diversity within variety B. Only 3 bands were polymorphic and the Nei's diversity was 0.0015 (0.0011 for variety B). This result is particularly important because one common objection to the use of molecular markers in DUS testing is that they would reveal heterogeneity even for varieties that were homogeneous on the basis of morphological traits.

Definition of a threshold for heterogeneity revealed by AFLPs

Our results showed that, AFLPs are very powerful to reveal morphological off type lines when the remaining lines of the variety is homogenous. But when the level of morphological heterogeneity is the cause of the rejection, it is necessary to define a threshold of heterogeneity. The comparison between variety A and variety C gives some ideas. In a primary approach, variety A is more heterogeneous than variety C (0.0706 for A and 0.0251 for C). But the PCA and the distribution of pairwise genetic distances allowed to reveal 6 off type lines for variety A and 2 for variety C. When the off type lines are removed from the calculation, the Nei's diversity of variety A become 10 times lower than Nei's diversity of variety C (0.0015 for A and 0.0147 for C). If a molecular uniformity threshold would be define, it value could be between these marks. A methodology to assess the uniformity of candidate varieties with molecular markers could be defined as followed. The distribution of Rogers' pairwise distances PCA could reveal the off types lines. If the number of off type lines exceeds the norm, the variety would be rejected. If not, the value of the Nei's diversity for the normal lines could be compared with an adapted threshold.

Conclusion

AFLP were very efficient to reveal all the morphological off types and only the former. The levels of molecular diversity are in agreement with those measured using morphological traits. Principal component analysis and distribution of pairwise distances between lines were adapted tools to separate off type lines from the ramaining lines of the varieties. In the case of globally heterogeneous varieties, the Nei's diversity value is useful to assess the molecular diversity within varieties. In the present work, we only compare three varieties and it is difficult to propose a molecular threshold for the rejection of candidate varieties with a large residual heterogeneity. Further studies on a larger set of candidate and registered varieties selected from various types of breeding programs would permit to effectively define a molecular threshold for uniformity testing.

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Table 1. Definition of measured characters. All the traits are noted on a 1 to 9 scale excepted flowering time (number of days after the first January) and plant height (cm).

	Traits
Leaf	- green color
	- number of lobes
	- dentation of margin
	- habit
Flower	- color of petal
	- length of petal
	- width of petal
	- flowering time
Siliqua	- habit
Plant height	

Table 2. Polymorphism and Nei's diversity index within each variety

Variety	Number of polymorphic markers	Nei's diversity index
A	54	0.0706
A (without the six off types)	3	0.0015
B	1	0.0011
C	29	0.0251
C (without the off type)	19	0.0192
C (without the off type and C12)	8	0.0147

Figure 1. associations among lines of 3 rapeseed varieties revealed by principal component analysis on the basis of 173 AFLP markers. A■, B■, and C■ were the gravity center of variety A, variety B, and variety C, respectively.

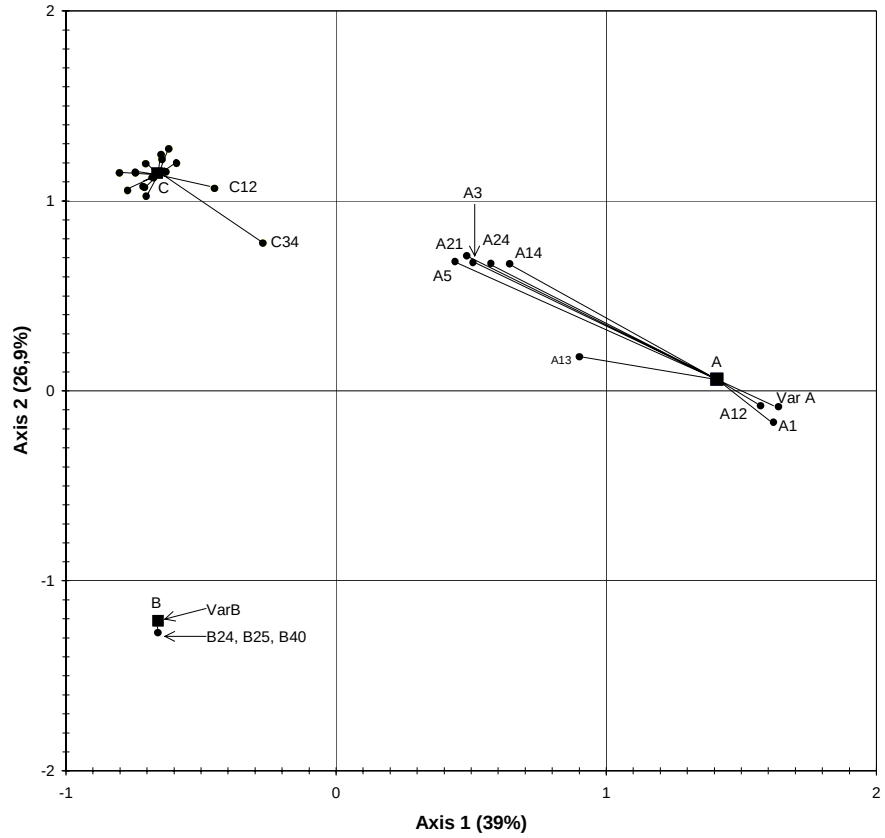
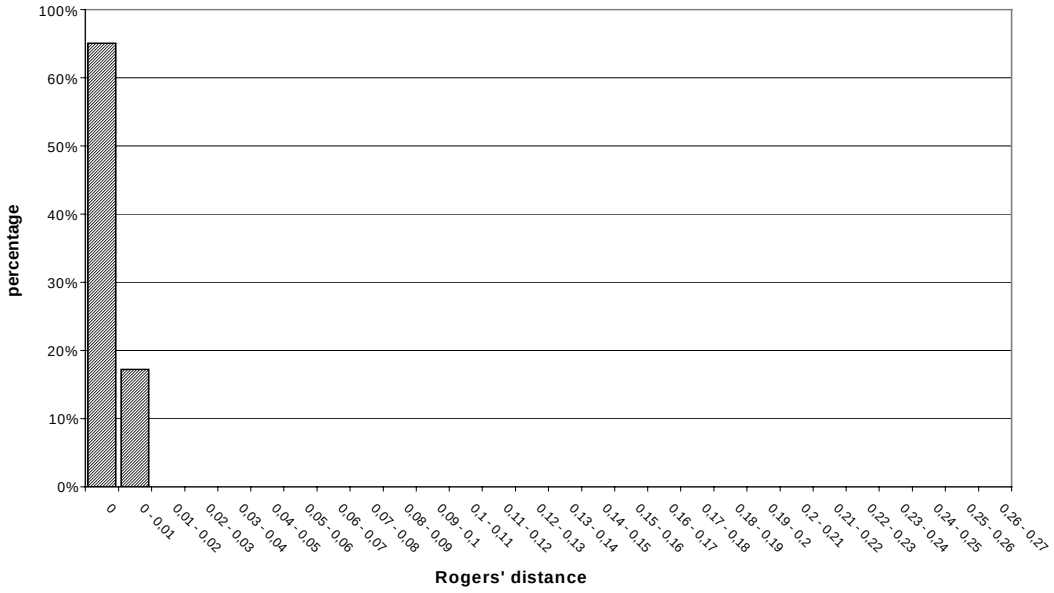
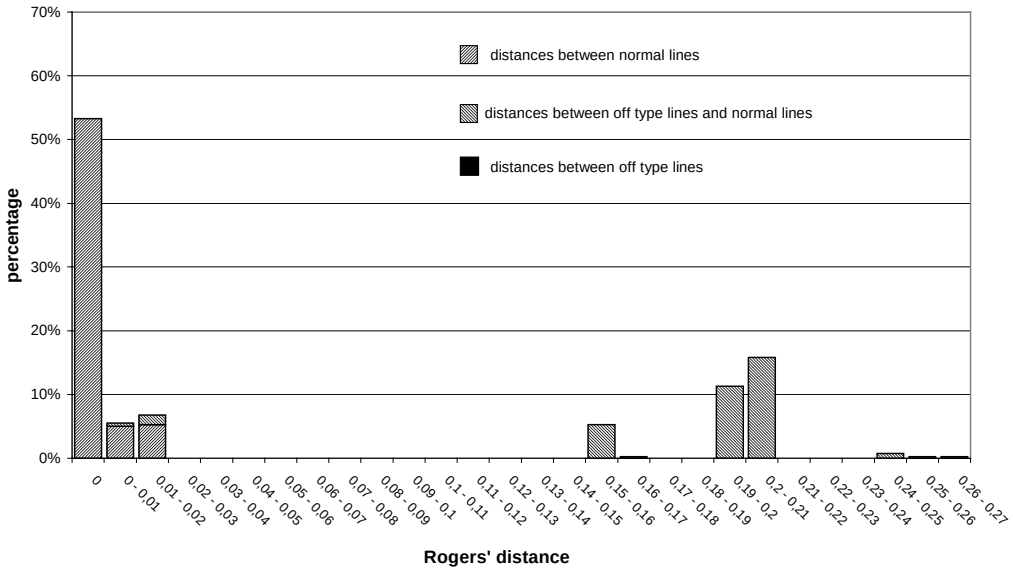
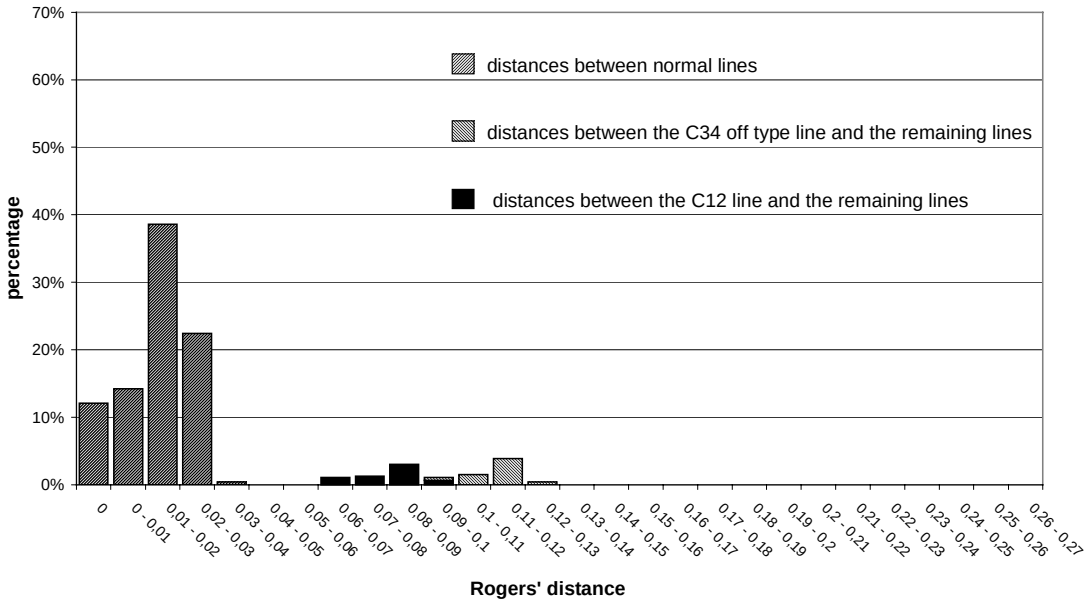


Figure 2. Distribution of pairwise Rogers' distances for variety B (*a*), for variety A (*b*), and for variety C (*c*).







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