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POSSIBLE USE OF MOLECULAR TECHNIQUES IN DUS TESTING ON MAIZE. HOW TO INTEGRATE A NEW TOOL TO SERVE THE EFFECTIVENESS OF PROTECTION OFFERED UNDER THE UPOV SYSTEM

Document prepared by experts from France

POSSIBLE USE OF MOLECULAR TECHNIQUES IN DUS TESTING ON MAIZE. HOW TO INTEGRATE A NEW TOOL TO SERVE THE EFFECTIVENESS OF PROTECTION OFFERED UNDER THE UPOV SYSTEM

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Why consider the use of using molecular techniques in maize DUS Testing?

1. This crucial question is the first one that should be addressed and answered, given that Maize is an "easy" crop to work on for DUS crop experts. Thanks to a large genetic and morphological variability, a high number of reliable and discriminating characteristics and a low genetic*environment interaction, it is easy to conduct a high quality assessment of new varieties for distinctness, uniformity and stability (DUS) as long as the number of varieties grown in the DUS trials remains reasonable (a few hundred). The existing Community Plant Variety Office (CPVO) list of characteristics is suitable for distinguishing maize varieties. New varieties are first described, then compared to the reference varieties. With the process we are using, the "super distinct" varieties are immediately identified and excluded from the field trial, whereas close varieties are identified and further compared, side-by-side, in the field trials. The final assessment of the distinctness of close varieties by maize experts in the DUS plots is for us the most powerful and reliable basis for decisions: it allows a precise comparison of the entire phenotype through successive observations throughout the development of the plants.

2. An important point in relation to these considerations and the possible introduction of molecular techniques is that we do not need to find new characteristics to establish the distinctness of the new candidates. Our concern is to find tools and procedures to continue to run distinctness tests in the way described above, giving the main role to the crop expert, handling a huge number of varieties, reducing the cost and improving the efficiency of the system.

3. Maize is a "huge" crop to work on for DUS crop experts: as an example, in 2005, we had 279 applications for new lines in their first year and 2,673 lines in our reference collection, resulting in 823,329 pair-wise comparisons to be made in order to establish the distinctness of the new lines.

4. The challenge we face is to maintain the high level of quality of our distinctness assessment whilst:

- considering several thousand varieties of common knowledge and candidates;
- avoiding prohibitive costs; and
- avoiding lengthening the duration of the tests.

5. The present situation is not new: we have been facing a regular increase of varieties for many years, and we have made regular changes and revision of our DUS testing system in order to be able to handle the ever larger number of varieties.

6. Looking at the recent past, the most significant steps have been the following:

(a) integration of characteristics derived from electrophoresis in combination with field characteristics;
(b) development of the concept of combination of differences observed on the different characteristics;
(c) development of the GAIA software to select the varieties which need to be grown in the field trials; and
(d) development of technical cooperation with Spain and Germany: construction of a common database for phenotypic data (field and electrophoresis characteristics, CPVO support)

7. So far, molecular analyses have only been applied to the control of the introduction of the transgene in GM varieties.

8. In a more general way, our present methodological work is orientated in the following directions:

(a) integration of genetic distances in combination with phenotypic characteristics to assess distinctness;
(b) integration of molecular techniques as tools to check the identity of lines and hybrids during the tests and for the maintenance of the reference collection

Experience and results of using molecular techniques in DUS testing on Maize

9. We focussed our work on the selection of reference varieties to be grown in the field trials, which is, for us, the major factor of quality of the distinctness assessment. The purpose is to avoid the need to compare in the field trials varieties which are "super distinct" and to concentrate on varieties which might be similar. We aim to eliminate as early as possible the pairs of varieties which are "super distinct" and to identify the pairs of varieties that are not "super distinct", which need to be further compared in the field.

10. The system currently used in France to select the varieties to be grown in the field trials uses a combination of differences between varieties observed on morphological and electrophoresis characteristics. Those differences are weighted (a more reliable difference is given greater weight than a less reliable one), and an index is calculated for each pair of varieties. All pairs of varieties which reach an index of 6 are considered as "super distinct" and not compared further. In the calculation of the index, the contribution of electrophoresis is intentionally limited to two-thirds of the final decision (4 points out of 6), which means that the morphology contribution has to be at least one-third of the final decision (2 points out of 6).

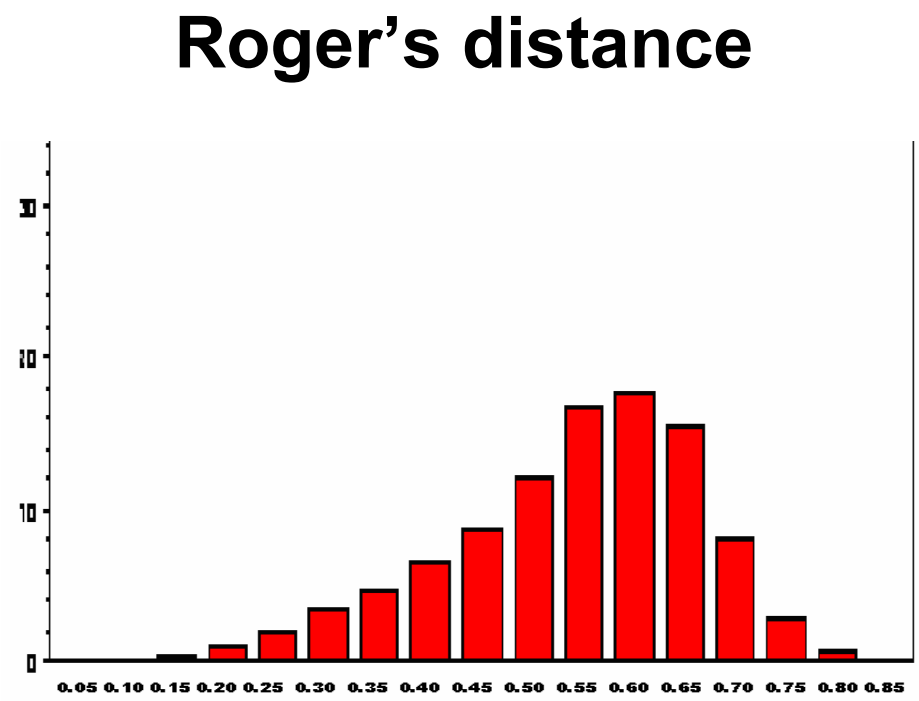
a) Development and evaluation of suitable molecular distances

11. We evaluated the potential of molecular distances to help in selecting reference varieties to be grown in the field trials. In particular, we studied the assumption that two varieties with a large molecular distance have a low risk of not being distinct according to the current UPOV procedure. A low risk means a risk which is lower than the risk taken when simply discarding a part of the reference varieties because of the workload.

12. After screening a large set of public markers and testing different distances, we decided, in the frame of this study, to work with the Rogers' distance. Distances were calculated using about 30 SSR markers chosen for their discrimination capacity and well spread over the genome. The Rogers' distance corresponds to the percentage of markers which differ between two lines (sum of the allelic differences on the tested loci).

13. The distribution of the frequencies of Rogers' distances, calculated on the 480 lines included in the test, showed that large distances were often observed (see graph below).

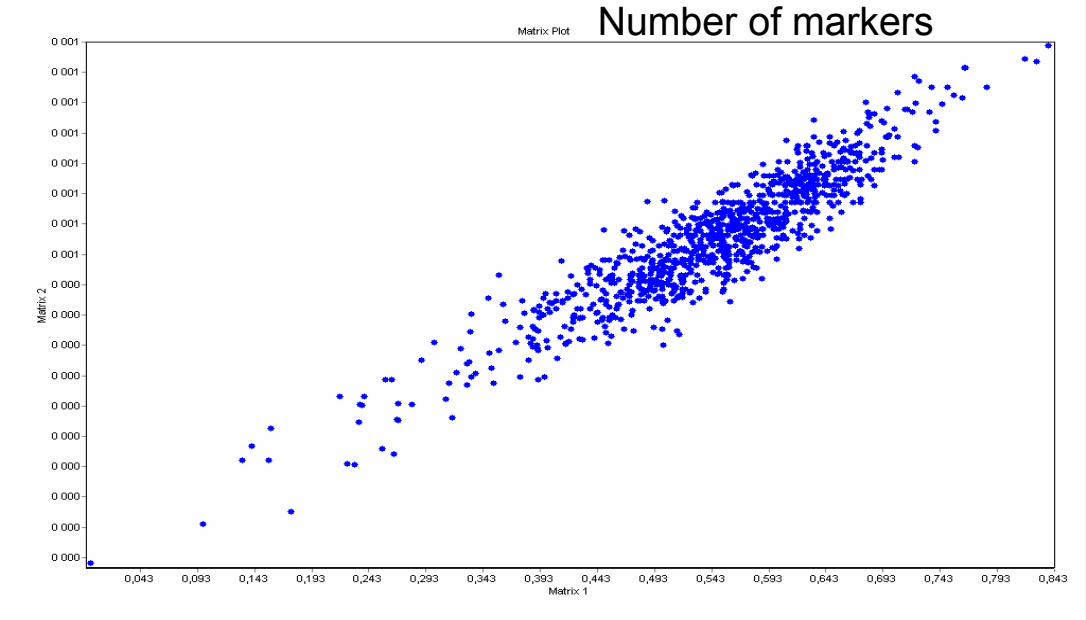
Figure1



14. The assessment of the correlation between the distances obtained using 36 markers and 51 markers showed that the number of markers did not need to be increased to improve the results (see Figure 1).

15. This first part of our studies showed that we could develop a system of calculation of molecular distances which could be used for structuring our reference collection. In addition, the markers we selected provided tools for checking the conformity of the formulae, as well as the identity of lines and hybrids under test, for the purpose of the maintenance of the reference collection. These results potentially allow the building of a complete DUS testing system integrating molecular data.

Figure 2



Correlation of genetic distances of maize lines analysed with 51 or 36 SSRs (r=0.93), from Mantel Test

b) Evaluation of the level of correlation between molecular and morphological data

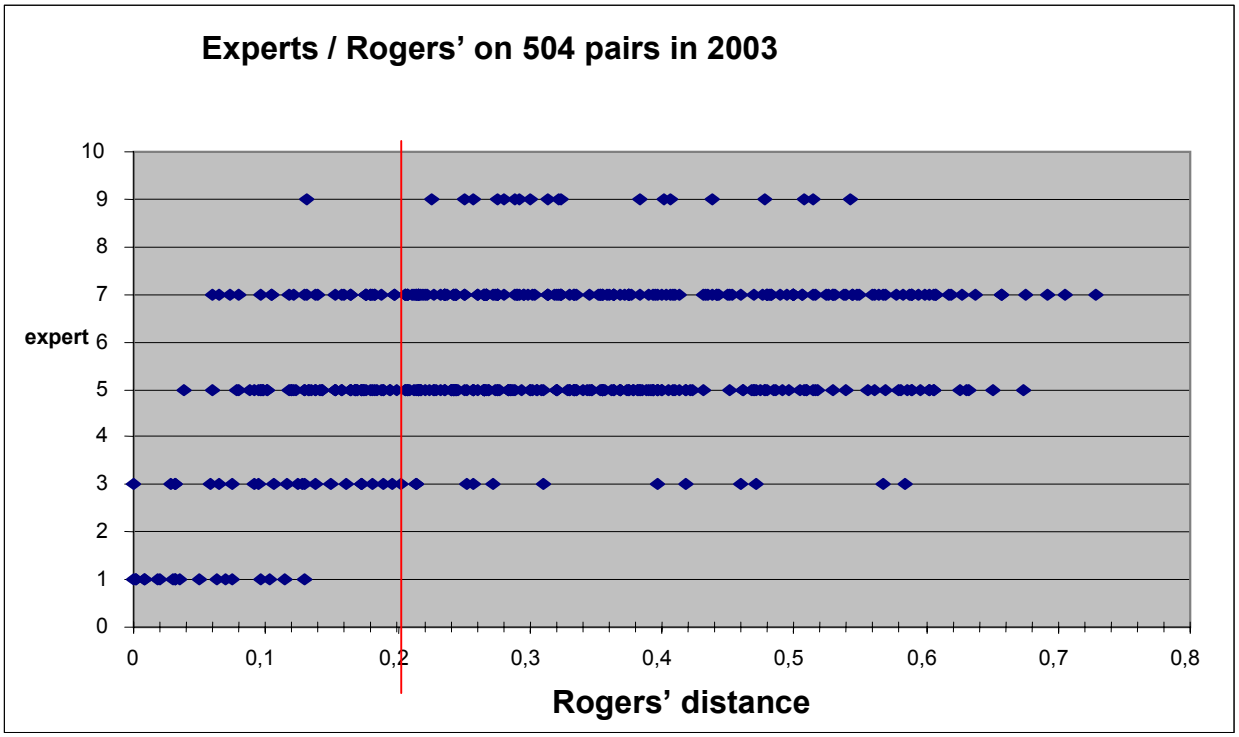
16. Previous studies have shown that the relation between genetic distances and phenotypic distances (Malahanobis distance, Gaia distance,…) is not linear. The main question was then to define an appropriate way of integrating molecular data into the decision without increasing the two risks of accepting a variety which was not distinct and of refusing a variety which was distinct.

17. We decided to use an approach previously experimented in 1994-1995 with RFLP markers, which showed that a visual appreciation by crop experts was an appropriate tool to investigate the correlation between morphological data and molecular data. This visual appreciation consisted of a global appreciation of the phenotype of the varieties, and not an individual appreciation of the characteristics of the varieties considered one-by-one. The crop experts were asked to observe different pairs of varieties grown side-by-side and to give a note using the following "scale of similarity":

1 the two varieties are similar or very close
3 the two varieties are distinct but close
5 the comparison was useful, but the varieties are clearly distinct
7 the comparison should have been avoided because the varieties are very different
9 the comparison should have been avoided because the varieties are totally different

18. We used this kind of experiment in 2003 and 2004 in our three DUS locations: 504 pairs of varieties, tested in parallel with molecular markers, were visually evaluated by our crop experts for their degree of morphological similarity/difference.

19. The experts' notes were compared with the Rogers' distances.



20. In these studies, the experts were able to distinguish between very similar varieties (Rogers' distance < 15%) and less related varieties. It appeared that those experts, thanks to their wide knowledge of the maize plant material, were able to reconstitute the degree of relationship between two lines from the observation of the phenotype. We noticed that all the pairs which were given note 1 by the experts had a genetic distance below 15%. This threshold could then be considered to progress in our work and different approaches could be developed.

c) Different ways of integrating molecular data and their comparisons with other existing systems

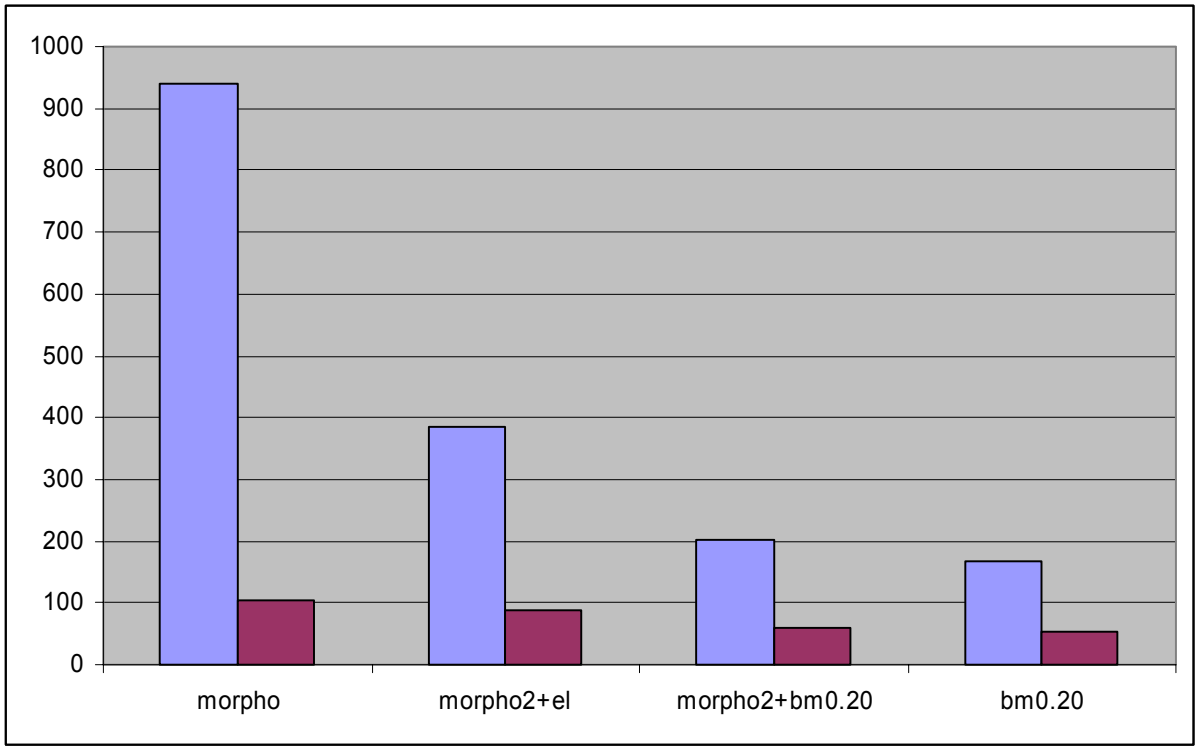
21. These approaches were devoted to compare the present system to alternative systems which would integrate molecular data. The present system has been applied for many years and has given very good results up to now (no mistake has been discovered after more than 15 years of application). To give an idea of its efficiency, we already mentioned that in 2005, 823,329 comparisons were made to assess the distinctness of our candidate varieties. These comparisons were made using our Gaia software with the morphological and electrophoresis data. The result was the following: 90 varieties were distinct after the first run of comparisons and 189 had to be studied further. These studies involved only 1,151 comparisons in the field trials for the next growing trials, which is very few indeed compared to the starting point of 823,329.

22. In our present system, the input of electrophoresis data is highly significant. Its contribution leads to a reduction of more than 50% of the comparisons to be made in the field trials. Integrating molecular data into the Maize DUS system should allow at least the same level of efficiency to be kept as in the present system and preferably to improve it, without increasing the cost of the tests.

23. We have been studying different possibilities using data available on a set of 430 lines which allowed the calculation of Rogers' distances on 940 pairs of varieties. The following graph illustrates the consequences of selecting different options:

- using only the morphological data
- using the morphological data and the electrophoresis data (present system used in France)
- using the morphological data and a molecular distance (20% chosen)
- using only a molecular distance (20% chosen)

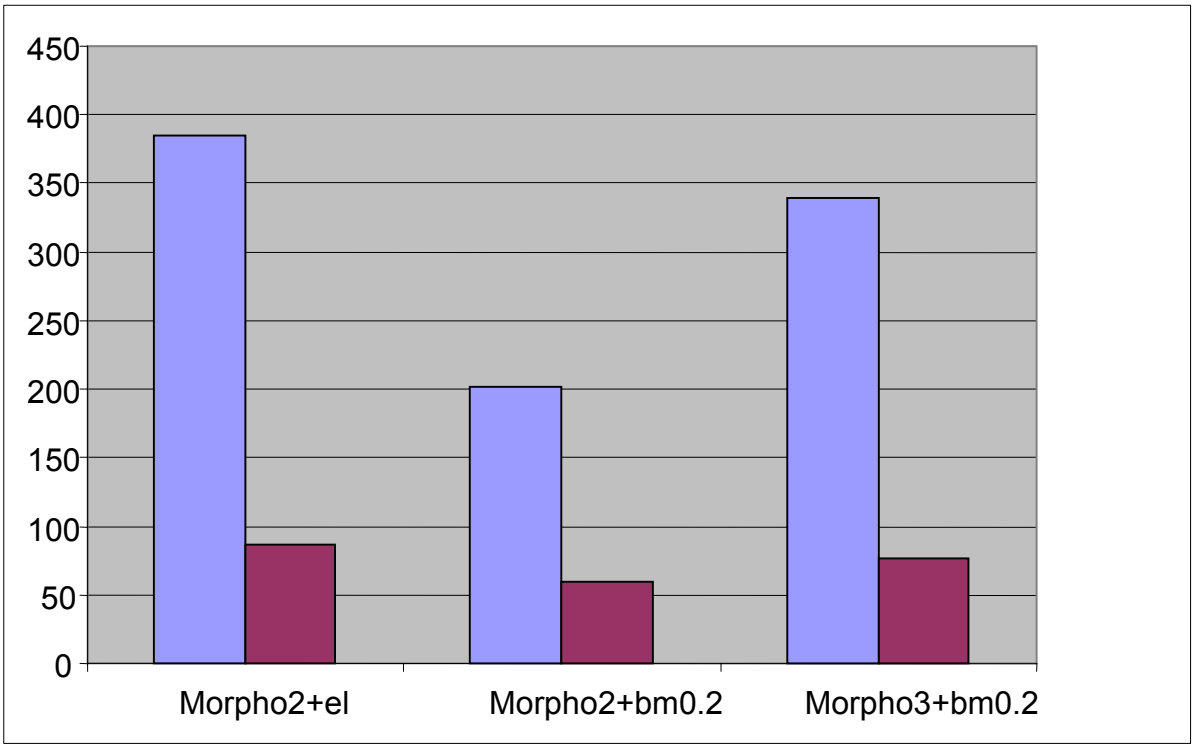
Graph 1: Number of pairs of varieties to be grown in the field trials (blue colour, left side) and number of varieties "non-super distinct" (index<6, red colour, right side)



24. In this particular example, using a molecular distance of 20% instead of using electrophoresis results, in combination with morphological data and without any change in the way the morphological data are considered (that is to say that morphological differences should contribute at least to one-third of the decision), gives a significant reduction of the number of comparisons to be made in the field. Using only a molecular distance of 20% would not decrease the field work much more compared to that option.

25. The next graph shows the impact of different levels of contributions of morphological data for a fixed molecular distance.

Graph 2



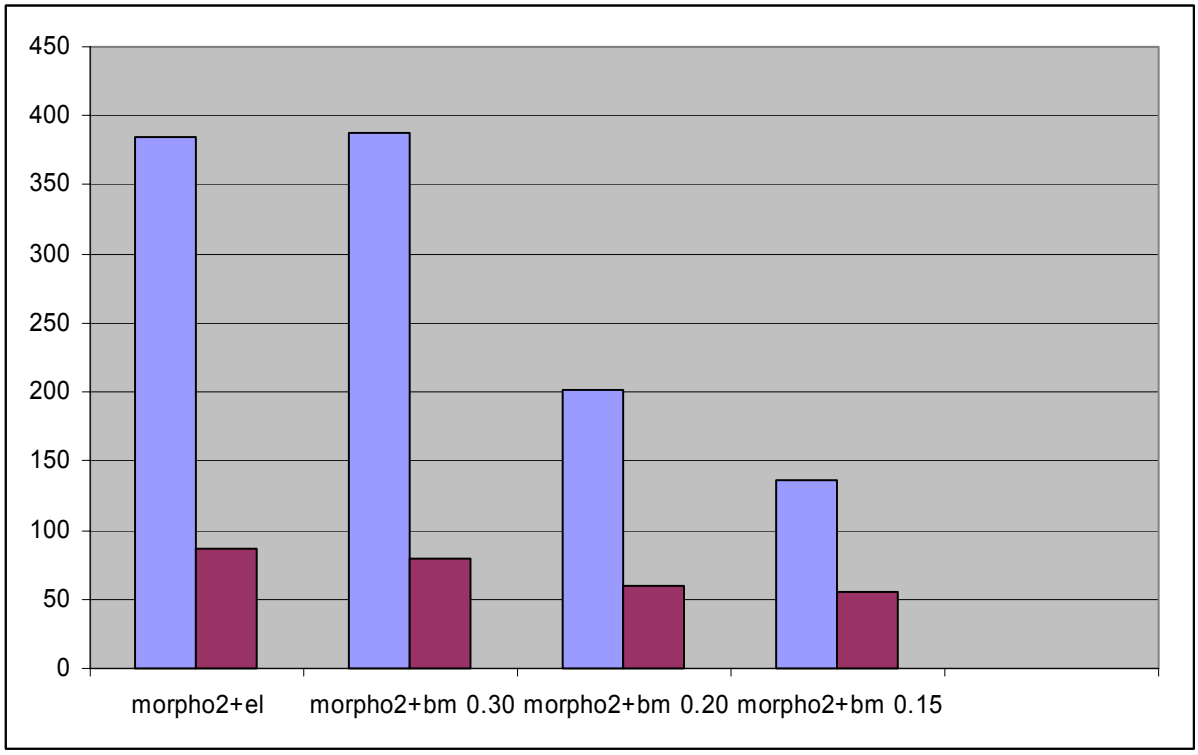
Morpho2+el: current system, including the condition that the morphological difference should contribute to at least one-third of the decision.

Morpho2+bm0.2: abandonment of electrophoresis and use of a molecular distance of 20% (same condition on the morphological data as above).

Morpho3+bm0.2: abandonment of electrophoresis and use of a molecular distance of 20% but the condition on the morphological difference is more stringent: it should contribute at least to half of the decision.

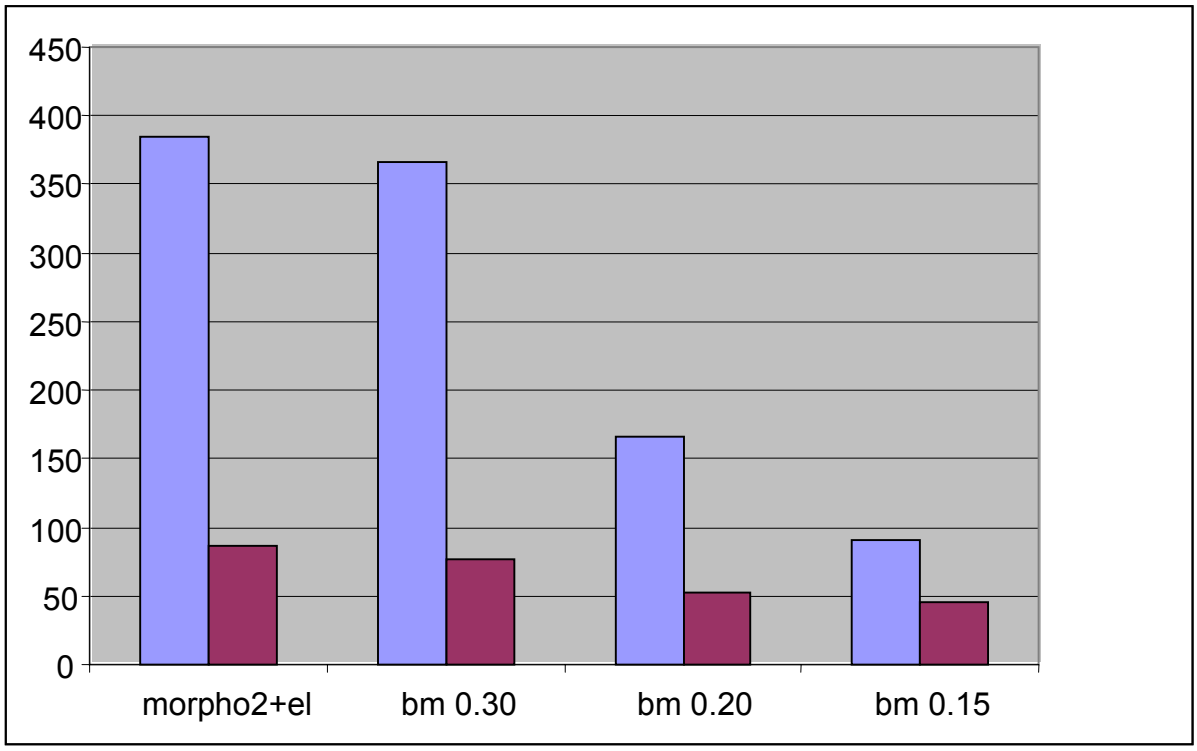
26. Graph 3 illustrates the impact of three different thresholds for molecular distances, used in combination with a fixed contribution of morphological data (one third of the final decision).

Graph 3



27. Graph 3 can be compared to the one obtained by using only molecular thresholds (see graph 4 below).

Graph 4



28. Graphs 1 to 4 show that the integration of a genetic distance as a component of the decision, or as the unique basis for decision, can lead to different strategies for the management of the field trials. Drastic reduction of the field work can easily be decided, but what would be the consequences for the increase in the risk of declaring a variety distinct when it was not, under the UPOV system? For us, the integration of a genetic distance in the distinctness process is promising but needs to be further studied in order to define the best procedures and thresholds which will ensure that the variety is distinct at the level of the phenotype. Such a demonstration is not easy and will need to be run in parallel with the current system and possible new systems with a comparative evaluation under real conditions of tests and practical situations. The potential savings in the field work also need to be precisely studied under the different options, given that there are fixed and variable costs and that varieties would need to be grown for the morphological description and the check of uniformity and stability.

Future work and perspectives

29. We are now testing the potential benefit of molecular techniques on a larger and real scale: we are currently analyzing the main part of the reference collection (3,000 lines) and expect to complete the results by summer 2007. The genetic distances will be calculated and we will then implement different scenarios. We expect to have a better idea of the number of varieties to include in our field trials with different molecular thresholds and different combinations of molecular distances and morphological characteristics. The balance between technical advantages, risks and costs will be evaluated in each situation, and discussed with the breeders and official bodies.

30. We are currently giving particular attention to the use of DNA-profiling for distinctness, but we want to underline again that we are in favour of a genetic distance approach combined with a morphological approach, bearing in mind that distinctness has to be established on a phenotypic basis. We are still convinced that the introduction of DNA markers in a "characteristic-by-characteristic" approach would undermine the quality of the protection granted to the varieties under the UPOV system.

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