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MOLECULAR AND OTHER MARKERS FOR ESTABLISHING ESSENTIAL DERIVATION IN CROP PLANTS (MMEDV) - AN OVERVIEW

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MOLECULAR AND OTHER MARKERS FOR ESTABLISHING ESSENTIAL DERIVATION IN CROP PLANTS (MMEDV)

AN EU FRAMEWORK 5 PROJECT

AN OVERVIEW

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1. Background

The concept of the Essentially Derived Variety (EDV) is now well known and arises from the 1991 UPOV Convention (Article 14):

> …(*b*) For the purposes of subparagraph *(a)*(i), a variety shall be deemed to be essentially derived from another variety ("the initial variety") when
>
> (i) 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,
>
> (ii) it is clearly distinguishable from the initial variety and
>
> (iii) 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.
>
> *(c)* Essentially derived varieties 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.

There has been much discussion about ED and how it might be assessed, and although certain suggestions and principles have been advanced for some species, there had been no agreed basis on which to proceed. Thus the overall objective of the MMEDV project was "*…develop a scientifically based framework to assist in the determination of the essential derivation of plant varieties…*".

The basic premise of the project is that demonstration of ED status is primarily concerned with an analysis of the degree of relatedness (genetic conformity) between varieties. This can be illustrated by the following diagram:

Table 1 Visual outline of the EDV concept.

Divergence (distance) from initial variety, X	Minimum distance ----------|----------|----------> Distance Limit of EDV		
Type of variety:	*X** - plagiarised X	*X′* - new, EDV (dependent)	*Y* - new (independent)
Differentiation from X	insignificant 'cosmetic' changes	clearly distinct, but retains 'essential characteristics'	clearly distinct
Eligibility for PBR	No	Yes (essentially derived)	Yes

The issue is to try to define in a meaningful and scientifically valid way where the boundary (threshold) lies between distinct, dependent varieties (EDVs) and distinct, independent varieties (not EDVs). As this will vary from crop to crop and also be dependent on how the measurements are made, a multi-species, multi-techniques approach was adopted.

2. Experimental Approaches

Three crops were used as models within the MMEDV project – barley, maize and roses. In outline, the project utilised specially produced genotypes of these crops arising from a range of situations which might potentially produce EDVs, i.e. repeated back-crossing, mutations, somaclonal variation, production of GMOs and hybrid production. In order to assess the "relatedness" between these genotypes, various approaches were used including examination of morphological characteristics and levels of heterosis. A major focus of effort was the use of molecular markers, including novel retrotransposon-based systems. Table 2 summarises the methods and the crops to which they were applied:

Table 2 – Summary of crops/markers within MMEDV

			Molecular Marker[1]				
Crop	Morphology	Heterosis	AFLP	S-SAP	REMAP	IRAP	SSR
Barley	*		*	*	*	*	*
Maize	*	*	*		*		*
Roses			*	*			*

[1]AFLP = Amplified Fragment Length Polymorphism; S-SAP = Sequence-Specific Amplification Polymorphisms; REMAP = Retrotransposon - Microsatellite Amplified Polymorphism; IRAP = Inter-Retrotransposon Amplified Polymorphism; SSR = Simple Sequence Repeat

These approaches were applied to defined sets of genotypes of the three model crops, as summarised in table 3:

Table 3 – Summary of material

	Approach/EDV issue				
Crop	Backcross	GM	Somaclonal variation	Methylation	Mutation
Barley	*	*	*	*	
Maize	*	*	*	*	
Roses				*	*

3. Statistical Concepts and Analysis

Clearly the assessments of relatedness between genotypes rely on the effective estimation of "distance"(or similarity), which in turn is dependent on the suitability of the markers (including morphology and heterosis) as a measure of distance. Thus a central part of the MMEDV project was to develop the statistical concepts and tools to evaluate data from the different kinds of approaches being taken in the three model crops. This includes issues such as (i) the sources of variation in a technique (and thus the statistical confidence levels), (ii) the suitability of the various molecular markers to measure genetic distances that reflect the known relationships between the genotypes, (iii) the precision of these distance measures, (iv) assessing the importance of the number of markers/datapoints used, and (v) assessing if the map position of the markers is important in the determination of distances.

4. Summary of Results

The central question addressed within the MMEDV project was "at what degree of relatedness for a given species is it considered and agreed by breeders that dependence (ED) of varieties is significant?" There were two related issues within this – (i) the effective and statistically valid estimation of distance as a measure of variety relatedness, and (ii) the estimation of an objective distance (relatedness) threshold against which to measure ED in different species.

4.1 Roses

The 1991 UPOV Convention states that mutation is one mechanism for obtaining essentially derived varieties. Since mutants or "sports" are frequently observed during multiplication in roses, it is thus a useful species for studying the ED concept. Implementation of the concept in rose necessitates that similarities between mutant varieties can be clearly separated from non-mutant varieties, and that experimental errors in determining the relatedness between varieties are small. The results obtained from all three marker systems analysed (SSR, AFLP, SSAP) showed that similarities between mutants and the variety from which they are derived were very high (close to 1.0) and clearly separated from similarities between non-mutant varieties. Experimental errors, even between laboratories, were very small. It was also clear that a "forensic" approach to the EDV issue is a viable one, which can be implemented at relatively low cost using a few highly informative microsatellite markers, opening the way for implementing the ED concept in rose. These results will be described in detail separately.

4.2 Maize

Maize "triplets" (male and female inbred lines and their F1 hybrid) were analysed. The results from all marker systems analysed (SSR, AFLP) and from heterosis studies indicated that a potential ED threshold at about the F2-BC1 level exhibited some overlap in terms of genetic distances. Morphological measures of distance were less informative, and also slower and more expensive than the other approaches. Therefore setting the ED thresholds will require further discussion of precise boundaries.

The aim of the second part of the part of the research with maize was to examine the extent to which somaclonal variation affects the genetic similarity between genetically modified (GM) maize and the donor lines. Six sets of T1, T2, T3 and T4 GM-seed, were considered, together with the original donor lines. A set of 24 AFLP primer combinations (PCs) were tested based on the commonly used EcoRI/MseI restriction enzymes and 20 AFLP PCs based on EcoRI

(rare cutter) and two isoschizomers (*Hpa*II and *Msp*I) with different sensitivity to cytosine methylation, a major cause of somaclonal variation. Since transposon-based activity is also probably an important cause of somaclonal variation in maize, 19 REMAP PCs were also considered. The estimates of genetic similarity and cluster analysis showed that the overall level of similarity revealed by AFLPs was higher than 0.99. The results obtained using cluster analysis with the REMAP markers also indicated a similarly high level (ca. 0.99) of genetic similarity. The dissection of the AFLP data verified the conclusion that both de-methylation and *de novo* methylation contributed to somaclonal variation. Based on these results, the polymorphisms attributable to somaclonal variation can be disregarded in ED considerations relating to GM maize. In particular, when GM maize hybrids are obtained using GM lines backcrossed with non-GM lines, they appeared to be unaffected by somaclonal variation.

4.3 Barley

The marker systems used in barley (IRAP, REMAP, S-SAP and AFLP) all gave useful levels of polymorphism when used to examine a representative set of varieties from Finland and the UK. Genetic distances between these barley varieties were generally below 95% similarity, but there were some pairs of varieties closer than this. Approximately 5% of the set of all possible pair-wise variety similarities were over 95% similar. This level has been suggested as a possible "zone" where potential EDV cases could arise. Study of the set of pairs of varieties with similarity coefficients greater than 0.95 showed that they comprised closely related sister lines, material from the same breeder, and varieties known to be morphologically very similar. Calibration of the similarity coefficients against systems where the relationship between the material is known showed that the 95% similarity threshold was crossed at the BC1-BC2 boundary. This means that at BC2 the distribution of genetic similarities, generated from a number of individuals, all achieved a similarity greater than 0.95. Corresponding results at BC1 showed that, while a small proportion of the set of pair-wise similarities had a similarity exceeding 0.95, the majority had lower similarity coefficients.

In a second phase of the barley work programme, lines generated through various tissue culture approaches (double haploid procedure, genetic transformation) were found not to differ significantly from the donor variety, when measured with molecular or morphological characters. Assessment of backcross lines using the standard DUS morphological characters revealed considerable numbers of non-conforming individuals within varieties as well as within backcross lines. This indicates that the quality of data provided by morphology was less suitable for determining genetic relationships for assessing ED

5. General Conclusions

The MMEDV project unequivocally established that molecular markers provide a means of examining ED by the establishment of genetic conformity between varieties. Methods for the assessment of ED in the three crops under examination were produced and a number of statistical tools for use in the measurement of ED, along with example sets of background data for comparison, developed. In a more generic sense, the project established a framework for the assessment of potential EDVs in any crop, giving principles of approach to the use of various analytical techniques. It is clear that morphological markers are generally inappropriate for the determination of genetic conformity in the ED context.

The UPOV Convention gives the following as examples of methods by which EDVs may be obtained: "…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". The project demonstrated that molecular markers can be used to detect the close similarities between EDVs and the donor variety in all these cases. Colour sports in roses were used to represent mutants, somaclonal variation was examined using DH barley lines, and the natural variation within varieties was studied to establish the potential for isolating natural variants from an initial variety. For backcrossing, each generation of lines in barley up to backcross four (BC4), and a separate population of near-isogenic lines, plus maize F2 and backcross one (BC1) lines, were examined. Finally, transformations by genetic engineering using GM barley and GM maize were studied.

The project showed that independently bred rose varieties are genetically more distant than independent barley varieties, perhaps reflecting the outbreeding nature of rose and the convergence for a limited number of ideotypes and shared pedigree materials within barley. Maize varieties fall between these extremes, being outbreeding but also sharing common ideotypes. Within varieties of rose there is no problem caused by genetic variation due to the vegetative propagation, whereas in species reproduced by seed, like maize and barley, considerable variation within varieties can exist. These factors make the detection of EDVs in rose, where colour mutants are derived from independent varieties, considerably more straightforward than in barley and maize. ED situations involving very small genetic changes such as tissue culture, transformation, and selection from a variety are detectable in maize and barley, but there is more of a challenge defining a potentially useful threshold in backcross situations, as there is overlap between individuals at each cross with individuals of the preceding and proceeding cross. The project paid particular attention to developing statistical tools to tackle this problem area. These observations will be relevant when considering ED models for other species, as the choice of species for the project has been made to encompass the majority of situations likely to occur in plant breeding.

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A more detailed analysis of many of the results and conclusions from the project will be made in separate presentations.

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