The establishment of 'essential derivation' among rose varieties, using AFLP

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Abstract In the International Union for the Protection of New Varieties of Plants Act of 1991, mutation is mentioned as one of the mechanisms to obtain an 'essentially derived' variety (EDV). For the implementation of the EDV concept in the case of mutation, it is important that the level of genetic relatedness between an initial variety and derived mutant varieties can clearly be distinguished from the level of relatedness between arbitrary pairs of varieties without a derivation relation. Conditions to be fulfilled for such a distinction include enough genetic differentiation in the germplasm pool of interest, sufficiently low levels of genomic sampling error and technical laboratory error and high reproducibility within and between laboratories. In rose, mutants or 'sports' are frequently observed during multiplication, making it a suitable crop for studying the possibilities for introduction of the EDV concept in ornamentals. We studied genetic similarities among 83 rose varieties, including 13 mutant groups. Twelve AFLP primer combinations generated 284 polymorphic markers and 114 monomorphic (fixed) bands. Pair-wise Jaccard similarities between original varieties and derived mutants were close to 1.0 (>0.96), whereas all similarities between original varieties were below 0.80, with 75% of the non-mutant similarities even being below 0.50. Values less than 1.0 for similarity among original varieties and their mutants were to a major extent due to scoring errors. Error rates in automated scoring proved to be lower than those in manually scored and transferred data. Experimental errors, even between laboratories, turned out to be very small. On the basis of a consistent and large difference between similarities, relations between an original variety and its mutants can easily be identified and distinguished from relations between original varieties. These results open the way for implementing the essential derivation concept in rose.

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Introduction

The occurrence of mutants is a common phenomenon among many ornamental plant species. Most easily detectable are mutations in the colour of the flower. Usually, such mutants or 'sports' are detected during the multiplication phase, i.e. when large numbers of plants are produced for marketing. In practice this means that mutants are often discovered by others than the breeder of the original variety. The discoverer can obtain plant breeders' rights for such mutants when they are shown to be distinct from all existing varieties, including the original variety. To protect the interests of the breeder of the original variety the International Union for the Protection of New Varieties of Plants (UPOV) has introduced the concept of an 'essentially derived variety' (EDV). This concept, which is described in the UPOV (1991) Act of 1991, extends the scope of protection of the initial variety to any variety essentially derived from it. Therefore, all rights given to the breeder of the initial variety also apply to the EDV (http://www.upov.int/). According to the text of the UPOV Act of 1991, a variety shall be deemed to be essentially derived from another variety ('the initial variety') when: (1) it is predominantly derived from the initial variety, or from a variety that is itself predominantly derived from the

initial variety, while retaining the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial variety; (2) it is clearly distinguishable from the initial variety; and (3) except for the differences which result from the act of derivation, it conforms to the initial variety in the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial variety.

EDVs may be obtained, for example, by the selection of a natural or induced mutant, or of a somaclonal variant, the selection of a variant individual from plants of the initial variety, backcrossing or transformation by genetic engineering (UPOV 1991).

The implementation of an essential derivation concept will entail the definition of a threshold level of genetic conformity, or relatedness, above which the owner of the putative derived variety should prove that his variety was obtained by accepted breeding practices. Conformity is estimated by a similarity coefficient calculated from a set of characters that can include both standard phenotypic characteristics, heterosis related characteristics and molecular marker information (http://www.worldseed.org/). There is considerable debate about which traits to include and what threshold levels for reversal of the burden of proof to use. Whether EDVs can be distinguished from non-EDVs will depend on a number of issues, like the breeding system, the size of the gene pool, the presence of selection and drift, and the existence of genetic heterogeneity within varieties. When molecular information is involved, additional issues concern the type of marker system (dominant vs co-dominant, bi-allelic vs multi-allelic), the coverage of the genome by markers, the precision and reliability of the marker system, etc. (Nuel et al. 2001; van Eeuwijk and Baril 2001; van Eeuwijk and Law 2004).

The standard morphological and physiological characters used for registration or granting plant breeders' rights are adequate for determining distinctness, uniformity and stability of the new mutant variety. However, these characters appear less suitable for relating mutants to the original variety, as they do not seem to allow an accurate determination of genetic conformity. Molecular markers then constitute a viable alternative (Debener et al. 1996; Debener et al. 2000; De Riek et al. 2001; Bredemeijer et al. 2002; Heckenberger et al. 2002; Röder et al. 2002; Esselink et al. 2003), as they provide a more accurate methodology for the determination of genetic similarity.

According to the examples given in the UPOV act, mutants should be considered as EDVs. As mutants usually are the result of just very few changes in the genetic makeup of a variety, the genetic similarity between original variety and mutant will be very high (close to 100%). This was shown to be the case in roses (Debener et al. 2000). Whether it will be possible to separate relations between original varieties on the one hand from relations between original varieties and their mutants (mutant groups) on the other, will depend on

the difference in genetic similarity between original varieties as compared to the genetic similarities observed in mutant groups. Additional prerequisites for separation are a relatively small standard error for the similarity and high reproducibility within and between laboratories. Jones et al. (1997) showed that results obtained with microsatellites as well as AFLP are in principle reproducible between laboratories. Heckenberger et al. (2002) demonstrated that variation in genetic distance estimates between different accessions of the same inbred line of maize can be caused by laboratory errors and heterogeneity in the seed sample. Distance stands here for the complement of similarity (distance = 1 − similarity). The effect of laboratory errors was marginal as compared to the effect of heterogeneity within maize inbred lines on the variation in genetic distance estimates.

In this paper we report on genetic similarities detected between original varieties of rose as compared to the genetic similarities observed in mutant groups, using the AFLP technique. Hybrid tea roses (*Rosa × hybrida*) were taken as a model; they are outcrossing, vegetatively propagated, and mutants occur rather frequently. Consequences for implementing the EDV concept will be discussed.

Materials and methods

Materials

In this study, 83 rose varieties were used. These consisted of 12 known mutant series groups (37 varieties in total, Table 1), and in addition, a set of 44 red and two yellow rose varieties. Leaf material was obtained from the breeding companies or from the Centre for Genetic Resources, The Netherlands. Young, still-folded leaves were sampled in liquid nitrogen, freeze-dried and ground. DNA was extracted using the Qiagen Dneasy isolation kit. Leaf material of a subset of 21 rose varieties was provided to Keygene in order to study interlaboratory differences in AFLP data production. DNA of this subset was isolated by use of a modified CTAB extraction procedure as described by Steward and Via (1993).

AFLP analysis

Varieties were fingerprinted using AFLP, essentially as described by Vos et al. (1995) and separated and detected according to Arens et al. (1998). The amplification product was labelled using [^{33}P]-labelled *Eco*RI primer and visualized using Kodak X-OMAT film (exposure for 1–3 weeks at room temperature). Gels were scanned and scoring of bands was performed using Quantar scoring software (Keygene). Only incidentally some bands were scored by hand. All automatic scores done by the Quantar package were

Table 1 Plant material used from different mutant groups

Mutant groups[a]		Variety identifiers							
Leonidas	g[1]	1	2	3	4	50			
Edith Piaf	g[2]	5	6						
Pretty Woman	g[3]	7	8						
Vivaldi	g[4]	9	10	11	12	14			
Prophyta	g[5]	13	15	16	45	46	47	48	79
Femma	g[6]	17	18	19	20	21	24	65	
Pistache	g[7]	22	23						
Jazz	g[8]	25	26						
Renate	g[9]	27	28	29	30				
Surprise	g[10]	31	32	38					
Lydia	g[11]	34	35	36	37	44			
Frisco	g[12]	39	40	41	42	43	49		
?	g[13]	51	70						

[a] Mutant groups are identified by the name of the original variety

controlled visually for proper interpretation. AFLP markers generated by Keygene (Vos et al. 1995) were exposed to Fujix phosphor image screens for 16 h; fingerprint images were produced using a Fujix BAS-2000 phosphor image analysis system and scored using proprietary software. Bands that were found in one variety only were considered as (potential) artifacts and not scored. All other bands were scored as '1' (present) or '0' (absent).

Initially approximately 150 AFLP primer combinations (PCs) were tested on four rose varieties. From these, the best 50 PCs were selected on the basis of the number of bands, clarity of the pattern and distribution over the gel. These 50 PCs were tested again in a second round using eight varieties. Finally, the 12 best AFLP PCs were selected (Table 2), using the same criteria mentioned above but even more stringent, for the set of 83 varieties. Primers used were *Eco* primer core sequence: 5′-GAC TGC GTA CCA ATT C . . . -3′, selective bases: E33 AAG and E35 ACA; *Mse* primer core sequence: 5′-GAT GAG TCC TGA GTA A . . . -3′, selective bases: M49 CAG, M52 CCC, M53 CCG, M54 CCT, M56 CGC, M57 CGG and M61 CTG.

Table 2 AFLP primer pairs used, number of polymorphic bands and number of monomorphic bands detected with each primer pair

Primer combination	Number of polymorphic markers	Number of monomorphic markers
E33 M52	27	16
E33 M53	31	7
E33 M54	32	22
E33 M56	22	5
E33 M57	26	6
E35 M49	30	11
E35 M52	17	10
E35 M53	25	5
E35 M54	26	11
E35 M56	13	6
E35 M57	18	3
E35 M61	17	12
All	284	114

Data analysis

Genetic conformity and similarity

Genetic conformity between varieties on the basis of AFLP bands was expressed in terms of Jaccard similarities (Digby and Kempton 1987), i.e. the number of shared bands between two varieties as a proportion of the total number of bands that were observed in at least one of the two varieties being compared.

Population structure

The population structure in our collection of rose varieties was visualized by an average linkage (UPGMA) dendrogram (Digby and Kempton 1987) and a non-metric, multi-dimensional scaling plot (Borg and Groenen 1997). Both techniques give an indication of groups of varieties being more closely related than others. In the dendrogram, similar or related varieties will occur together below a particular node. In the scaling plot, similar varieties occur closer together than dissimilar varieties. The scaling plot can be rotated over any angle without the interpretation changing. The quality of representation of distances by non-metric plot was estimated by calculating the stress value, which is expressed on a 0 (perfect fit)-to-1 scale. The objective of dendrogram and scaling plot was to quickly and informally check the extent to which mutant groups were genetically homogeneous and the extent to which mutant groups were genetically different from each other and from the other varieties.

Distributions of similarities

The most important aspect of this paper concerns the study of the distributions of similarities between, on the one hand, initial varieties and their derived mutants, and, on the other, between original varieties. These two distributions were compared using all 12 PCs for the calculation of the similarities. Similarities as obtained from calculations on individual PCs were also studied.

First, similarities were calculated on the basis of the markers corresponding to the individual primer combinations. Then, the similarities belonging to a particular PC were replaced by ranks. Next, a matrix of ranks was constructed of which the rows were indexed by (non-mutant group) variety pairs and the columns by PCs. On this matrix of ranks a principal components analysis was performed, and a plot was constructed that portrayed the (rank) correlations between the PCs.

Standard errors for similarity estimates

First, standard deviations were calculated for each of the 83×82/2 variety-pair similarities across the 12 similarity estimates obtained from the individual PCs. From those standard deviations, standard errors for similarity estimates were obtained in the usual way, i.e. by dividing the standard deviations by the square root of the number of observations, where the latter is 12 (= the number of PCs). These standard errors will depend on the magnitude of the similarity estimates. For the group of similarities between members of mutant groups and the group of similarities between original varieties, a typical standard error was calculated as the median of the standard errors for the similarities within the group.

Reproducibility within and between laboratories

Errors in the determination of genetic conformity can have various origins. First, there are the technical errors in the laboratory. To study technical errors, the marker scores on 17 mutant varieties from four mutant groups were compared to the corresponding ideal, error-free profiles. The error-free profiles were obtained from the median profiles for the mutant groups, i.e. within each mutant group the error-free score for individual markers was taken equal to the median of the scores (1, 0) across the varieties belonging to that group. The median profile is effectively equivalent to the rounded-off average profile across the varieties of a mutant group. We compared the variety profiles within a mutant group with the ideal profile. The technical error was the average number of discrepancies within the corresponding ideal profiles across the 17 mutant varieties. In this way the technical error within the laboratories of Plant Research International (PRI) and Keygene was calculated, where both laboratories used independently of each other the same 12 PCs, which did not necessarily produce the same individual markers.

In addition to the 17 varieties from four mutant groups, four other varieties were fingerprinted by both laboratories. Standard errors for the similarity estimates for the original varieties, excluding mutants, were compared between the two laboratories to estimate the magnitude of technical and genomic sampling error together.

Finally, to estimate between laboratory reproducibility, Pearson rank correlations between the similarity

estimates obtained by the two laboratories were calculated, where mutant groups are represented by their median profiles. For comparison, the intra-class correlations were calculated for the similarities within both laboratories. The intra-class correlation provides an estimate for within-laboratory reproducibility, so the correlation to be expected between repeated similarity estimates in the same laboratory. Intra-class correlation was estimated by the ratio of the variance for individual pair-wise similarity estimates (square of standard error) to the variance of all pair-wise similarities

Results

Selection of primers and distribution of bands

To select appropriate PCs for AFLP analysis, initially four varieties (two original varieties and two mutants of one of them) were characterized using 150 PCs. Out of these 150 PCs, a total of 119 PCs resulted in scorable, good-quality AFLP fingerprints. With these 119 PCs, a total of 5,841 bands were generated (on average 49 per primer pair). Of these, 1311 (22%) were polymorphic between the two original varieties. The patterns obtained for the mutant group (consisting of an original variety and its two mutants) showed differences in only four out of the 1,311 bands (0.3%). PCs producing them were (for other reasons) not selected for the remainder of the study.

From the set of 119 PCs, 12 were selected (Table 2) to genotype the larger set of 83 varieties. In total 284 polymorphic markers and 114 monomorphic (fixed) bands were scored in this set of varieties (Table 2). The number of polymorphic markers per PC varied from 13 to 32. Figure 1 shows the distribution of band frequen-
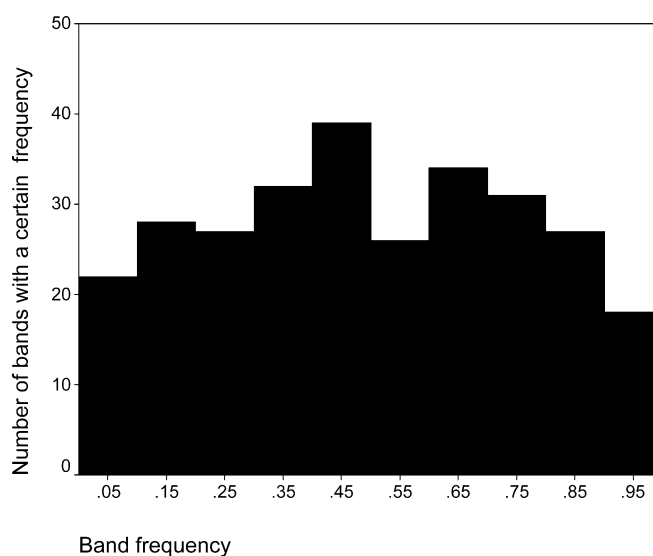

Fig. 1 Band frequencies for polymorphic markers

cies for the polymorphic markers. This distribution appears to be remarkably uniform.

Relationship between varieties

On the basis of the 284 polymorphic markers, Jaccard similarities were calculated between all pairs of varieties. The structure in the collection of varieties as arising from the Jaccard similarities is presented in the form of a dendrogram (UPGMA) in Fig. 2. The compactness of the mutant groups in the dendrogram reveals the ease with which relations between varieties within mutant groups can be distinguished from relations between varieties that do not share membership of a mutant group. Note that similarities within mutant groups were not necessarily equal to 1.0, although they were generally close to 1.0. Figure 3 shows a non-metric, multi-dimensional scaling plot in which all original varieties are included. The closer varieties are in the plot, the more alike were their genetic profiles. Mutant groups (filled circles) were represented by their median profiles (see 'Materials and methods'). The mutant groups seem to represent extreme genotypes, as they appear principally in the periphery of the cloud of circles representing the varieties. In addition, there is a cluster of varieties forming mutants at the right of the plot. Figure 3 had a stress of 0.25, which means that the distances were ra-

ther poorly represented. However, the main feature of Fig. 3, the relative 'extremeness' of the mutant groups, can be trusted, as the varieties furthest away from the centre of a scaling plot are typically the best represented. Furthermore, various alternative forms of scaling produced almost identical configurations.

Mutants versus non-mutants

The distribution of pair-wise similarities is most easily assessed using a histogram in which all pair-wise similarities are plotted. Figure 4 shows the histogram for mutant (108 variety pairs) and non-mutant similarities (3,295 variety pairs). All similarities between individuals belonging to a mutant group were greater than 0.96, whereas pair-wise similarities among non-mutant group members were smaller than 0.8. From Fig. 4 it is evident that there is a complete separation between mutant group and non-mutant group similarities. The exception was formed by two evidently outlying mutant similarities (too low), which could later be identified as being derived from a contaminated DNA sample. In the non-mutant group of varieties, the varieties 51 and 70 showed, unexpectedly, identical AFLP profiles. At a later stage, when new material was obtained from the breeder, it became evident that the original material had been labelled incorrectly, and that both varieties had
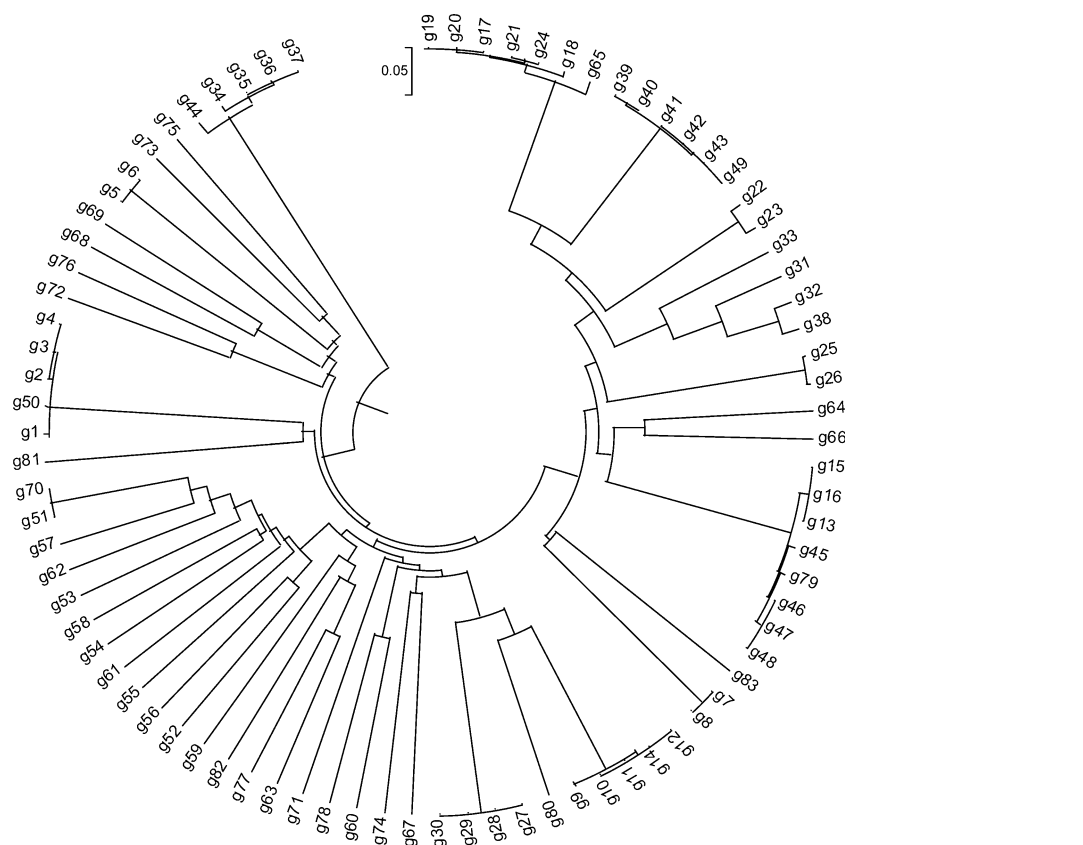

Fig. 2 UPGMA dendrogram on the basis of Jaccard similarities, including all genotypes, i.e. original varieties and mutants. Fitted distances (1.0 Jaccard similarity) between varieties can be calculated from the scale indication at the *top of the figure*
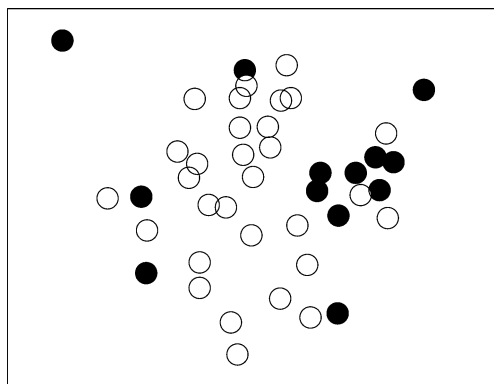
Fig. 3 Multidimensional scaling plot showing the relations between mutant families, represented by their original variety (*dark*) and other varieties (*light*)

indeed identical genotypes. They could therefore be treated as a mutant group as well. After these adjustments on the contents of the mutant and non-mutant groups, we conclude that the 12 PCs (284 polymorphic markers) seem to provide a reliable basis for distinguishing between mutant and non-mutant similarities. As mentioned above, mutant similarities were close to 1.0 (> 0.96), with the explainable exception of two outliers, whereas non-mutant similarities were below 0.80, with 75% of the non-mutant similarities even being below 0.50. It is remarkable that the non-mutant similarities were so far apart from unity. A reason for this observation may be found in the size of the rose gene pool and the absence of selection towards a common phenotype.

Effect of primer combinations

The relations between the similarities as generated by individual PCs underlie the graph in Fig. 5, where the
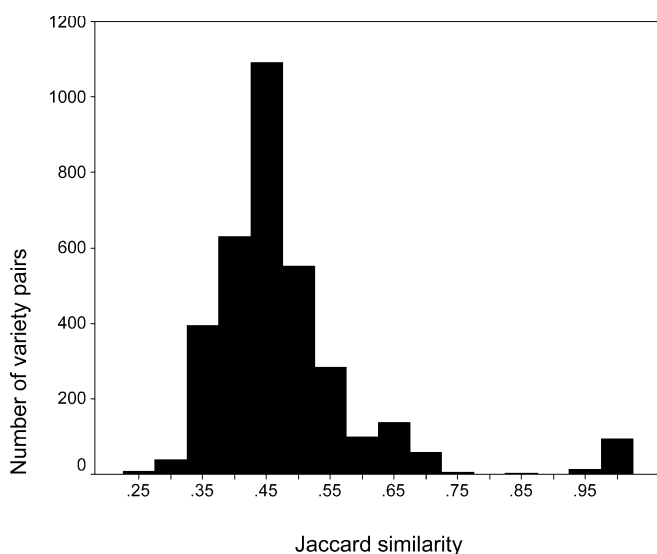

Fig. 4 Distribution of similarities across all variety pairs. Note that all mutant similarities are found between 0.95 and 1.0

Spearman rank correlations between the vectors of similarities corresponding to individual PCs are portrayed (see 'Materials and methods'). We concentrate on the non-mutant similarities, as the mutant similarities were all close to 1.0 anyway. In Fig. 5, the [cosines of the] angles between the vectors representing the PCs are proportional to the rank correlation. An angle of 90° means a correlation of 0; an angle of 0° stands for a correlation of 1, while an angle of 180° indicates a correlation of −1. For ease of visual inspection, Fig. 5 is oriented such as to let the positive *y*-axis coincide with the similarities as calculated across all PCs jointly, i.e. PCs whose similarities are highly correlated with the similarities across all PCs will have directions closely coinciding with the positive *y*-axis (note that the interpretation of Fig. 5 is not influenced by interchanging left and right in the figure). As can be observed in Fig. 5, most individual PCs produced similarities closely related to those produced by all PCs jointly. The most deviating PCs in Fig. 5 are E35 M56 and E35 M57, which both had a relatively low number of polymorphic bands, 13 and 18, respectively. A low number of polymorphic bands, however, do not automatically have to lead to a low correlation with the overall similarities, as E35 M52 and E35 M61, with 17 polymorphic markers each, aligned well with the overall similarities. The conclusion from Fig. 5 is that as soon as PCs produce more than 20 polymorphic bands, the similarities start to give a reasonably unbiased, i.e. on average correct, impression of the genetic relatedness between rose varieties.

Comparison between laboratories

For a reliable impression of genetic relatedness, not only a sufficient number of markers are required, but also sufficient precision, i.e. a small standard error, where the standard error for similarity estimates comprises both technical laboratory errors and genomic sampling errors. It is obvious that the genomic sampling error will decrease with the use of increasing numbers of markers.

Technical errors for both laboratories involved in the reproducibility study, PRI and Keygene, were very low and comparable. Both laboratories scored on average per mutant variety profile (= around 300 polymorphic markers) about one band different from the median profile. Upon closer inspection of gels and scoring patterns, manual scoring and transfer errors were found to constitute the major cause of technical errors. The technical error rate could be reduced to practically zero by the use of automated scoring.

The small technical errors make evident that the standard errors of the non-mutant similarities were almost exclusively determined by the sampling of the genome. Like the technical errors, the standard errors (= technical error + genomic sampling error) of non-mutant similarities for both laboratories were very close. The standard error in the PRI study was 0.0361, implying a total margin of error of 0.0707. The Keygene
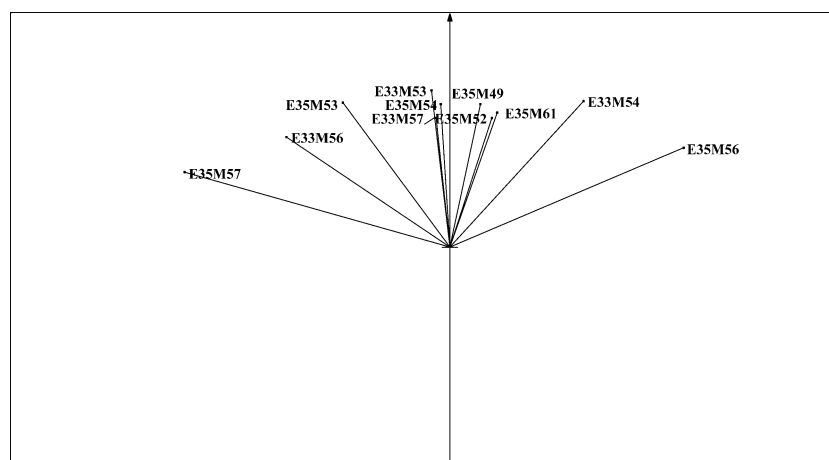
Fig. 5 Relations between similarities obtained from individual primer combinations and relation of individual primer combinations to similarities across all primer combinations. *Acute angles* indicate positive rank correlation between primer combinations. *Orthogonal angles* indicate null rank correlations. *Obtuse angles* indicate negative rank correlations. The positive *y*-axis (*vertical direction*) represents the similarities as calculated from all 12 primer combinations

study led to almost the same standard error, 0.0363. When the differences between mutant and non-mutant similarities (>0.15) are compared with the standard errors for non-mutant similarities (mutant similarities had almost negligible standard errors), it can be safely concluded that the AFLP marker system used in our study, based on the selected 12 PCs, provides a reliable system for the identification of putative EDVs in rose.

Finally, the correlation between the similarity estimates from both laboratories was, as expected, high: 0.93 ($P < 0.001$). The correlation between repeated estimates from the same laboratory was found to be 0.96 (average of both laboratories), so about the same as the observed correlation between laboratories. This result once more suggests that the precision of similarity estimates for non-mutant pairs is almost exclusively determined by genetic sampling errors. From these results we can again conclude that AFLPs provide a highly reproducible methodology for establishing relationships between rose cultivars.

Discussion

For implementation of the concept of 'essential derivation', it is vital that a clear distinction can be made between original varieties and putatively EDVs, using highly reproducible methodology. In practice this means that one needs to be able to define a threshold beyond which varieties coming from independent breeding programs are suspiciously closely related, and where one variety may be thought of as being essentially derived from the other, unless the breeder of the putatively derived variety can prove otherwise. Where this threshold is drawn has to be decided by the breeders. Its position will depend on a range of factors, such as overall genetic diversity available to breeders in that crop, genetic distance between present

varieties, breeding practice in that crop, propagation method, etc. A threshold should be based on genetic similarity measurements, preferably on the basis of molecular markers. Molecular markers are environmentally insensitive and can be generated in large amounts to provide sufficient characters for a statistically sound evaluation.

In the UPOV (1991) act, mutants are mentioned as examples of EDVs. The genetic similarity between mutants and the variety they are derived from is expected to be very close to 100%, since random molecular marker analyses are extremely unlikely to detect the few differences between original varieties and mutants thereof.

The main objective of this study was to determine whether varieties belonging to a mutant group could be distinguished from random pairs of varieties (not coming from the same mutant group). The possibility to separate pairs of original varieties from pairs of varieties that consist of initial and derived variety will depend on the difference in genetic similarity between original varieties as compared to the genetic similarities observed within mutant groups. In the set of original varieties, not a single pair-wise relation was found with a similarity greater than 0.80. This is in sharp contrast to the genetic similarities within mutant groups that all turned out to be greater than 0.96. These results clearly show that AFLP can be successfully used to distinguish between similarities originating from variety pairs belonging to the same mutant group and similarities of random pairs of varieties, provided that suitable PCs (both in quality and quantity) are chosen. A similar observation was made by Debener et al. (2000), who compared sports and seedling populations of two cut rose varieties. In their study no reproducible polymorphisms were detected between the sports using RAPD and AFLP, whereas up to 22.5% polymorphic bands were observed in the seedling populations using RAPD. The high similarities between mutants are comparable with the

values found in clonal analysis studies (Arens et al. 1998). The consequence for the assessment of essential derivation cases in rose is that mutant groups are immediately identified by similarities very close to 1.0. Deviations from unity may be caused by scoring errors. We have shown that scoring errors are very small (<1%), and that different experienced laboratories have scoring errors that are very comparable. We also noticed that automated scoring is advisable above manual scoring.

Assuming that the only way to obtain an EDV in rose is by mutation, it is feasible to distinguish between EDVs and non-EDVs. Based on the results presented in this paper, a safe separation line between EDVs and non-EDVs can be drawn at a Jaccard genetic similarity of 0.95. A threshold of 0.95 allows some variation in genetic similarities as result of experimental errors as well as from the existence of original varieties that are closer to each other. This threshold would also fit to the results obtained by Debener et al. (2000). Although a very representative set of varieties was used, some more closely related varieties might be found in the future when many more varieties are analyzed. However, we do not expect closely related non-mutant varieties to come close to the 0.95 separation line. Since their introduction in 1867 more than 10,000 hybrid tea rose varieties have entered the market (Cairns 2000).

From the data presented above, it can be concluded that mutants in rose do not impose a major problem for introducing the EDV concept and setting a possible threshold. This is due to the fact that mutant groups and original varieties are separated so evidently. Whether the results can be transferred to other crops will strongly depend on the breeding practice in these other crops. For cross-pollinating species with a broad genetic base and no convergent breeding, a similar situation can be expected, provided that the end product is propagated vegetatively, as is the case for many ornamentals. When backcrossing or breeding for a common type is practice, one can expect a less clear separation between mutant groups and original varieties. Introduction of the EDV concept needs to be looked at on a crop-by-crop basis.

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