4. Identification of essentially derived varieties (EDVs) derived from biparental crosses of homozygous lines. I. SSR data from maize inbreds

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Abstract

Genetic distances (GDs) based on molecular markers such as simple sequence repeats (SSRs) have been proposed as an appropriate tool to assess the genetic conformity between putative essentially derived varieties (EDVs) and their initial varieties (IVs). However, for maize and other crops no consensus has been reached regarding GD thresholds for identification of EDVs, because reliable benchmark data are lacking. The objectives of this study were to (1) estimate the variation in the parental contribution (*p*) to the genome of homozygous progeny lines derived in recycling breeding programs, (2) investigate the power of SSR-based GD estimates for discriminating between progeny lines derived from F_2, BC_1, and BC_2 populations, (3) exemplify the theoretical and simulated results of a companion study with experimental data, and (4) draw conclusions with regard to various EDV thresholds suggested hitherto. A total of 220 European and U.S. maize inbred lines comprising 163 triplets were genotyped with 100 uniformly distributed SSRs. A triplet consisted of one F_2-, or BC_1-progeny line and both parental lines. SSR-based estimates of *p* varied from 0.25 to 0.74 for F_2-derived lines with a mean (0.49) close to the expectation (0.50) and ranged from 0.51 to 0.80 for BC_1-derived lines with a mean (0.66) significantly smaller than the expectation (0.75). Relative to the variation in *p*, the GD between progeny lines and parents was less influenced by the variation in the GD between the parents, particularly for BC_1-derived lines. Suggested GD thresholds T for EDVs using a fixed GD yielded considerably different values for Type I (α) and Type II ($1-\beta$) errors among different gene pools and material groups. Therefore, we recommend germplasm specific thresholds with fixed α or $\alpha=1-\beta$.

Introduction

Legal regulations for plant variety protection (PVP) should secure the reward for past breeding efforts but also sustain future breeding progress. Registered plant varieties need to be protected against plagiarism and misuse on the one hand, but protected germplasm should be accessible for the development of new varieties on the other hand. The latter was warranted by the concept of "breeder's exemption" or "breeder's privilege" in the original convention of the Union for the Protection of New Varieties of Plants (UPOV, 1978).

The advent of new methods such as genetic engineering and marker-assisted backcrossing, however, has provided the basis to undermine the breeder's exemption in its original intention. These tools make it possible to add a few new genes to a protected variety or to select deliberately for lines that are very similar to one of their parents and apply for PVP for this "new" variety. Therefore, the investments made in breeding the original variety can be exploited by the breeder of the plagiarized variety without indemnification for the breeder of the original variety.

The concept of essentially derived varieties (EDVs) was implemented into the revised UPOV convention (UPOV, 1991) and several national PVP acts to cope with this new situation. Accordingly, a variety is deemed to be essentially derived from an initial variety (IV), if it (i) was predominantly derived from the IV, (ii) is clearly distinguishable from the IV, and (iii) is genetically conform to the IV. However, breeding companies have not agreed on specific breeding procedures that are considered to yield independently derived varieties (IDVs) or EDVs (*e.g.*, the number of acceptable backcross generations to a protected variety). In addition, no official guidelines or appropriate methods have been fixed to assess the genetic conformity between IVs and potential EDVs. Hence, crop-specific thresholds for the discrimination between EDVs and IDVs have not yet been defined.

In principle, the coefficient of parentage (*f*) introduced by Malécot (1948) could serve for identification of EDVs, because it reflects the degree of relatedness between two genotypes on the basis of their pedigree. In the case of a suspected EDV, however, pedigree

data are usually not available for the breeder of the IV. In addition, *f* is an indirect measure of genetic similarity based on several simplifying assumptions such as equal parental genome contributions and absence of selection, mutation, or drift (Messmer et al., 1992).

Molecular markers such as simple-sequence repeats (SSRs) or amplified fragment length polymorphisms (AFLPs) allow to determine the parental origin of the chromosomal segments in a progeny. Therefore, genetic distances (GDs) based on molecular markers were proposed as an appropriate tool to determine the genetic conformity between an IV and putative EDVs and, consequently, to distinguish between EDVs and IDVs (ASSINSEL, 1999; International Seed Federation, 2002). In maize, GDs between lines based on AFLP and SSR data were tightly correlated with each other and with *f* estimates (Lübberstedt et al., 2000; Smith et al., 1997), suggesting that the degree of relatedness of two genotypes can be inferred from their GD. However, distributions of GDs for F_2- and BC_1- derived progenies showed a substantial overlap (Bernardo et al., 1997).

In a companion paper, we proposed a conceptual framework, based on principles of statistical test theory, for identification of EDVs with molecular markers (Bohn et al., 2003). Accordingly, for a progeny line derived from bi-parental crosses, the GD to each parent depends on the GD between the two parents and *p*, the parental genome contribution transmitted to the progeny. Experimental estimates of *p* for F_2- and BC_1-derived progenies were reported by Bernardo et al. (1997; 2000). Moreover, formulas for the variance of *p* for both types of progeny were derived by Wang and Bernardo (2000). None the less, further experimental data are required to verify the approach of Bohn et al. (2004) and quantify the influence of the above mentioned factors with regard to consequences for potential EDV thresholds.

In this study, we investigated a large number of triplets in maize, each consisting of homozygous progeny lines derived from F_2, BC_1, or BC_2 populations and their parental inbreds. Our objectives were to (1) estimate the variation in the parental contribution to the genome of the progeny, (2) investigate the power of SSR-based GD estimates for discriminating between progenies derived from F_2, BC_1, and BC_2 populations, (3) exemplify the theoretical and simulated results of Bohn et al. (2004) with experimental data, and (4) draw conclusions with regard to various EDV thresholds suggested in the literature.

Materials and Methods

Plant Materials

A total of 220 elite maize inbred lines were analyzed comprising 89 European flint, 74 European dent, 14 U.S. dent, and 43 introgression lines. These lines originated from the maize breeding programs at the University of Hohenheim (Stuttgart, Germany), Iowa State University (Ames, USA), and three commercial breeding companies in Germany. The 220 lines comprised 163 triplets. A triplet consisted of one progeny and both parental lines. The materials consisted of 118 intra-pool triplets of European dent or flint lines and 45 inter-pool triplets, each consisting of one European and one U.S. line with an introgression line as progeny. Altogether, 83% of the progenies were derived from F_2 populations and 17% were derived from BC_1 or BC_2 populations (Table 1). Detailed information on all 163 triplets and the 220 maize inbreds included in this study is available in Tables A and B in the appendix of this thesis.

Molecular Analyses

All lines were genotyped with a set of 100 SSR markers uniformly covering the entire maize genome as described in detail by Heckenberger et al. (2002). Briefly, DNA samples were analyzed using an ABI Prism™ 377 DNA Sequencer with 96 lane polyacrylamid gels. Internal fragment size standards were used in each lane to increase accuracy of DNA fragment size determination. The size of each DNA fragment was determined automatically by using the GeneScan® software and assigned to specific alleles by the Genotyper® software. The 100 SSRs were selected on the basis of reliable single-locus amplification, absence of null alleles, high degree of polymorphism, and high reproducibility of the bands. Seventy of the 100 SSRs contained di-nucleotide repeat motifs, whereas the other 30 markers consisted of tri- to octa-nucleotide repeats. SSR analyses were performed on a commercial basis by Celera (1756 Picasso Avenue, Davis CA 95616, USA). Non-parental alleles were defined as alleles present in the progeny line, but absent in each of the parents.

Statistical Analyses

Malécot's (1948) coancestry coefficient (*f*) was calculated between all pairwise line combinations. Genetic distances (GDs) between lines were estimated using Rogers' distance (Rogers, 1972). In the case of missing values in one of the two inbreds compared, the corresponding alleles of the other accession were not used for GD calculation. Standard errors (SEs) for GDs were estimated using the jackknife procedure (Efron, 1979) with resampling over primer pairs (Tivang et al., 1994). Coefficients of correlation between GD_{SSR} and *f* were calculated using simple correlation coefficients (Snedecor and Cochran, 1980). In addition, the linear relationship between *f* and GD was tested with a lack-of-fit test. Calculation of GDs were performed with the PLABSIM software (Frisch et al., 2000). All other statistical calculations were performed with the R software package (Ihaka and Gentleman, 1996).

Suppose progeny line O is derived from a biparental cross (*e.g.,* F_2, BC_1, or BC_2 generation) between the homozygous parent P1 and P2 and the GDs between P1 and P2 or O are denoted by $GD_{(P1,P2)}$ and $GD_{(P1,O)}$, respectively. When O was an F_2-derived homozygous progeny line, P1 was the first parent listed in the pedigree record of O. When O was a BC_1-derived inbred, P1 was the recurrent parent, whereas P2 was the donor parent. If GD is determined by a large number of polymorphic markers with uniform coverage of the entire genome, we obtain the following equation:

$$GD_{(P1,O)} = (1-p)GD_{(P1,P2)}, \qquad [1]$$

where *p* denotes the proportion of the genome transmitted from P1 to O.

Solving Eq. [1] for *p* yields

$$p = 1 - \frac{GD_{(P1,O)}}{GD_{(P1,P2)}}, \qquad [2]$$

which can be used for estimating *p*. Similar formulas were given by Bernardo et al. (1997, 2000) on the basis of the number of common bands between P1 and O or the simple matching coefficient (Sneath and Sokal, 1973). Since the latter is based on single alleles without weighting of multiple bands within a marker locus, we chose the Rogers' distance for this study.

In the absence of selection, *p* is a random variable with distribution properties depending on (a) the degree of relatedness between P1 and O and (b) the number and length of the chromosomes (Wang and Bernardo, 2000). If P1 and P2 are unrelated ($f_{(P1,P2)}$=0), then the expected value μ_p of *p* corresponds to the coancestry $f_{(P1,O)}$ and, thus, μ_p=0.500, 0.750, and 0.875 for F_2-, BC_1-, or BC_2-derived progeny lines of P1, respectively.

Formulas for the variance σ_p^2 of F_2- or BC_1-derived progeny lines were given by Wang and Bernardo (2000). In addition, numerical values for maize were obtained for F_2-, BC_1-, or BC_2-derived progeny lines from stochastic simulations by Bohn et al. (2004). The latter were based on a genetic model allowing for genetic drift but neither selection nor mutation. Hence, empirical and simulated frequency distributions of *p* values were compared with a Kolmogorov-Smirnov test (Lehmann, 1986) to check for significant deviations caused by selection or mutations. Equality of variances of empirical and simulated frequency distributions of *p* was evaluated with Levene's test (Levene, 1960).

If progeny lines are derived from a large number of bi-parental crosses with different pairs of parents P1 and P2 representative for a germplasm pool, then $GD_{(P1,P2)}$ can be regarded as a random variable with mean $\mu_{GD_{(P1,P2)}}$ and variance $\sigma^2_{GD_{(P1,P2)}}$. Since the value of *p* for a specific progeny is completely unrelated to the GD of its parent lines, $GD_{(P1,P2)}$ and *p* are stochastically independent. Thus, we obtain from Eq. [1] the following equations (Bohn et al., 2004):

$$\mu_{GD_{(P1,O)}} = \mu_{GD_{(P1,P2)}} - \mu_{GD_{(P1,P2)}}\mu_p \qquad [3]$$

$$\sigma^2_{GD_{(P1,O)}} = (1-\mu_p)^2\sigma^2_{GD_{(P1,P2)}} + \mu^2_{GD_{(P1,P2)}}\sigma^2_p + \sigma^2_{GD_{(P1,P2)}}\sigma^2_p, \qquad [4]$$

where $\mu_{GD_{(P1,O)}}$ and $\sigma^2_{GD_{(P1,O)}}$ are the mean and variance of $GD_{(P1,O)}$, respectively, for a given relationship between O and P1. By inserting experimental estimates for $\sigma^2_{GD_{(P1,P2)}}$ and estimates for μ_p and σ_p^2 determined (a) either from computer simulations (Bohn et al., 2004) or (b) the formulas given by Wang and Bernardo, (2000), we were able to calculate predicted values for $\sigma^2_{GD_{(P1,O)}}$ and compare them with estimated values for F_2- or BC_1-derived progeny lines from unrelated parents. In addition, Eq.[4] permits to compare the relative

influence of σ_p^2 and $\sigma_{GD_{(P1,P2)}}^2$ on the variance of $GD_{(P1,O)}$ for F_2- or BC_1-derived progeny lines, which is of importance for the question of EDV thresholds. In addition, simulated $GD_{(P1,O)}$ values were calculated with Eq. [1] for each material group on the basis of simulated *p* values and $\hat{\mu}_{GD_{(P1,P2)}}$ and $\sigma_{GD_{(P1,P2)}}^2$ for observed $GD_{(P1,P2)}$ values of unrelated lines.

Threshold Scenarios

To increase the sample size, not only GD values obtained within triplets were used for evaluation of potential thresholds (T), but all GD values of the dataset with corresponding *f* values of 0.500, 0.750, and 0.875 for F_2-, BC_1-, or BC_2-derived progeny lines. The frequency distributions of empirical $GD_{(P1,O)}$ values for F_2-, BC_1-, or BC_2-derived progeny lines were approximated by beta distributions (Johnson et al., 1995) with parameters chosen such that the mean and variance of the original distribution were conserved. Based on these distributions, we calculated Type I (α) and Type II (1-β) errors for various EDV thresholds T and various types of populations. Here, α corresponds to the probability that a true IDV will be wrongly judged as EDV and 1-β corresponds to the probability that a true EDV will not be recognized as such and judged as IDV (Fig. 1). First, we considered the situation that an F_2-derived progeny will be regarded as IDV, but a BC_1-derived progeny as EDV. Second, we assumed that a BC_1-derived progeny will be regarded as IDV, but a BC_2-derived progeny as EDV.

SSR- or RFLP-based GD values of 0.25, 0.20, 0.15, and 0.10 were suggested as possible EDV thresholds T by ASTA (Smith and Smith, 1989), ASSINSEL (2000), SEPROMA (Leipert, 2003, personal communication) and Troyer and Rocheford (2002), respectively. For all thresholds, the corresponding α and 1-β values were calculated for homozygous progeny lines derived from F_2, BC_1, and BC_2 populations. In addition, other thresholds T with fixed α=0.05 ($T_{0.05}$) or α=1-β ($T_{\alpha=1-\beta}$) were tested.

Results

Genetic Variation for SSRs

A total of 1099 SSR alleles were observed with the 100 SSRs on the set of 220 inbred lines. The number of alleles per marker varied from 3 to 26. PIC values ranged from 0.10 to 0.88 with a mean of 0.71. Only 3.7% of all marker data points were missing due to amplification failure or null alleles. Correlations between GD and *f* were highly significant ($P<0.01$) for all three material groups and highest for dent lines (r=-0.90**), intermediate for flint lines (r=-0.75**) and lowest for introgression lines (r=-0.58**). In addition, we observed a linear relationship between *f* and GD for all three material groups. A detailed description of the genetic diversity of the germplasm is given elsewhere.

Parental Contributions (*p*) for F_2- and BC_1-derived Progenies

The three material groups did not differ from each other in their means $\hat{\mu}_p$ for both the F_2- and BC_1- derived progenies. Hence, the data from all three groups were pooled for further analyses. For F_2-derived progenies, SSR-based estimates of *p* ranged from 0.25 to 0.74 with $\hat{\mu}_p$=0.49 (Fig. 2), close to the expectation of 0.50. Variances for observed and simulated values of *p* (σ_p^2) did not differ significantly ($P<0.05$) (Table 2). Frequency distributions for observed and simulated estimates of *p* were significantly different ($P<0.05$) from each other due to a higher kurtosis of the former.

SSR-based estimates of *p* for BC_1-derived progenies varied from 0.51 to 0.80 with a mean $\hat{\mu}_p$=0.66, which was significantly smaller than the expectation of 0.75 (Fig. 2). Variances for observed and simulated values of *p* (σ_p^2) were not significantly ($P<0.05$) different from each other (Table 2). Frequency distributions for observed and simulated estimates of *p* showed significant differences ($P<0.01$) due to the shift to smaller values, the lower skewness and the higher kurtosis for the distribution of observed *p* values.

Genetic Distances among Unrelated Parental Inbred Lines

GDs among unrelated ($f_{(P1,P2)}$=0) flint lines ranged from 0.23 to 0.79 with $\hat{\mu}_{GD_{(P1,P2)}}$=0.58 (Fig. 3). GDs for unrelated dent lines varied from 0.25 to 0.85 with a significantly (P<0.01) larger mean $\hat{\mu}_{GD_{(P1,P2)}}$=0.61. Unrelated parents of introgression lines, consisting of pairs of European and U.S. maize lines, had by far the largest range from 0.22 to 0.93 and also a significantly (P<0.01) higher mean $\hat{\mu}_{GD_{(P1,P2)}}$=0.74 than the intra-pool pairs of the other two material groups.

Subdivision of the Variance of $GD_{(P1,O)}$ for F_2- and BC_1-derived Progenies

Observed values of $\sigma^2_{GD_{(P1,O)}}$ obtained directly from experimental data were in close agreement with the predicted $\sigma^2_{GD_{(P1,O)}}$ values calculated with Eq.[4] on the basis of simulated values of μ_p and σ^2_p as well as experimental estimates of $\hat{\mu}_{GD_{(P1,P2)}}$ and $\sigma^2_{GD_{(P1,P2)}}$. Further analysis revealed that for F_2-derived progenies 65% of $\sigma^2_{GD_{(P1,O)}}$ could be explained by σ^2_p and 34% by $\sigma^2_{GD_{(P1,P2)}}$. For BC_1-derived progenies, 94% of $\sigma^2_{GD_{(P1,O)}}$ were explained by σ^2_p and only 5% by $\sigma^2_{GD_{(P1,P2)}}$. The contribution of the product $\sigma^2_p\sigma^2_{GD_{(P1,P2)}}$ to $\sigma^2_{GD_{(P1,O)}}$ was less than 1% for both F_2- and BC_1-derived progeny lines (Table 2).

Evaluation of EDV-Threshold Scenarios

Observed frequency distributions of GD values for F_2-, BC_1-, and BC_2-derived progenies fitted well the approximated beta distributions for flint and dent lines, but only moderately for introgression lines (Fig. 4). For all three material groups, considerable overlaps between the frequency distributions of GDs for F_2- *vs.* BC_1- as well as for BC_1- *vs.* BC_2-derived progenies were observed. Within each generation, $\hat{\mu}_{GD_{(P1,O)}}$ was significantly higher (P<0.05) for the dent lines than for the flint lines. In addition, $\hat{\mu}_{GD_{(P1,O)}}$ for the introgression lines was always significantly higher (P<0.01) than $\hat{\mu}_{GD_{(P1,O)}}$ for the flint and dent

lines. Estimates of $\sigma^2_{GD_{(P1,O)}}$ for the same generation were not significantly different (P<0.01) between flint and dent lines but significantly (P<0.01) larger for introgression lines.

Given α=0.05 for F_2-derived lines, the power β to classify a BC_1-derived progeny line as EDV amounted to 77%, 63%, and 15% for the particular thresholds determined for flint, dent, and introgression lines, respectively (Table 3.). Corresponding values of β for BC_2-derived lines, assuming α=0.05 for BC_1-derived lines were smaller for flint and dent lines, but larger for introgression lines. The power β for thresholds determined for α=1-β to classify BC_1- or BC_2-derived progenies as EDVs increased considerably compared to the values for α=0.05. This increase in the power β, however, is associated with higher values for α. Therefore, this leads to a considerably higher frequency of F_2- or BC_1-derived progenies incorrectly classified as EDVs.

For T=0.25, 0.20, or 0.15, the corresponding α levels for F_2-derived lines varied between α=0.18 and α=0.00 (Table 3). Corresponding β values ranged between 7% and 92%. For T=0.15 and T=0.10, the power β to detect a BC_2-derived line as EDV varied from 10% to 99% with corresponding α values for BC_1-derived lines ranging from 0.02 to 0.07. For each T substantial differences for α and β between flint, dent, and introgression lines were observed.

For α=0.05 and α=1-β, T values obtained from simulated data were lower than from observed data with the exception of α=0.05 for introgression lines (Table 3.). For all these scenarios, the power β to classify BC_1- or BC_2-derived progeny lines as EDVs was similar between thresholds based on observed and simulated values of $GD_{(P1,P2)}$ for both flint and dent lines. For introgression lines, however, β was substantially higher for T values based on simulated data than those based on observed data. Considerable differences existed also between observed and simulated data regarding values of α and β for T=0.25, 0.20, 0.15 and 0.10.

Discussion

Our study was initiated by commercial breeding companies to derive EDV thresholds in maize based on scientifically reliable criteria, as requested by UPOV and ASSINSEL. Representative germplasm for each material group was taken from public and private breeding programs. SSRs were chosen as a suitable marker system due to their known map positions, high degree of polymorphism and suitability for automated high-throughput analyses. Therefore, our results are relevant for the definition of EDV thresholds and provide a general overview on putative essential derivation scenarios in European maize germplasm and the power of SSRs for identification of EDVs.

Use of SSR-Based GDs for Identification of EDVs

The rationale for using SSR-based GD estimates for identification of EDVs is their close relationship to *f*. Therefore, they can be used to uncover close pedigree relationships between pairs of inbred lines. Correlations between GDs and *f* calculated across the entire data set (r=0.77) and separately for each material group were similar or higher than reported in previous studies with maize (Lübberstedt et al., 1999; Pejic et al., 1998). This reflects the broad basis of germplasm in this study ranging from unrelated to closely related combinations of lines. Moreover, the linear relationship of GD and *f* corroborates that GDs based on SSRs faithfully reflect the genetic diversity of the germplasm. In spite of the observed high correlations, considerable variation was observed for GD values obtained for the same *f* values and, thus, overlaps in the frequency distributions of GDs occurred for *f*=0.50, 0.75, and 0.88. Therefore, F_2-, BC_1-, and BC_2-derived progenies could not be distinguished unambiguously by their $GD_{(P1,O)}$.

Factors Influencing $GD_{(P1,O)}$

According to Eq. [1], $GD_{(P1,O)}$ is influenced by two factors: $GD_{(P1,P2)}$ and *p*. Assuming the ideal case that unrelated lines ($f_{(P1,P2)} = 0$) show a GD of 1.0, $GD_{(P1,O)}$ yields an estimate of 1-*p,* wich theoretically results in the highest discrimination ability between different types of progeny. However, even for this most favorable case, considerable overlaps

between the frequency distributions of F_2- and BC_1-derived or between BC_1- and BC_2-derived lines were found in simulations (Fig. 1).

Means and variances for distributions of observed *p* values for F_2-derived progenies were to a large extent identical with the distribution of simulated *p* values. However, the observed $\hat{\mu}_p$ for BC_1-derived progenies was substantially lower than the expectation (Table 2). This shift towards the distribution of F_2-derived progenies is very likely attributable to the selection of the most vigorous BC_1 plants in the development of improved progeny lines. Due to the phenomenon of heterosis, such BC_1 plants are more heterozygous and consequently have a higher proportion of donor genome than the average. Obviously this selection for more heterozygous plants would result in an increased overlap in the frequency distributions of GDs between F_2- and BC_1-derived or between BC_1- and BC_2-derived lines, compared to the simulated data shown in Figure 1.

Further comparison of the above-mentioned ideal case with authentic data revealed that $GD_{(P1,P2)}$ between unrelated lines was lower than 1.0 and showed a considerable variance $\sigma^2_{GD_{(P1,P2)}}$. This leads to condensed and more flat frequency distributions for $GD_{(P1,O)}$ values of F_2-, BC_1-, and BC_2-derived progenies and, therefore, to a further increase of the overlaps. The magnitude of the overlaps is mainly caused by the parameters $\hat{\mu}_{GD_{(P1,P2)}}$ and $\sigma^2_{GD_{(P1,P2)}}$ of unrelated lines. Due to different levels of genetic diversity among breeding germplasm of crops, $\hat{\mu}_{GD_{(P1,P2)}}$ and $\sigma^2_{GD_{(P1,P2)}}$ vary considerably among different crop species. For example, the GDs between unrelated barley (Melchinger et al., 1994) or tomato cultivars (Grandillo et al., 1999) were substantially lower than observed in maize (Messmer et al., 1993). This underlines the necessity of crop-specific thresholds T for the discrimination of EDVs and IDVs.

Power of SSR-based GDs to Detect EDVs

For fixed thresholds of T=0.25, 0.20, 0.15, and 0.10, substantial differences for the Type I error α and the Type II error $1-\beta_T$ were found between the three material groups. Further analyses revealed that pooling of flint and dent data would lead to a significant increase of flint lines in the fraction of EDVs (data not shown). Moreover, developing a

joint threshold for intra-pool and inter-pool progenies would result in a substantially greater risk of developing an EDV from intra-pool than inter-pool crosses. Consequently, a pool-specific approach is more fair in terms of α and $1-\beta_T$ than fixed GD thresholds. Therefore, thresholds T need to be gene pool specific and different thresholds must be developed for potential EDVs from intra-pool crosses than for progenies from inter-pool crosses.

The thresholds calculated for simulated $GD_{(P1,O)}$ values were generally lower than from observed data. This can be partially explained by the occurrence of non-parental alleles. The most probable reason for this shift, however, is the fact that simulated GD values were based on $\hat{\mu}_{GD_{(P1,P2)}}$ and $\sigma^2_{GD_{(P1,P2)}}$ of all pairwise distances between unrelated (*f*=0) lines within a material group. But breeders often prefer using genetically diverse inbred lines within a gene pool as parents for their recycling breeding. This iplies that the parental lines used in breeding programs may not be a random sample of all unrelated lines of a germplasm pool. When the generation of simulated GD values was repeated with the mean and variance of only the GDs of parental lines actually used, this resulted in a shift of the thresholds towards the corresponding experimental values.

Precision of GDs and Number of Markers Required

Apart from their Type I and Type II errors, the robustness of $GD_{(P1,O)}$ against addition, substitution, or removal of markers is an important factor to be considered for the development of appropriate thresholds. Standard errors (SEs) attached with GD values were of considerable size across all scenarios and material groups, but decreased with decreasing GD thresholds. Assuming a 95% confidence interval (CI) for GD thresholds, this would range from –2 SEs to +2 SEs and, *e.g.*, from 0.13 to 0.29 for $T_{0.05}$ (F_2 *vs.* BC_1 for flint lines) and from 0.04 to 0.14 (BC_1 *vs.* BC_2 for flint lines). Thus, a number of 100 SSRs seems to be at the lower limit for identification of EDVs, as high SEs for GDs increase the probability of Type I or Type II errors. Hence, we recommend a two-stage procedure for identification of EDVs with SSRs in which a set of 100 SSRs uniformly distributed across the genome is analyzed initially, and if there are doubts about the relationship of an IV and a potential EDV, a second set of 100 or more SSR markers is analyzed subsequently.

Given a maximum SE of GDs, one can calculate the necessary number of markers to reach this SEs depending on the mean number of alleles per marker. Minimizing the mean SE for GDs to acceptable values of 0.02 or 0.01 in our study would require a substantial increase in the necessary number of SSRs. For example, a minimum of 260 SSRs would be required to reduce the average SE to 0.01 at a GD level of 0.20. As an alternative, the SE of GDs can be reduced by the choice of highly polymorphic SSR markers. The effective number of alleles (n_e) in our study was 4.2. If it could be doubled to n_e=8.4 by an appropriate choice of highly polymorphic SSR markers only ~120 SSRs would be required to reduce the average SE to 0.01 at a GD level of 0.20. As this high degree of polymorphism is rather unrealistic in connection with an equal distribution of markers over the maize genome, different types of markers seem more promising. This includes the use of a standard DNA chips for EDV identification with the extended large-scale use of expression patterns, the high-throughput application of newly developed marker systems like single nucleotide polymorphisms (SNPs), or the targeted use of allelic information such as the generation of haplotypes.

In addition, intra-varietal variation caused by heterogeneity within lines and lab errors must be considered for the development of thresholds. If ignored, this leads to an overestimation of GDs and, therefore, a bias to the benefit of the breeder of the potential EDV (Heckenberger et al., 2002). Hence, thresholds should be adapted accordingly to reduce this bias. In addition, thresholds must be specific according to a particular set of markers, as the specific choice of markers should be neutral with regard to the conclusion, whether a variety is deemed as an EDV or not.

Appropriate Distance Measures

It is desirable that the GDs between the progeny and either parent add up to the GD between the parental lines (Melchinger, 1993). From all commonly used genetic distance measures, this criterion holds generally only true for the Rogers' (1972) and the Nei and Li (1979) distance. In addition, a linear relationship to *f* is requested, which is fulfilled by both GD measures. Coefficients like Dice (1945), Jaccard (1908), or simple matching (Sneath and Sokal, 1973) are based on single bands, irrespective of the marker to which they belong. Therefore, heterozygous loci are overweighted. In contrast, Rogers' distance

is based on the frequencies of alleles of each marker. Therefore, multiple alleles for a particular marker are weighted in comparison with homozygous alleles of another marker. In addition, frequency-based distance measures could be applied for population varieties and it would be possible to include codominant data from dominant marker systems (Jansen et al., 2001; Piepho and Koch, 2000). Therefore, we recommend the Rogers' distance for identification of EDVs with SSRs. Moreover, we conclude that EDV thresholds must be specific for the distance measure used.

Conclusions

Our results showed that GDs based on SSRs are suitable tools to distinguish between progenies derived from F_2, BC_1 or BC_2 source populations, however, associated with a certain error rate. Due to the observed overlaps in the frequency distributions of $GD_{(P1,O)}$ for F_2-, BC_1-, and BC_2-derived progenies, the choice of an appropriate threshold T is a crucial issue to minimize the Type I (α) and the Type II ($1-\beta_T$) errors. Whereas the GD threshold suggested by ASSINSEL (0.20) results in fairly acceptable α and $1-\beta_T$ values for flint lines, but fairly low $1-\beta_T$ values for dent and introgression lines, we recommend crop- and genepool-specific thresholds on the basis of a fixed α level or $\alpha=1-\beta_T$. Furthermore, the threshold should depend on the marker set and distance measure chosen. Implementation of the EDV concept in practical plant breeding requires a standard set of a large number of highly polymorphic markers for reliable determination of GDs. In addition, we strongly recommend replications of lab assays to minimize lab errors. The frequency distributions of GDs used in this study were based on unrelated parental lines. Obviously, use of related parents for the development of new varieties by recycling breeding will increase the probability of breeding an EDV from an accepted breeding procedure.

Acknowledgements

We are indebted to the 'Gesellschaft zur Förderung der privaten Pflanzenzüchtung' (GFP), Germany for a grant to support Dr. Bohn and the SSR analyses of the present study. Financial support for M. Heckenberger was provided by a grant from the European Union, Grant No. QLK-CT-1999-01499 (MMEDV).

References

ASSINSEL. 1999. Essential derivation and dependence. Practical Information.

ASSINSEL. 2000. DUS testing: phenotype *vs.* genotype. Position Paper adopted at the Rome Congress in May 2000.

Bernardo, R., A. Murigneux, J. P. Maisonneuve, C. Johnsson, and Z. Karaman. 1997. RFLP-based estimates of parental contribution to F_2- and BC_1-derived maize inbreds. Theor. Appl. Genet. 94:652-656.

Bernardo, R., J. Romero Severson, J. Ziegle, J. Hauser, L. Joe, G. Hookstra, and R.W. Doerge. 2000. Parental contribution and coefficient of coancestry among maize inbreds: pedigree, RFLP, and SSR data. Theor. Appl. Genet. 100:552-556.

Bohn, M., M. Frisch, and A. E. Melchinger. 2004. A statistical framework for identification of essentially derived varieties obtained from biparental crosses of homozygous lines. Statistical theory. In Preparation.

Dice, L. R. 1945. Measures of the amount of ecologic association between species. Ecology. 26:297-302.

Efron, B. 1979. Bootstrap methods: another look at the jackknife. Ann. Stat. 7:1-26.

Frisch, M., M. Bohn, and A. E. Melchinger. 2000. Plabsim: Software for simulation of marker-assisted backcrossing. J. Hered. 91:86-87.

Grandillo, S., H. M. Ku, and S. D. Tanksley. 1999. Identifying the loci responsible for natural variation in fruit size and shape in tomato. Theor. Appl. Genet. 99:978-987.

Heckenberger, M., M. Bohn, J. S. Ziegle, L. K. Joe, J. D. Hauser, M. Hutton, and A.E. Melchinger. 2002. Variation of DNA fingerprints among accessions within maize inbred lines and implications for identification of essentially derived varieties. I. Genetic and technical sources of variation in SSR data. Mol. Breed. 10:181-191.

Ihaka, R. and R. Gentleman. 1996. R: A language for data analysis and graphics. J. Comp. Graph. Stat. 5:299-314.

International Seed Federation. Chicago, May 2002. ISF view on Intellectual Property.

Jaccard, P. 1908. Nouvelles recherches sur la distribution florale. Bull. Soc. Vaud. Sci. Nat. 44:223-270.

Jansen, R.C., H. Geerlings, A.J. Van Oeveren, and R.C. Van Schaik. 2001. A comment on codominant scoring of AFLP markers. Genetics. 158:925.

Johnson, N. L., S. Kotz, and N. Balakrishnan. 1995. Continuous Univariate Distributions Vol.2. p. 210-275. J. Wiley and Sons, Inc., New York.

Lehmann, E.L. 1986. Testing Statistical Hypotheses. J. Wiley and Sons, New York.

Levene, H. 1960. Robust tests for equality of variances. p. 278-292. *In* Contributions to probability and statistics: Essays in honour of Harold Hotelling. Stanford University Press, Stanford, CA.

Lübberstedt, T., A. E. Melchinger, C. Dussle, M. Vuylsteke, and M. Kuiper. 2000. Relationships among early european maize inbreds: iv. Genetic diversity revealed with AFLP markers and comparison with RFLP, RAPD, and pedigree data. Crop Sci. 40:783-791.

Malécot, G. 1948. Les Mathematiques de l'Heredité. Masson & Cies, Paris.

Melchinger, A. E., A. Graner, M. Singh, and M. M. Messmer. 1994. Relationships among European barley germplasm. 1. Genetic diversity among winter and spring cultivars revealed by RFLPs. Crop Sci. 34:1191-1199.

Melchinger, A. E. 1993. Use of RFLP markers for analysis of genetic relationships among breeding materials and prediction of hybrid performance. *In* Int.Crop Science I. CSSA. Madison, WI. p.621-628.

Messmer, M. M., A. E. Melchinger, J. Boppenmaier, E. Brunklaus Jung, and R.G. Herrmann. 1992. Relationships among early European maize inbreds. I. Genetic diversity among flint and dent lines revealed by RFLPs. Crop Sci. 32:1301-1309.

Messmer, M. M., A. E. Melchinger, R. G. Herrmann, and J. Boppenmaier. 1993 . Relationships among early European maize inbreds. II. Comparison of pedigree and RFLP data. Crop Sci. 33:944-950.

Nei, M. and W. H. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Soc. USA. 76:5269-5273.

Pejic, I., P. Ajmone-Marsan, M. Morgante, V. Kozumplick, P. Castiglioni, G. Taramino, and M. Motto. 1998. Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs, and AFLPs. Theor. Appl. Genet. 97:1248-1255.

Piepho, H.-P. and G. Koch, 2000. Codominant analysis of banding data from a dominant marker system by normal mixtures. Genetics. 155:1459.

Rogers, J. S. 1972. Measures of genetic similarity and genetic distance. Studies in Genetics VII. Univ. Texas Publ. 7213:145-153.

Smith, J. S. C., E. C. L. Chin, H. Shu, O. S. Smith, S. J.Wall, M. L. Senior, S. E. Mitchell, S. Kresovich, and J. Ziegle. 1997. An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.) - comparisons with data from RFLPs and pedigree. Theor. Appl. Genet. 95:163-173.

Smith J.S.C. and O.S. Smith. 1989. The description and assessment of distances between inbred lines of maize: II. The utility of morphological, biochemical, and genetic descriptors and a scheme for testing of distinctiveness between inbred lines. Maydica. 34:151-161.

Sneath, P. and R. Sokal. 1973. Numerical taxonomia. WH Freeman & Co, San Francisco.

Snedecor, G. and W. Cochran. 1980. Statistical Methods. Iowa State University Press, Ames, IA.

Tivang, J. G., J. Nienhuis, and O. S.Smith. 1994. Estimation of sampling variance of molecular marker data using the bootstrap procedure. Theor. Appl. Genet. 89:259-264.

Troyer, A. F. and T. R. Rocheford. 2002. Germplasm ownership: related corn inbreds. Crop Sci. 42:3-11.

UPOV. 1978. International convention for the protection of new varieties of plants.

UPOV. 1991. International convention for the protection of new varieties of plants.

Wang, J. K. and R. Bernardo. 2000. Variance of marker estimates of parental contribution to F_2- and BC_1-derived inbreds. Crop Sci. 40:659-665.