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UPOV

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

# WORKING GROUP ON BIOCHEMICAL AND MOLECULAR TECHNIQUES AND DNA-PROFILING IN PARTICULAR

## Fifth Session
## Beltsville, United States of America, September 28 to 30, 1998

REPORT

*adopted by the Working Group on Biochemical and Molecular Techniques and DNA-Profiling in Particular*

Opening of the Session

1. The Working Group on Biochemical and Molecular Techniques and DNA-Profiling in Particular (hereinafter referred to as "the Working Group") held its fifth session in Beltsville, Maryland, United States of America, from September 28 to 30, 1998. The list of participants is reproduced in Annex I to this report.

2. Mr. Willam J. Franks, Jr., Deputy Administrator, Agricultural Marketing Service Science and Technology, U.S. Department of Agriculture (USDA) welcomed the participants to Beltsville, and provided an overview of activities in the Beltsville Agricultural Research Center. The session was opened by Mr. Joël Guiard (France), Chairman of the Working Group.

3. Before opening the session, the Chairman gave an overview of the previous four BMT sessions as follows:

4. First of all, he observed that much information on different molecular techniques and statistical methods had been provided in the sessions. The DNA profiling methods presented in the BMT sessions quickly shifted from RFLP and RAPD to more advanced techniques with higher polymorphism and reproducibility, such as AFLP and microsatellite. He also referred to the usefulness of document BMT/3/2, introducing definitions and nomenclature for DNA profiling methods.

5. The Working Group had provided a forum for an exchange of views and information between molecular biology researchers, statisticians, and UPOV experts. It had discussed the use of molecular techniques in the context of the UPOV Convention. It had noted the risks involved in the use of DNA profiling data without sound knowledge of the genetic background. It had noted the need to identify precise statistical methods and the error risks involved.

6. The Working Group had agreed that several technical problems had to be solved before any introduction of DNA profiling for DUS testing. In particular, the uniformity and stability of varieties for molecular markers was still an open question. Variability within and between varieties for molecular markers needed to be studied intensively. In addition, the standardization of reproducible molecular techniques would be indispensable for the introduction of these techniques.

7. The Working Group had also discussed the use of DNA profiling for prescreening and in disputes on essential derivation. It had introduced a new notion "genetic distance" for the UPOV framework. The concept of genetic similarity or conformity had demonstrated the potential use of molecular techniques for the judging of essential derivation. The Working Group had agreed that the criteria of distinctness and of essential derivation should be kept separate.

8. Finally, the Chairman referred to the main aim of the work of the Working Group and the conclusion reached at the third session as reproduced in document BMT/3/18, paragraph 36, 38 and 39.

> "36. Final Conclusions: The Working Group agreed that the new techniques for DNA-profiling were a powerful tool to provide detailed information on the relationship between varieties. They supplied considerable background on a variety and were also very useful for the identification of existing varieties. They would be very useful for the estimation of essential derivation together with other sources of data (e.g. breeding history). The Working Group was, however, not in a position to recommend its use for distinctness purposes. [...]. It therefore finally proposed that the Technical Committee not recommend the use of DNA-profiling for DUS purposes before all these open points had been clarified or before harmonized protocols had been established for the use of DNA-profiling (if its use was ever accepted for DUS testing)."
>
> "38. The Working Group favored the approach of ASSINSEL which was to keep the judgment of essential derivation as far as possible separate from the DUS testing and that the criteria of essential derivation had to be judged species by species. At present information on DNA-profiling should only be complementary

information which may help the expert in the testing but which would not be used for distinctness testing."

"39. The Working Group agreed that UPOV should not feel under pressure to accept the new methods just for fear of being regarded as old-fashioned. It had the task of defending the efficacy of the plant variety protection system and of defending it against the introduction of unsuitable tools which might affect its functioning. [...]"

Adoption of the Agenda

9. The Working Group unanimously adopted the Agenda as reproduced in document BMT/5/1 Rev. after having agreed on the documents to be discussed under each item.

Short Presentation of Biochemical and Molecular Techniques: New Techniques, Advantages and Limits of Different Techniques

10. Mr. Robert J. Cooke (United Kingdom) introduced document BMT/5/2 on "The Use of Molecular Markers for Variety and Seed Testing: A Summary of Research at NIAB," prepared by him.

11. Mr. Cragan (United States of America) presented his research result entitled "The Use of Simple Sequence Repeats (SSR's) in Documenting Distinctness and Identification in Plant Variety Protection for Soybean." The transparency sheets used in this presentation are attached to this document as Annex II.

12. Mr. Ben Vosman (Netherlands) introduced document BMT/5/8 on the "Standardization of Molecular Marker Systems for Variety Testing," prepared by him together with Robert Cooke (United Kingdom), Martin Ganal, Roger Peeters, Peter Issac (Netherlands) and Gerard Bredemeijer (France).

13. Mrs. Joëlle Lallemand (France) introduced document BMT/5/9 on the "Applicability of Amplified Fragment Length Polymorphism Markers for Strawberry Variety Identification," prepared by Gemma Arnau and Mireille Bourgoin (France).

14. Mr. Yoshiyuki Ban (Japan) introduced document BMT/5/10 on the "Genetic Identification of Morphological Mutants of Strawberry Characterized by AFLP Analysis," prepared by him together with T. Kimura (Japan).

15. Mrs. Joëlle Lallemand (France) introduced document BMT/5/11 on the "Potentiality of STS for Variety Distinction in Ryegrass," prepared by her together with Patricia Lem, Marc Ghesquiere, Gilles Charmet and François Balfourier (France).

Discussion on Short Presentation

16. In the discussions following each individual report as well as in the discussions after all reports of research results, several main topics were discussed which can be summarized as follows:

17. Reproducibility: The Working Group noted that the reproducibility of AFLP markers could be significantly improved if the markers and the appropriate DNA preparation procedures were carefully chosen. The studies also showed high consistency in the results of STMS markers conducted in different institutes by the standardized DNA preparation procedure.

18. Stability on molecular markers: The results of estimated mutation rate on SSR alleles in soybeans indicated that in some cases there might be high mutation rates of molecular markers. In principle, if information obtained with molecular markers was used for DUS testing, that information should satisfy not only distinctness, but also uniformity and stability criteria. Through further empirical studies on variability in molecular markers over the generations, the criteria of stability applied to molecular markers for DUS testing needed to be studied. New molecular characteristics, if unstable, might force breeders or maintainers of the protected varieties to additional selection work to keep the characteristics stable. Therefore, breeders insisted that stability criteria for molecular markers should be carefully discussed so that they did not create an extra burden.

19. Access to molecular techniques: The expert from ASSINSEL referred to the importance of access to molecular techniques. He warned that many molecular techniques were proprietary and not freely accessible. If the use of a certain kind of molecular technique was recommended, the technique should be freely available (if necessary, against payment) to plant variety protection offices worldwide. In addition, if a national office permitted the use of a particular molecular technique for DUS testing, the information of such molecular technique and the resulting data should be publicly available and accessible to other countries. The Working Group reaffirmed the necessity to take into account access to molecular techniques as well as their costs.

20. Databases of DNA profiles of varieties: The Working Group noted the future need for the construction and standardization of databases of DNA profiles of varieties. In the light of the likely future use of DNA profiling, the construction of standardized database should start as soon as possible in order to utilize the burgeoning data efficiently. However, prior to such construction, the molecular methods to be used for the future must be identified; the robustness of reference markers and their stability over generations should be considered. For example, one expert questioned how large a population of existing varieties had to be for deriving robust reference markers. UPOV had to recommend a set of techniques to collect data for such a database especially as so many data were already available.

21. The Working Group also discussed difficulties in freely accessing such databases. If molecular markers were used for DUS testing, the free access to databases of those molecular markers would be necessary. However, the confidentiality of certain variety information should also be taken into account.

22. Development of microsatellite markers: The Working Group heard of several projects to develop new microsatellite markers. The problems of microsatellites, however, was that microsatellite markers were still developed only for major species and their development was very costly. In this connection, the use of genes known in one species to derive microsatellite markers for other closely related species seemed to be a useful method to develop markers for minor species.

23. Use of molecular techniques for DUS testing: The Chairman summarized the discussions on the research reports as follows. More and more information had become available on different methods giving good results. The question was what was one to do with all that information. The Working Group had to think of ways how that information could be used in the testing of varieties, especially in the light of the speed with which molecular markers provided variety identification. It was necessary to make concrete recommendations to avoid repeating the errors made in the past with respect to the use of electophoretic characteristics. There were, however, still many unsolved problems, such as the lack of repeatability, where some progress had been made, but not enough. The next problem was the testing of uniformity and stability. Some results were available but they were far from sufficient. The future research had to concentrate in this area. In addition, the introduction of molecular characteristics might reduce the minimum distance between protected varieties. At present, it was still dangerous to use molecular markers to establishing distinctness.

24. Following a question on the present position of the Plant Variety Protection Office of the United States of America on molecular characteristics in DUS testing, the experts from USA answered that the Office had not yet received any application for a new variety which had been distinct from another variety only in molecular characteristics. There had always been differences in some other characteristics and thus rights had been granted on the basis of morphological characteristics complemented by molecular characteristics.

## Assessment of Variability Within Varieties and Between Varieties

25. Mrs. Claire Baril (France) introduced document BMT/5/4 on "The Potential of AFLP Markers for Distinguishing Between Ryegrass Varieties," prepared by her together with P. Dubreuil (France), F. Van Eeuwijk (Netherlands), Ch. Dillmann (France), M. De Loose (Belgium), J. Law (United Kingdom) and I. Roldan-Ruiz (France).

26. Mr. David Zhang (France) introduced document BMT/5/6 on the "Assessment of Molecular Variability Between and Within Varieties by AFLP in Rose," prepared by him together with E. Germain, M. Q. Cao and Marie-Hélène Gandelin (France).

27. Ms. Mercedes Echaide (Argentina) presented the preliminary result of her studies, carried out in cooperation with GEVES, France, on "Homogeneity in Maize Lines: Utility of Molecular Markers for Variability Assessment."

Discussion: Uniformity

28. In the case of roses, the uniformity level was very high as had been expected, but for sexually reproduced species, further studies had still to be made. Results were highly dependent on the choice of molecular method.

29. The Working Group discussed the detection of phenotypic mutations by molecular methods. Because molecular markers could not cover all genetic information, some phenotypic mutations, especially those caused by a change in a single gene, might not be detected by molecular markers. Several molecular researchers suggested that the causes of mutations and the preparation procedures might influence whether a phenotypic mutation could be detected or not.

30. The Working Group discussed the correlation between the uniformity of phenotypic and biochemical and molecular characteristics. Some experts reported cases in which a variety with high uniformity in isozyme analysis did not show uniformity in phenotypic characteristics observed in the field, so the same situation can occur in the case of molecular markers.

31. The Working Group discussed how to assess uniformity in molecular markers, for example, how many samples were necessary and how much variability within a variety should be allowed. The Chairman reminded the participants of the following four options concerning the acceptable level of uniformity for characteristics obtained with molecular markers in paragraph 34 of document BMT/3/18:

> "..... There are a number of ways of approaching this problem [of the uniformity requirement]:
>
> (i) it could be decided that this lack of uniformity precludes the use of such profiling techniques;
>
> (ii) it could be accepted that the level of non-uniformity exhibited by currently registered cultivars (which would need to be determined systematically and empirically) represented a baseline which candidates in the future would not be allowed to exceed;
>
> (iii) it could be suggested that from a certain date, all future candidates would have to be uniform in the particular profiling character;
>
> (iv) it could be accepted that the repeatability (i.e. stability) of the differences between cultivars is more important than the insistence on plant to plant uniformity. Thus if the variability within a cultivar, as estimated whether by single plant analysis or by a bulk analysis, is maintained from generation to generation (is stable) then this could be accepted as evidence of sufficient uniformity within that cultivar. This proposition would be recognizing that the examination of uniformity is at least partly to ensure that the distinguishing features of a cultivar are maintained during multiplication and commercialization. Hence it is stability rather than uniformity *per se* which is essential."

32. One expert suggested that the minimum level of uniformity required for the use of molecular markers should be derived from variability within existing reference varieties, which was the same method as that used for electrophoretic characteristics.

33. The Working Group reaffirmed that the greatest remaining shortcoming was the checking and control of uniformity in characteristics obtained with molecular markers. It agreed that the four options mentioned above would have to be discussed in the next session together with research results on more and different species.

Reports on Statistical Methods

34. Mr. David Zhang (France) introduced document BMT/5/5 on the "Comparison of Genetic Distances Between Rapeseed Cultivars Characterized by AFLP Markers" prepared by him together with V. Lombard, C.P. Baril P. Dubreuil, and F. Blouet (France).

35. Mrs. Claire Baril (France) introduced document BMT/5/7 on the "Phenotypic Distance Prediction According to Molecular Data" prepared by her together with G. Nuel and S. Robin (France). The information in document BMT/5/7 was enlarged by several overhead tables and diagrams used and are reproduced in Annex II to this report.

36. Mr. Jan De Riek (Belgium) introduced document BMT/5/16 on the "Comparison of AFLP Data with Pedigree (Azalea) or Morphology (Flax and Linseed)" prepared by him together with Johan Van Waes, Isabelle Everaert, Erik Van Bockstaele and Marc De Loose (Belgium).

Discussion on Statistical Methods

37. Choice of genetic estimator, molecular markers and diversity in sample varieties: The Working Group noted that, although different methods of estimating genetic distance showed different figures, the results from different methods were often strongly correlated. On the other hand, the Working Group noted that the choice of the method of estimating molecular distance, the weighting of markers, and the genetic diversity of the tested variety population should depend on the objectives for which the molecular distance is to be used. Which markers should be chosen for the molecular marker set, frequently observed markers or rarely observed markers? For the estimation of the genetic distance, is it worthwhile to weight the markers according to their frequency and to take into account the genetic map? Which set of varieties (full reference collection or a subset of close varieties) should be chosen for establishing the marker system? The answer should depend on whether the purposed use was for essential derivation or for distinctness.

38. In the discussion of the choice of molecular markers, several experts suggested that the markers related to morphological information should be given the highest importance. The choice of markers relating to morphological characteristics would be very important for the purposes of prescreening. One molecular scientist reported from his practical point of view

that he had chosen molecular markers by discarding markers highly correlated to other markers.

39. Precision of molecular distance: The Working Group also noted that further studies were needed to develop a more precise system for the estimation of molecular distance. The high standard deviation observed in the molecular distance estimations indicated the difficulty of using this method to judge essential derivation. The effects of sample size and number of markers on the accuracy of the molecular distance estimate should also be studied.

40. Comments from the Chairman of the TWC: The Chairman of the Technical Working Party for Automation and Computer Program (TWC) reported that several studies on statistical methods for DNA profiling data were in progress in the TWC and that the discussions in the TWC on optimum precision would also be able to contribute to the discussions of the Working Group. He also reported that an electronic bulletin board for participants in Technical Working Parties as well as in this Working Group had been established by experts from the United Kingdom. He warned against the risk of using one or two graphs of the Principle Components Analysis (PCA). He reminded the participants that, although that type of graph showed the relationship of different varieties clearly and graphically, it represented only a part (for example, 40%) of variability. He also suggested that the combination of diverse data, for example AFLP and microsatellite data, should be explored in view of its precision. Finally, he requested that good firm data sets including not only molecular data, but also morphological and pedigree data were needed for assessing the advantages and disadvantages of different statistical methods.

41. Following his request, the Working Group discussed the establishment of complete data set of molecular markers, pedigree and morphological characteristics in cooperation with member States and breeders in order to ask the TWC to assess different statistical methods. The expert from ASSINSEL stated that they were pleased to cooperate in the project under the condition that the objective of the project and the necessary data were clearly defined. Finally, experts from the United Kingdom proposed to establish a data set by using data available in their institute, but asked that other experts should supply data on different species: e.g., ryegrass, mutants in ornamental crops and oilseed rape, as the problems involved in the three examples mentioned would be completely different.

## Definition of Variety

42. The Chairman reported on the discussion on the definition of "variety" held by a Working Group which met on February 12, 1998. Part of the overheads used are reproduced as Annex III to this document. He briefly explained the four options discussed in the said Working Group. The first and second option were strict interpretations of Article 1 of the 1991 Act of the UPOV Convention, while the third and fourth options were its wide interpretation, allowing the use of molecular characteristics. The first option was that establishment of distinctness was only based on phenotypic characteristics and that no molecular characteristics were admitted except if they were strictly linked with phenotypic characteristics. The second option was that information obtained using a molecular tool could not be used alone for a conclusion on clear distinctness, but only as a complementary help to confirm a clear phenotypic difference (especially for use in otherwise not easily observable

phenotypic differences). In the third option DNA polymorphism would be considered as a result of the expression of the genotype or combination of genotypes and their molecular characteristics would be used for establishing distinctness. Referring to the lengthy discussions that had taken place on minimum distances of morphological characteristics, it would be difficult to define a minimum distances (a clear difference) based on molecular characteristics. This was essential, however, if a drastic erosion of the rights were to be avoided. It would also be difficult to judge uniformity and stability. In the fourth option, every difference in molecular markers could be used as basis for establishing distinctness. The Chairman also briefly explained the advantages and disadvantages of each option. He reported that most participants in the said Working Group had favored the second option.

43. The Office of UPOV introduced paragraph 20 of document CAJ/38/7 Prov., the conclusion of the Chairman of the Administrative and Legal Committee (CAJ) on the discussions on "Characteristics Used in Distinctness Test" in the CAJ in its spring session of this year, which reads as follows:

> "20. ..... It seemed to him [the Chairman of the CAJ] that the following conclusions could be drawn from the documentation and the discussions:
> (a) One should not reject the use of molecular tools out of hand in the examination of distinctness.
> (b) It was not possible, at the present stage at least, to allow information obtained using a molecular tool to serve alone as the basis for a conclusion on the clear distinctness of two varieties.
> (c) The use of molecular tools could only be contemplated if there was a guarantee that the minimum distances between varieties would not be made smaller.
> (d) The risk of "mini systems of protection" evolving from different examination practices, mentioned at the previous session of the Committee, could not be ruled out, but everything should be done to avoid them.
> To that end, it was particularly appropriate that the Working Group on Biochemical and Molecular Techniques, and DNA Profiling in Particular, should continue its work."

In addition, it asked to take a cautious approach in view of the rather similar wording and especially the use of the wording "the expression of the characteristics resulting from a given genotype or combination of genotype" in the definition of variety and for essential derivation.

44. Most participants basically supported the conclusion of the CAJ and favored the second of the four options that information obtained using a molecular tool could not be used alone for a conclusion on clear distinctness, but only as a complement to phenotypic differences, thus confirming the opinion of the crop expert.

45. The expert from ASSINSEL, recalling the discussion in the Diplomatic Conference in this respect, stated that, in his opinion, the difference between these two concepts had been clear despite the same wording finally used in the Convention. The basic concepts were that the "variety" was defined by phenotypic expressions and that essential derivation was assessed by conformity of genotypes. He also emphasized that essential derivation was part of the scope of the breeders' right.

46. The Working Group discussed the application of the second option by using the case of disease resistance. One expert asked how to deal with a potato variety which was known to have a disease resistance gene, but whose disease resistance could not be observed. Several experts insisted that the genetic evidence alone was not enough to establish distinctness for the variety; therefore, such a variety without sufficient differences in phenotypic characteristics should not be protected. Another expert quoted the case of a variety which was known to have a disease resistance gene, but whose disease resistance could be observed in some cases, but not in others. Some experts indicated that in this case because of its lack of uniformity the characteristic of disease resistance could not be used for distinctness.

47. Several experts emphasized that the purpose of plant variety protection was in particular to protect the rights of breeders of existing protected varieties and not just to grant a right for breeders of a new variety. The Working Group reaffirmed that the introduction of new techniques should not lead to a reduction of minimum distance and to the erosion of existing plant breeders' rights.

48. The Working Group also reaffirmed that many technical questions, such as uniformity and stability in the use of molecular tools for DUS testing were still open and needed to be solved before any recommendation on the use of those tools could be made.

49. The expert from the Community Plant Variety Office explained the position of his office on this subject. At present, the CPVO did not accept a difference based on molecular techniques for DUS purposes. Therefore, the CPVO favored at present the first option discussed in the said Working Group. However, in the future, the CPVO might have to shift to the second option. The expert from the CPVO believed that, before the introduction of molecular techniques, the cases where molecular characteristics could be used for the establishment of distinctness should be clearly defined and that detailed technical guidelines, including protocols for molecular methods and the assessment of uniformity and stability, should be described in the Test Guidelines.

Position of the Breeders vis-à-vis DNA Profiling

50. Mr. Bernard Le Buanec (ASSINSEL) introduced document BMT/5/14, "ASSINSEL Position on Characteristics for DUS Testing," adopted by the General Assembly of ASSINSEL on May 30, 1997. In that document, ASSINSEL proposed the classification of characteristics used for DUS testing in the following three groups: (1) UPOV characteristics (Test Guidelines); (2) Additional "phenotypic" characteristics, such as yield, sugar content, disease resistance, combining capacity and herbicide resistance; (3) Additional non-phenotypic convincing evidence. The third group of characteristics should be used with the agreement of applicants, if all other characteristics failed to establish sufficient distinctness, despite some evidence and if a test procedure has been agreed upon between the competent authority and the applicants. He stated that the proposal made by ASSINSEL was almost the same as the second option discussed in the Working Group and the CAJ. He repeated that the problem of the introduction of new characteristics for DUS testing should be solved without placing new obligations on the holders of an already protected variety. He added that the group of breeders of ryegrass had opposed the use of "additional non-phenotypic convincing evidence" for ryegrass varieties and that a special working group was organized to discuss

which species should be excluded from the application of "additional non-phenotypic convincing evidence."

51. One expert insisted that electrophoretic characteristics should be dealt with in the same way as disease resistance and should be treated differently from information obtained with molecular markers. The Office of UPOV explained that the electrophoretic characteristics had already been included in the Annex of several Test Guidelines. Another expert stated that there should be a clear border line between morphological characteristics and biochemical/molecular characteristics, including electrophoretic characteristics, when considering on whether they could be used as independent characteristics or complementary information. In this context, the word "non-phenotypic" was liable to cause misunderstanding.

52. One expert pointed to the difficulty of applying new biochemical and molecular techniques to varieties of cross-fertilized species with relatively low stability. In such varieties, the breeders and maintainers may not be able to maintain the population with the same molecular characteristics generation after generation and submit the same samples in response to a request from the national office.

53. Several experts made comments on "additional phenotypic characteristics." They pointed out that characteristics, such as yield, disease resistance and sugar content, were dependent on the environment and therefore less reliable for distinctness testing. In addition, these were often observed on bulk samples and thus the checking of uniformity was difficult if not impossible. Other experts warned against the risk of using this group of characteristics to make cosmetic modifications to existing varieties.

54. Biochemical scientist asked for the position of ASSINSEL on the rapid introduction of molecular techniques for variety identification. The expert from ASSINSEL answered that the requirement of variety identification was completely different from that of plant variety protection. Variety identification techniques could be used for seed quality and for certification, but could not directly be used for plant variety protection. One big point of difference was the concept of minimum distance.

The Use of DNA-Profiling Methods by Expert Witnesses in Disputes on Essential Derivation

55. Mr. Bernard Le Buanec (ASSINSEL) introduced document BMT/5/13 on the "Assessment of Essential Derivation Using Molecular Markers: A Tomato Pilot Study," and document BMT/5/15 on the "Assessment of Essential Derivation," both prepared by ASSINSEL.

56. The expert from ASSINSEL repeated that, while the decision on DUS was taken by the competent national PVR authorities, the decision on the essential derivation was taken by arbitrators or courts and not by the PVR Offices. However, he stated that that did not mean that the PVR Offices did not need to do anything. Courts needed the guidance of technical experts and would probably approach PVR offices for advice. Because the definition of essential derivation was not defined in detail in the 1991 Act, UPOV and the PVR Offices should establish clear and detailed definitions of the provision for its application. In

particular, he stressed that the meaning of "predominantly derived from the initial variety" should be clarified by establishing threshold levels.

57. The expert from ASSINSEL further explained some problems in essential derivation. The "spirit" of the provisions of essential derivation was clear for breeders. The most important point to judge essential derivation would be "the intention of the second breeder." It was, however, impossible for the PVR Office to prove "the intention." Instead, the PVR Office could establish technical tools to be utilized for assessment of genetic conformity.

58. The Chairman stated that, in the Diplomatic Conference, UPOV had been requested to establish guidelines on essential derivation. The discussion on essential derivation in the Working Group could be considered as a part of activities of UPOV to establish such guidelines. However, defining the interpretation of the words on essential derivation was not the task of UPOV. The Working Group should focus on technical aspects, for example, identifying the methods and tools to assess essential derivation and providing technical information on how to use molecular markers to assess genetic conformity.

59. The Working Group agreed that the task of the Working Group was to discuss the technical tools for assessment of essential derivation, for example, molecular techniques, statistical methods and their accuracy. In addition, the Working Group noted that the further studies, especially by extending to the other species, were required for further discussion on essential derivation.

60. The Working Group discussed the establishment of threshold levels. Some experts insisted that, because the molecular distance was different depending on species concerned as well as on the molecular techniques and molecular distance estimators used, the threshold level of molecular distance for judgment of essential derivation had to be determined case by case. Some breeders insisted that the threshold level would be indispensable for the application of the concept of essential derivation in practice despite the difficulties in its establishment. However, the Working Group agreed that the threshold level for the judgment of essential derivation was not to be determined by UPOV, but by breeders or courts.

The Use of DNA-Profiling for Prescreening as a Possible Tool in DUS Testing

61. Mr. John Law (United Kingdom) introduced BMT/5/3 on the "Prediction of Variety Relatedness: Most Similar Variety Comparison as a Pre-screening Tool," prepared by him together with Robert Cooke (United Kingdom) and Stephan Smith (United States of America).

62. Mr. Huib Ghijsen (Netherlands) introduced BMT/5/12 on the "Prescreening of Varieties (With the help of electrophoresis): Progress Report of a Case Study in *Poa pratensis and Solanum tuberosum* (TWA/26/10 and TWA/27/20) and Report of the Discussion the Technical Working Party for the Agricultural Crop."

63. The result of document BMT/5/3 showed that the molecular distance was poorly correlated with the morphological distance, while similarity by pedigree showed higher consistency with that by molecular distance than that by morphological distance. Several

testing experts insisted that the result meant that the molecular distance was no useful tool for prescreening, since the aim of prescreening was to identify the varieties which were similar in morphological characteristics. Moreover, some experts questioned the basic idea that morphological distance could be substituted by molecular distance in absence of any systematic linkage between these distances. However, some molecular scientist explained that the result totally depended on the species and the choice of the markers. The results with azalea showed high correlation between molecular distance and morphological distance. The choice of markers relating to morphological characteristics may make effective pre-screening by molecular distance possible.

64. The Working Group agreed that the size of the reference collection was getting larger and larger, testing authorities needed more efficient methods, such as molecular techniques, in order to search for reference varieties effectively and to minimize the number of the reference varieties grown in tests for DUS.

65. One expert insisted that prescreening was an important part of DUS testing. Therefore, if molecular techniques could be used for prescreening, they should be clearly defined in the Test Guidelines themselves. He suggested that the Working Group should concentrate on a few species and discuss the protocol of molecular techniques for prescreening.

66. The Working Group stressed that tools for prescreening needed to be reliable as the discarded varieties would never be compared with the candidate variety. Some experts therefore suggested that, if molecular methods were not absolutely reliable, the results of the molecular techniques could not be used alone, but only together with morphological characteristics.

67. The Working Group concluded that further studies were needed and that discussion had to continue on the choice of molecular markers linked to morphological characteristics and on the use of molecular markers combined with morphological characteristics for prescreening.

Future Program, Date and Place of the Next Session

68. The Working Group discussed whether it should continue as a separate working group. The Working Group was the only forum where testing experts, molecular scientists, statistician and breeders were able to exchange their views and opinions on the use of molecular techniques for DUS testing as well as essential derivation and prescreening. Continuation of these discussions was needed for further progress. It had to continue its discussion on statistical improvements and the precision of methods and especially on the question of uniformity and stability. It also needed to consider how to introduce molecular markers in "option two" and how to use them for prescreening. In addition, the conclusions of the CAJ requested the Working Group to continue its work. Therefore, the Working Group proposed to have further sessions as a separate working group.

69. One expert suggested that more focused discussion was necessary in order to make real progress and to elaborate methods for practical use in DUS testing. He proposed that the Working Group concentrate on a few species, such as oilseed rape, and discuss its methods and protocol for application. That could be done in his view in a smaller adhoc group

concentrating on a few species. The Working Group decided not to follow the proposal of a small adhoc group because of the difficulty of choosing a limited list of species and the necessity for a broad information exchange. On the other hand, the Working Group decided to ask each Technical Working Party to choose one or two priority species to be taken up in the Working Group.

70. The experts from the Community Plant Variety Office offered to host the sixth session. The Working Group accepted that offer and agreed to hold its sixth session in Angers, France, towards the end of February or beginning of March 2000, about two to three weeks before the Technical Committee.

71. During the session, the Working Group planned to discuss the following items:

(a) Short presentation of biochemical and molecular techniques: new techniques, advantages and limits of different techniques (this item could be illustrated with experimental data obtained in different species)

(b) Assessment of variability within varieties and between varieties, in particular, uniformity and stability in molecular markers with reports on

- Cucumber (document to be prepared by experts from Spain)
- Rape seed and chrysanthemum (document to be prepared by experts from the United Kingdom)
- Ryegrass (documents to be prepared by experts from France and ASSINSEL)
- Rose (documents to be prepared by experts from France)
- Strawberry (documents to be prepared by experts from France )
- Sugar beets (document to be prepared by experts from Belgium )
- Tomato and wheat (document to be prepared by experts from the Netherlands)
- Influence of sampling of materials on the results (document to be prepared by experts from France)

(c) Construction and standardization of databases of DNA profiles of varieties (document to be prepared from experts from France)

(d) Statistical methods (this item could be illustrated with experimental data obtained in different species)

(i) Confidence intervals and improvement of precision of distance estimates (documents to be prepared by experts from the TWC and by experts from France and Germany)

(ii) Graphic representation of genetic distances (document to be prepared by experts from the TWC)

(iii) Comparison of genetic distances with phenotypic distances (document to be prepared by experts from France (rape oilseed)

(iv) Combination of information from diverse data types (AFLP, SSR, morphological data, etc.)

(e) Possibilities and consequences of the introduction of DNA profiling methods for DUS testing

(f) Position of the breeders vis-à-vis DNA profiling (report from ASSINSEL)

(g) The use of DNA profiling as a possible tool for prescreening in DUS testing

(h) The use of DNA profiling methods by expert witnesses in disputes on essential derivation with reports on

- Tomato (document to be prepared by ASSINSEL)
- Testing $H_0$ hypothesis on the threshold level (document to be prepared by experts from France)

(i) Future program, date and place of the next session

72. The Chairman requested that documents for the next session should be submitted to the Office of the Union by the end of 1999 in order to provide enough time for their preparation and distribution by the Office and for the reading by participants before the next session.

Visits

73. During the session, the Working Group visited in small groups Soybean and Alfalfa Research Laboratory, USDA-ARS, and was given information on the recent research on SSR markers to assess genetic variation in soybean by Dr. Cregan.

*74. This report has been adopted by correspondence.*

[Four annexes follows]

ANNEX I

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[Annex II follows]

# The Use of Simple Sequence Repeat (Microsatellite ) DNA Markers for Soybean Variety Identification

Perry Cregan and Charles Quigley
Soybean and Alfalfa Research Laboratory
USDA, Agricultural Research Service
Beltsville, Maryland USA

## Research Objectives

Develop an integrated genetic linkage map of soybean based upon Simple Sequence Repeat (SSR) DNA markers

Apply the map and markers for purposes of

- Marker assisted plant improvement
- QTL (Quantitative Trait Locus) Analysis
- Gene discovery
- Germplasm characterization
  - Variety Identification
  - Assays of genetic diversity

# TERMINOLOGY

- Simple Sequence Repeat (SSR)
- Microsatellite
- Short Tandem Repeat (STR)
- Sequence Tagged Microsatellite Site

# CHARACTERISTICS OF SIMPLE SEQUENCE REPEAT DNA MARKERS

- Highly informative
  - Multi-allelic
  - Co-dominant inheritance
- Single locus
- PCR based
- High level of reproducibility
- Abundant in plant genomes
- No probe maintenance or distribution

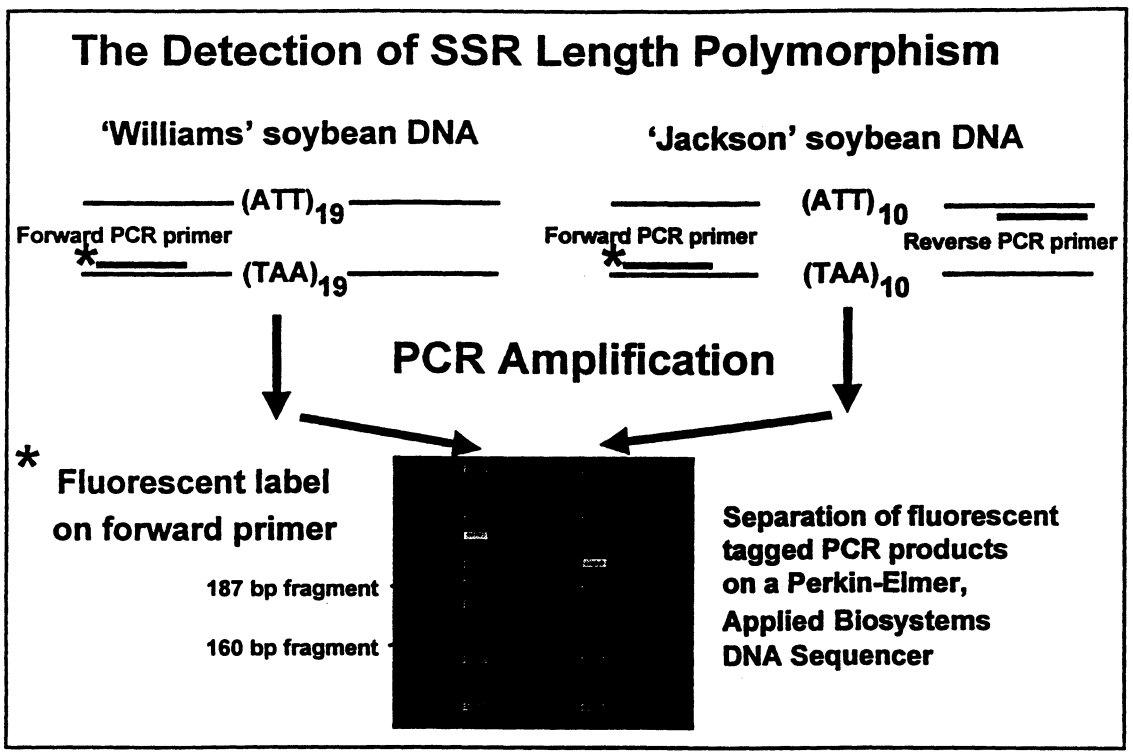
# Soybean SSR Marker and Linkage Map Development

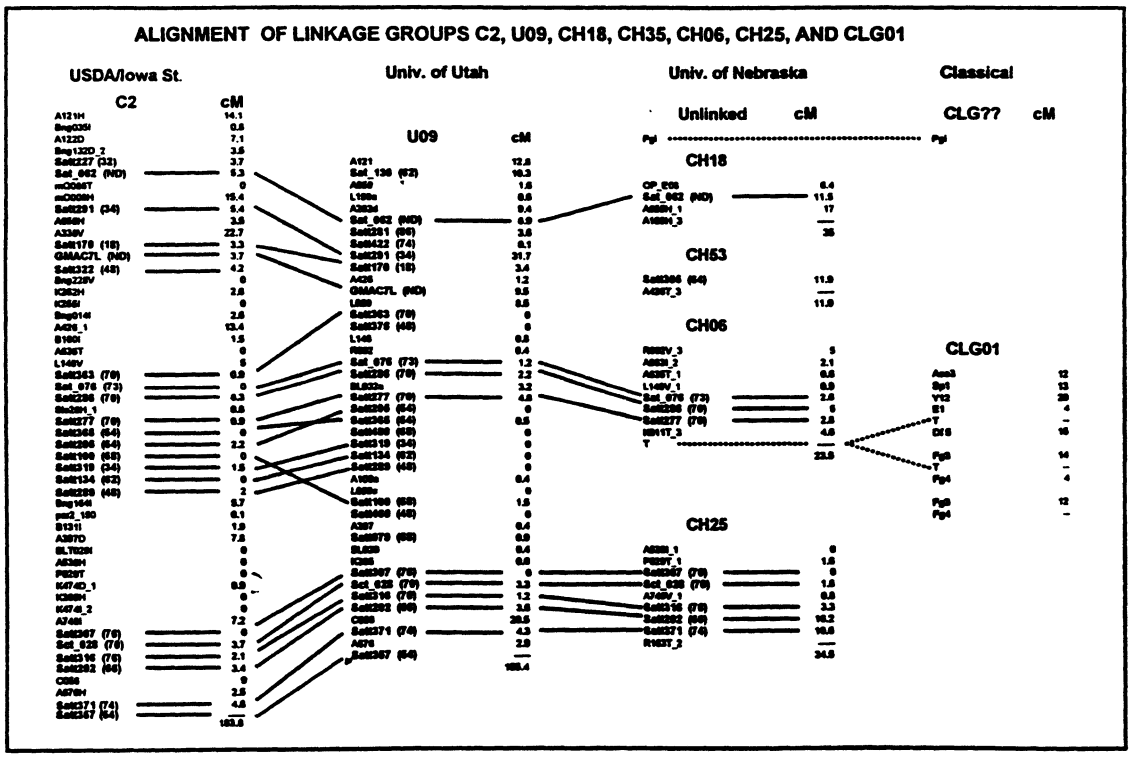
USDA-ARS, Beltsville, MD
University of Utah
BioGenetic Services, Inc.
Univ. of Nebraska
USDA-ARS, Ames, IA

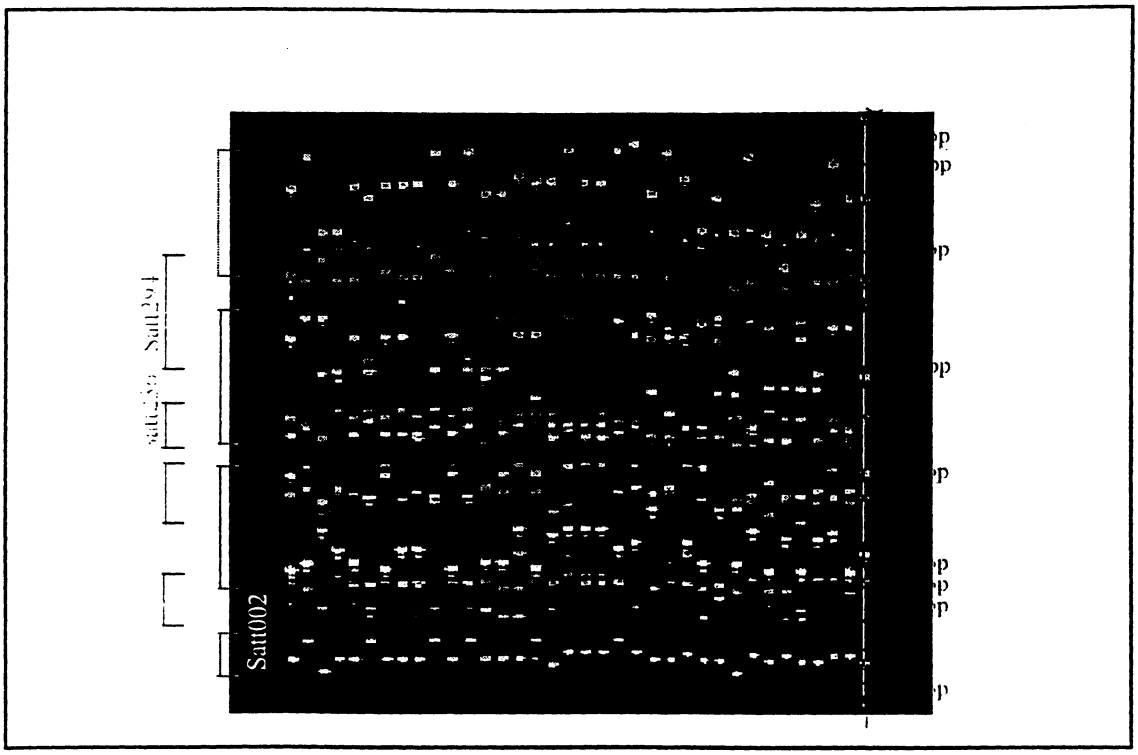
- Developed approximately 650 SSR loci
- Each mapped in one, two or three mapping populations
- Established 20 linkage groups corresponding to the 20 sets of soybean chromosomes

# Selection of a Subset of SSR Loci for Germplasm Classification and Variety Identification

- High level of allelic diversity among the N. American ancestral cultivars
- Good genome coverage with minimal genetic linkage among loci
- Robust amplification with a minimum of stuttering or extraneous products


The Detection of SSR Length Polymorphism
'Williams' soybean DNA
'Jackson' soybean DNA
(ATT)19
(TAA)19
(ATT)10
(TAA)10
Forward PCR primer
Forward PCR primer
Reverse PCR primer
PCR Amplification
* Fluorescent label on forward primer
187 bp fragment
160 bp fragment
Separation of fluorescent tagged PCR products on a Perkin-Elmer, Applied Biosystems DNA Sequencer


ALIGNMENT OF LINKAGE GROUPS C2, U09, CH18, CH35, CH06, CH25, AND CLG01
USDA/Iowa St.
Univ. of Utah
Univ. of Nebraska
Classical
C2
cM
U09
cM
Unlinked
cM
CLG?? cM
CH18
CH53
CH06
CLG01
CH25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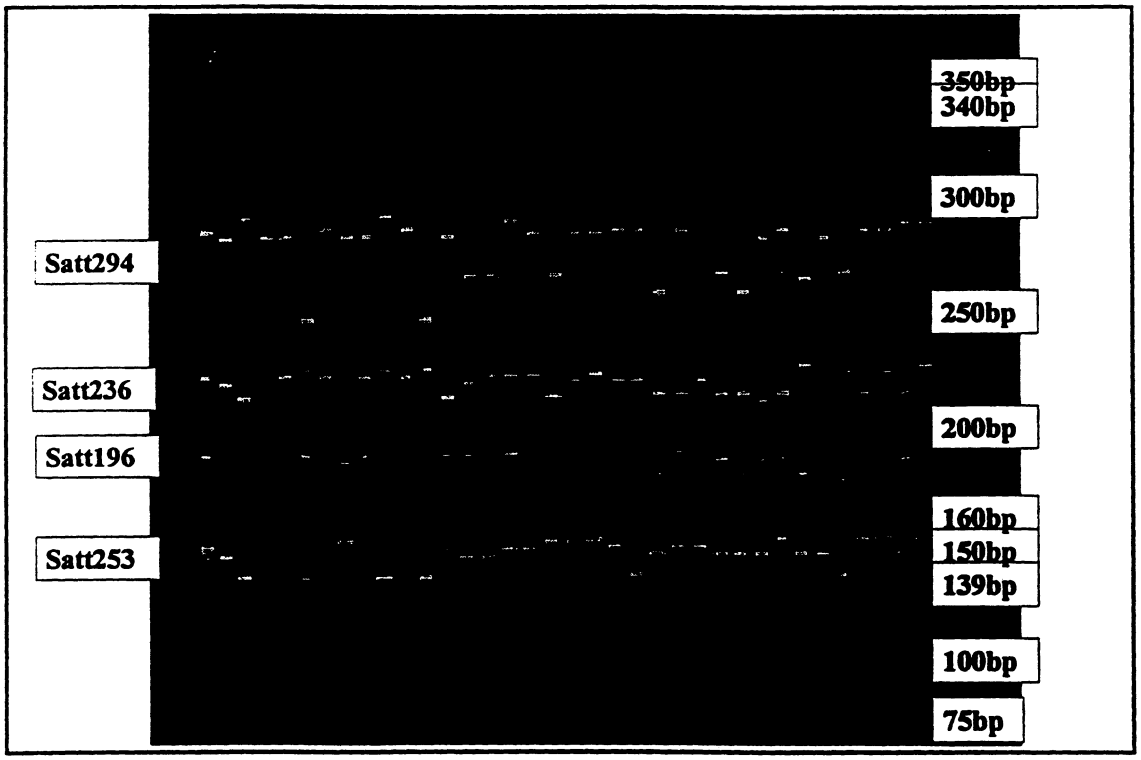
Satt002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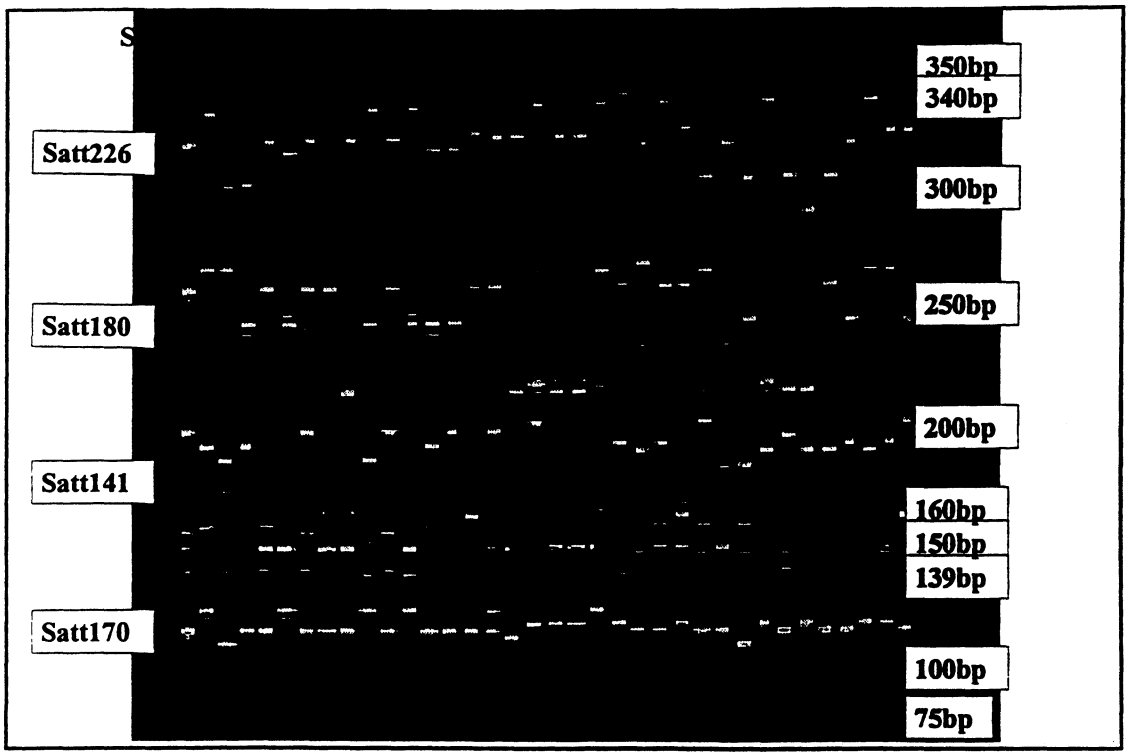
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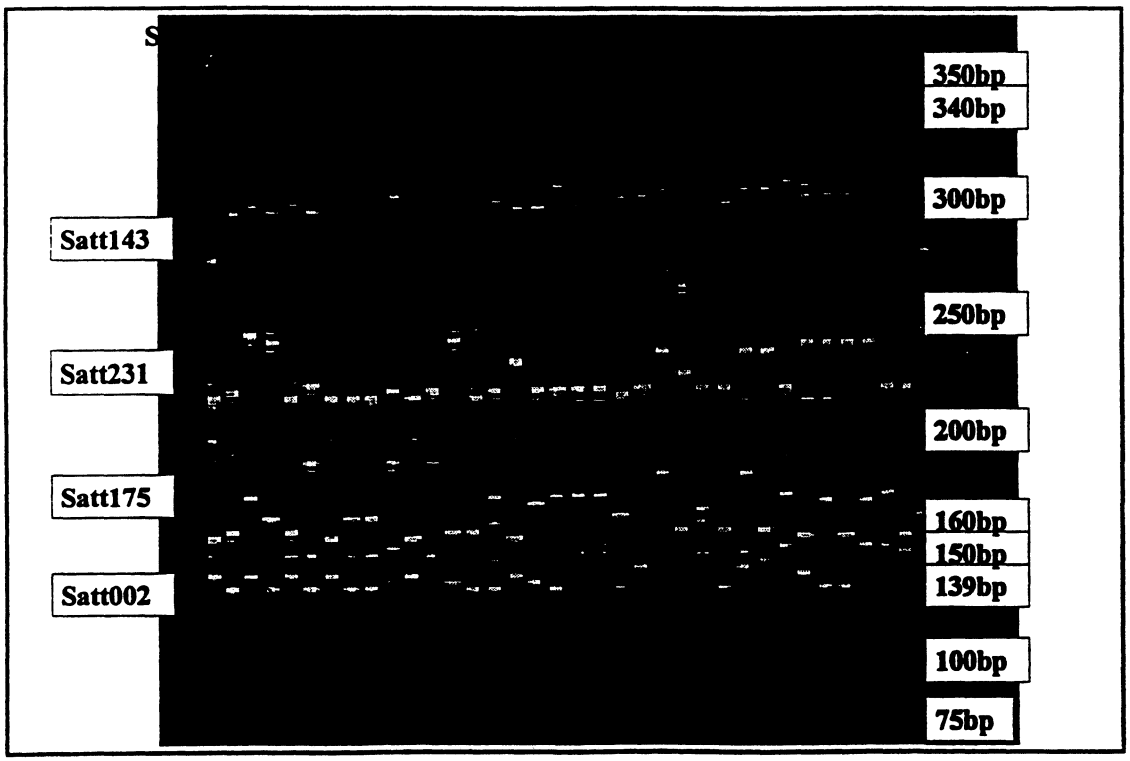
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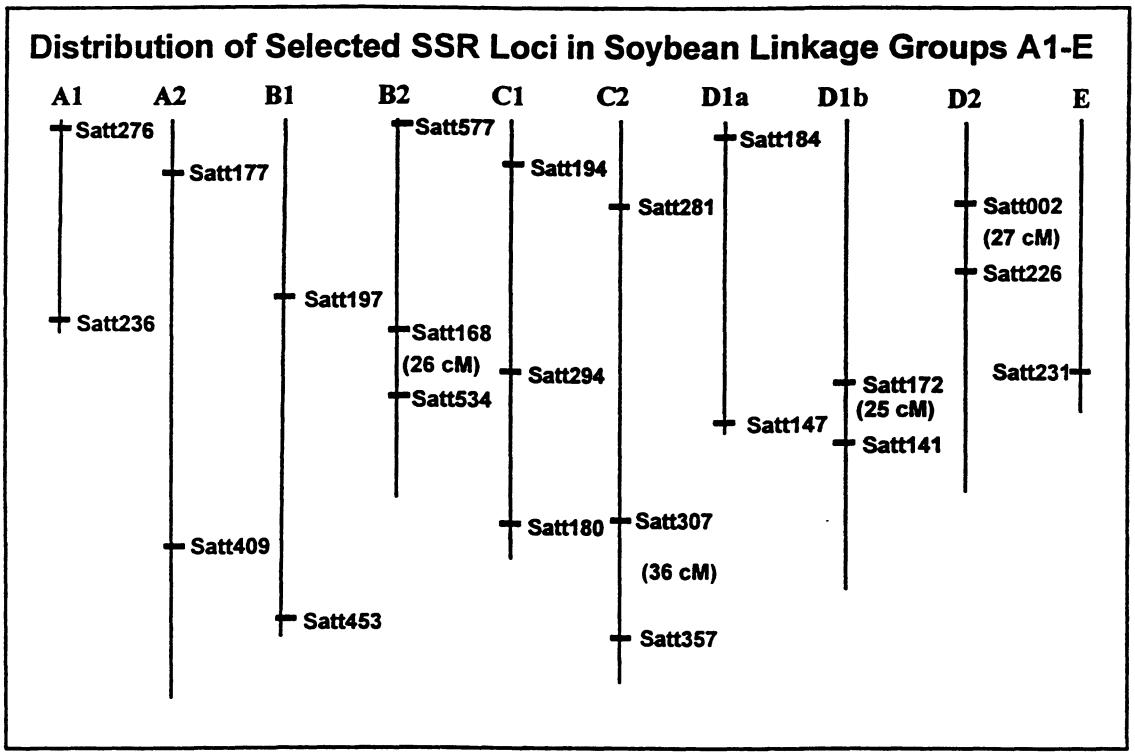


## Contents of a Rudimentary Soybean Genotyping Database

Genotype information for each of the 35 ancestors of N. American soybean cultivars at 48 SSR loci

Genotype information for each of 70 publicly released cultivars from Maturity Groups 000-IX at 48 SSR loci

## Maturity Group IV Soybean Cultivars with Identical Pigmentation and Morphological Characteristics

### 10 Cultivars

Black hila
Yellow cotyledon
Ovate leaflet shape
White flower
Tan pod
Tawny pubescence
Indeterminate growth habit

## Pedigree Analysis of Soybean Genotypes Using 20 SSR Loci: The Number of Alleles in the Progeny That Can Be Explained by Those Present in the Parents

| Progeny and ♀ and ♂ parents | Alleles present in progeny derived from | | |
|---|---|---|---|
| | ♀ Parent | ♂ Parent | Neither |
| Forrest = Dyer x Bragg | 20 | 20 | 0 |
| Bragg = Jackson x D49-2491 | 20 | 18 | 2 |
| D49-2491 = S-100 x CNS | 24 | 14 | 2 |
| Lee = S-100 x CNS | 20 | 18 | 2 |
| Adams = Illini x Dunfield | 20 | 18 | 2 |
| Clark = Lincoln x (Lincoln x Richland) | 27 | 11 | 2 |
| Amsoy = Adams x Harosoy | 16 | 22 | 2 |

# Reported Range of Mutation Rates for Human Microsatellite Loci

From:

Edwards et al. (1993)
Weber and Wong (1993)

$2.3 \times 10^{-5}$ to $5.6 \times 10^{-4}$

For 10 meiotic events this would produce a range of mutation rate from:

$10 \times 2.3 \times 10^{-5}$ to $10 \times 5.6 \times 10^{-4}$

Or

$2.3 \times 10^{-4}$ to $5.6 \times 10^{-3}$

Per 10 meiotic events or generations

For 20 loci, this would give a range of mutation rate from:

$$20 \times 2.3 \times 10^{-4} \text{ to } 20 \times 5.6 \times 10^{-3}$$

Or

$4.6 \times 10^{-3}$ to 0.112 rate of new allele formation per pedigree

Or

**0.46 to 11.2 % rate of new allele formation per pedigree**

**A rate of 0.46 to 11.2 % would generate from 0.092 to 2.24 new alleles per pedigree:**

**We observed seven (7) new alleles or one (1) per pedigree**

[Annex III follows]

# Prediction of phenotypical distances through molecular data

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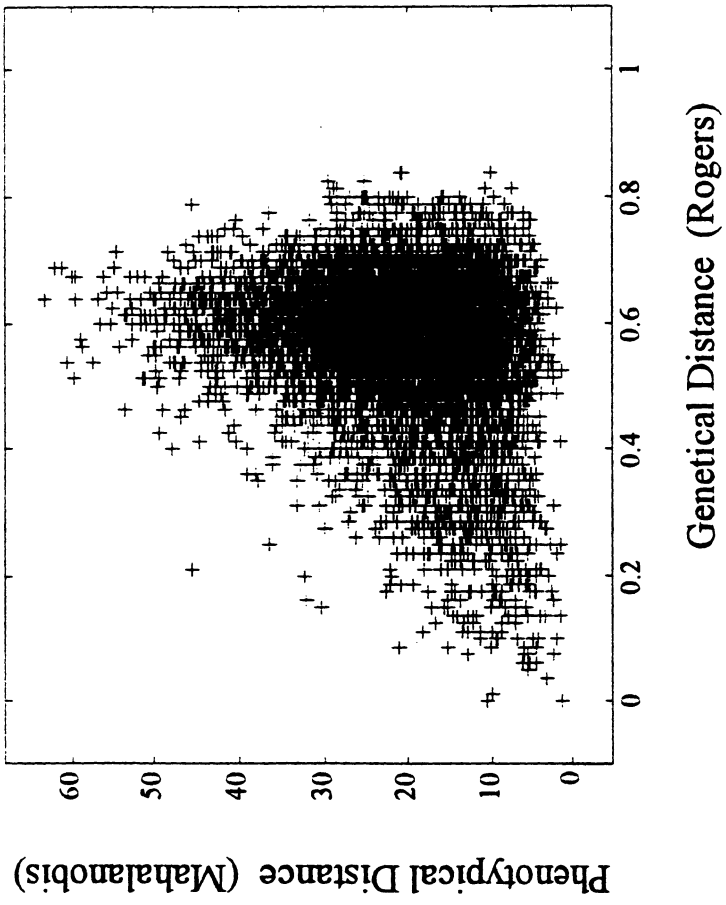
# CONTENTS

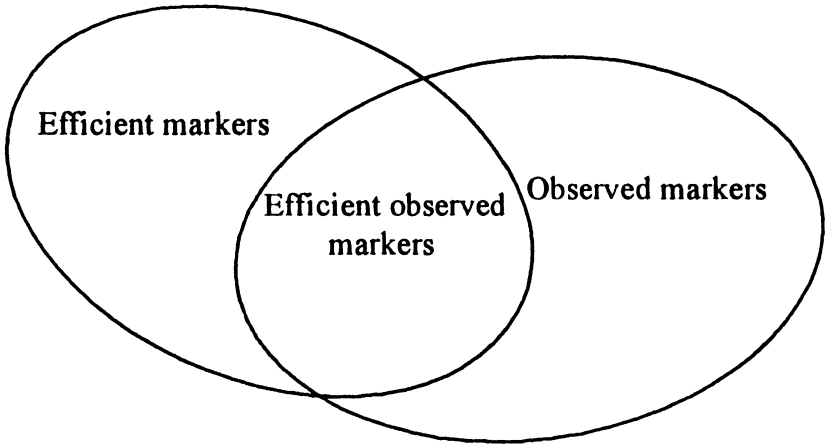
# I - Relation between Genetical and Phenotypical Distances



# II - Prediction of Phenotypical Distances :

## Model

Linear Model linking quantitative phenotypical variables with molecular markers




$$Y_{ij} = \mu_j + \sum_{\text{Efficient markers}} \alpha_{kj}(X_{ik}) + F_{ij}$$

$$Y_{ij} = \mu_j + \sum_{\text{Efficient observed markers}} \alpha_{kj}(X_{ik}) + E_{ij}$$

$$\underset{(1,p)}{\mathbf{Y}_i} = \underset{(1,p)}{\mu} + \underset{(1,M)}{\mathbf{X}_i} \times \underset{(M,p)}{\Theta} + \underset{(1,p)}{\mathbf{E}_i} \qquad \mathbf{E}_i \sim \mathcal{N}_p(\mathbf{0}, \Sigma)$$

Prediction of phenotypical distances

$$d_{\mathbf{P}}^2(i,i') = \frac{1}{2}\left(\mathbf{Y}_i - \mathbf{Y}_{i'}\right)\Sigma^{-1}\left(\mathbf{Y}_i - \mathbf{Y}_{i'}\right)'$$

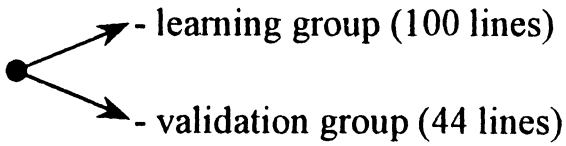
$$d_{\mathbf{P}}^2(i,i') \sim \chi_p^2(\text{predicted distance between i and i'})$$

$$\text{predicted distance between i and i'} = \frac{1}{2}\left(\mathbf{X}_i - \mathbf{X}_{i'}\right)\Theta\Sigma^{-1}\Theta'\left(\mathbf{X}_i - \mathbf{X}_{i'}\right)'$$

# II - Prediction of Phenotypical Distances :

## Material

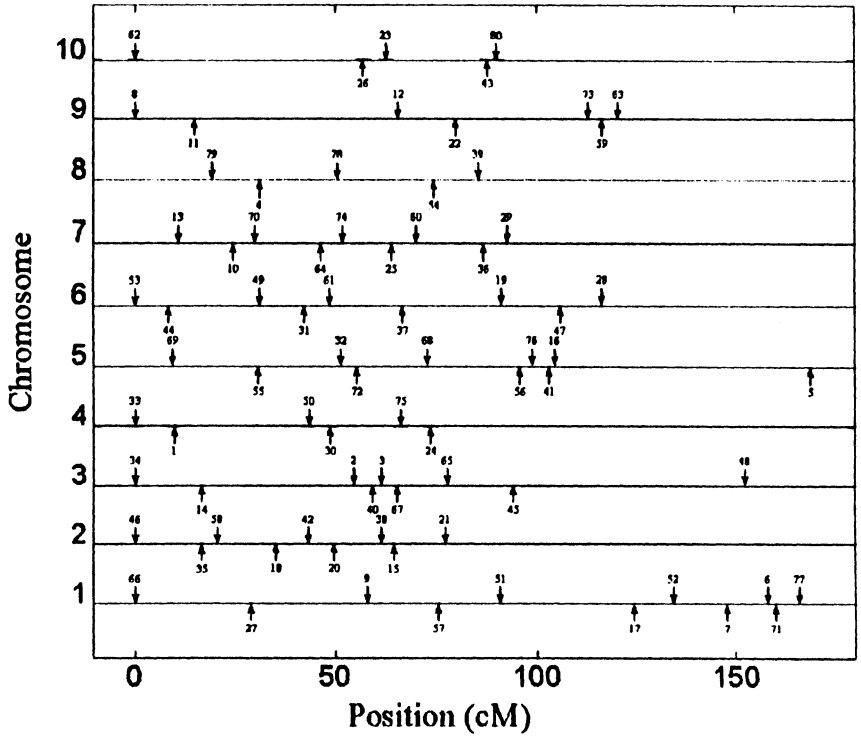
- 144 parental lines of maize split into two groups
- 10 phenotypical variables
- 80 molecular markers RFLP




### Phenotypical Variables

| Variable | Trait |
|---|---|
| $Y_1$ | Date of male flowering (jours) |
| $Y_2$ | Total plant length (cm) |
| $Y_3$ | Height of ear (cm) |
| $Y_4$ | Width of blade (mm) |
| $Y_5$ | Length of ear (mm) |
| $Y_6$ | Diameter of ear (mm) |
| $Y_7$ | Diameter of cob (mm) |
| $Y_8$ | Nomber of rows of seeds |
| $Y_9$ | Length of main axis above lowest side branch (cm) |
| $Y_{10}$ | Length of main axis above highest side branch (cm) |

### Molecular markers

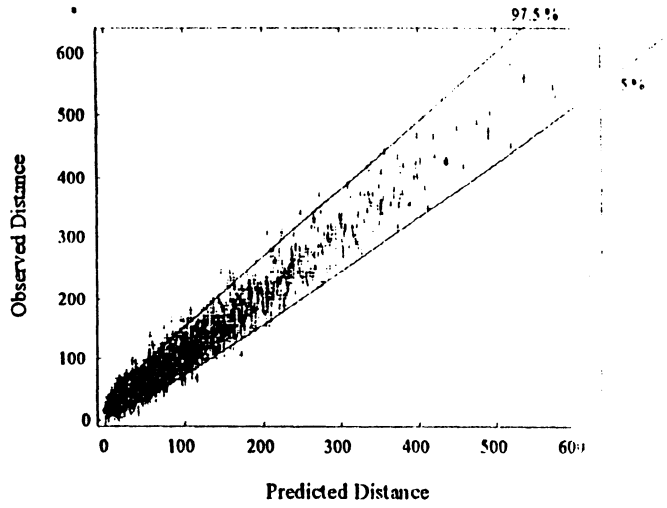


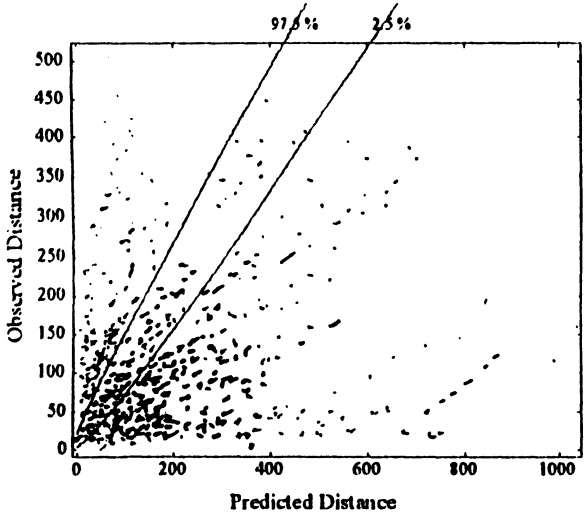
# II - Prediction of Phenotypical Distances :

# Results, 95% Confidence Intervals

[redacted]

| | | |
|---|---|---|
| bootstrap | 4262 | 86.1 % |
| Genetic map | 4678 | 94.5 % |



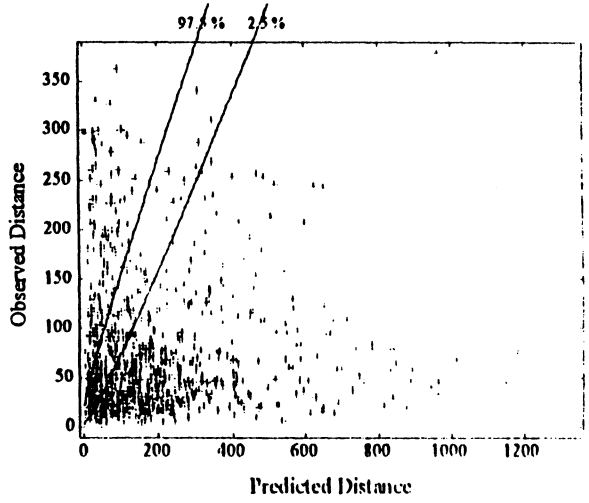




| | | |
|---|---|---|
| bootstrap | 2112 | 48.0 % |
| Genetic map | 1342 | 30.5 % |

Type 1 : 4400

[redacted]

| | | |
|---|---|---|
| bootstrap | 339 | 35.8 % |
| Genetic map | 204 | 21.6 % |



# II - Prediction of phenotypical Distances :

# Choice of efficient markers

Using «bootstrap» method

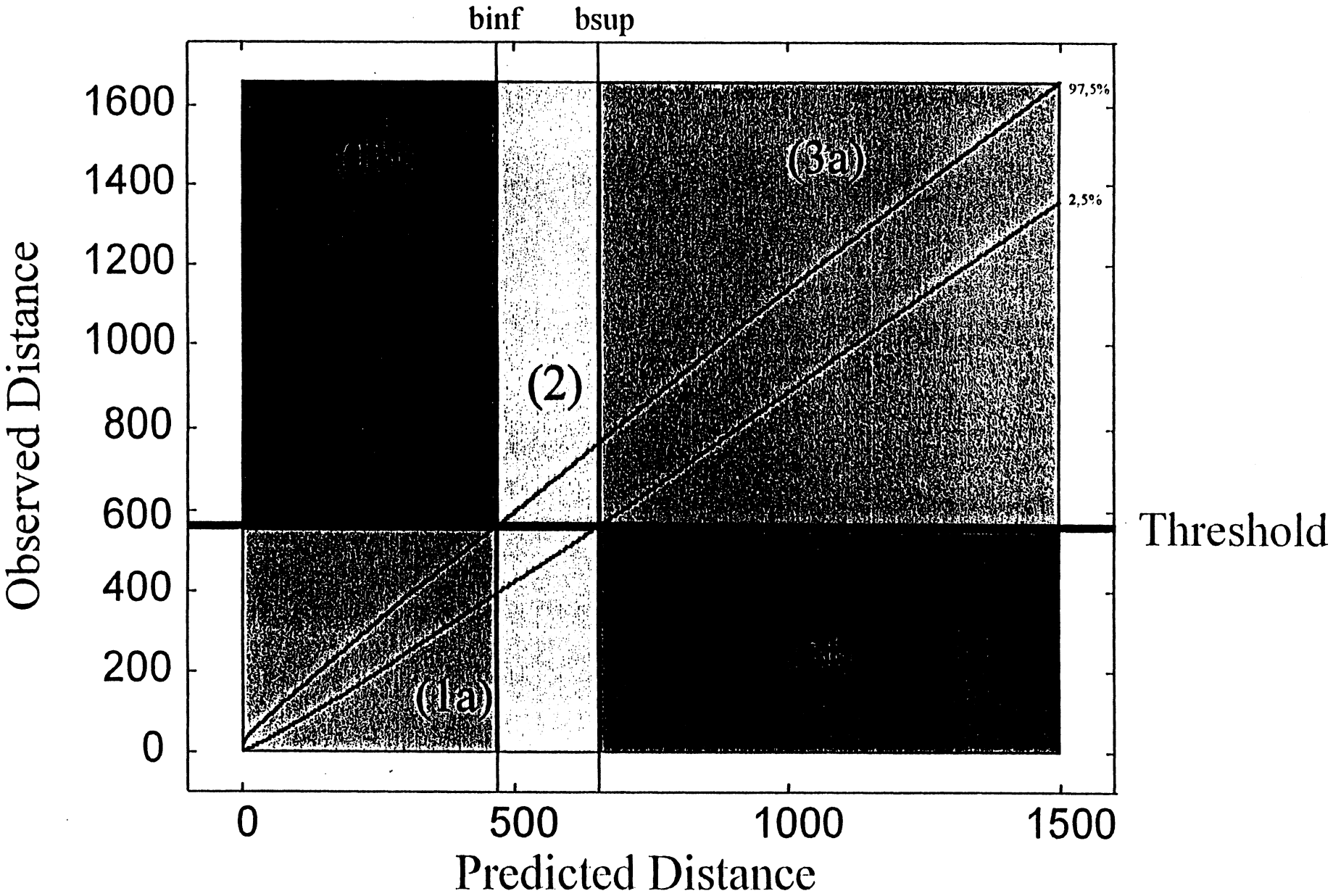
| **Variable** | $Y_1$ | $Y_2$ | $Y_3$ | $Y_4$ | $Y_5$ | $Y_6$ | $Y_7$ | $Y_8$ | $Y_9$ | $Y_{10}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| **$R^2$ ( % )** | 73.06 | 81.02 | 71.97 | 83.46 | 80.29 | 76.96 | 76.97 | 85.79 | 70.28 | 87.57 |

Using «genetic map» method

| **Variable** | $Y_1$ | $Y_2$ | $Y_3$ | $Y_4$ | $Y_5$ | $Y_6$ | $Y_7$ | $Y_8$ | $Y_9$ | $Y_{10}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| **$R^2$ ( % )** | 75.50 | 78.45 | 40.20 | 72.58 | 83.73 | 73.94 | 47.60 | 70.32 | 48.59 | 97.16 |

# III - Using these Predictions in the Distinction procedure:

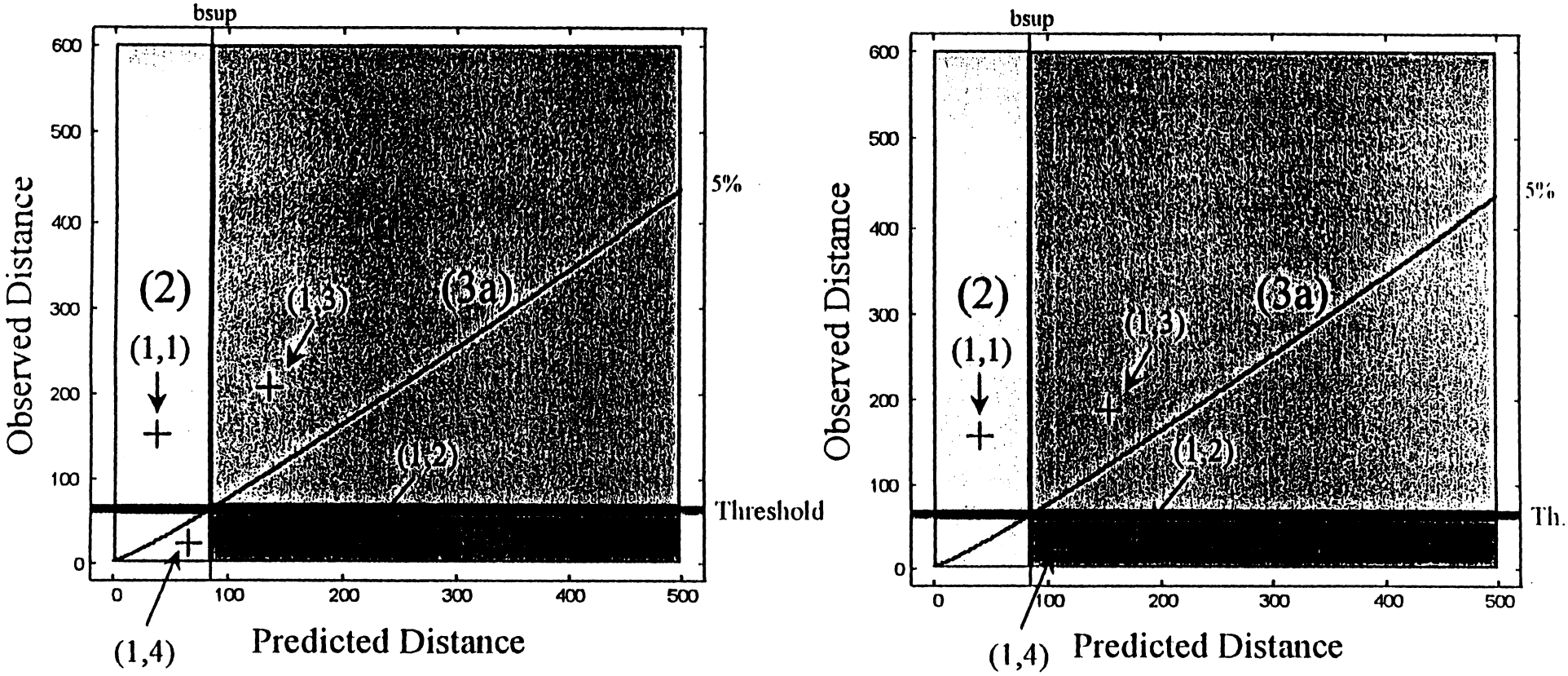
## Method



# III - Using these Predictions in the Distinction procedure :

# Errors' Nature

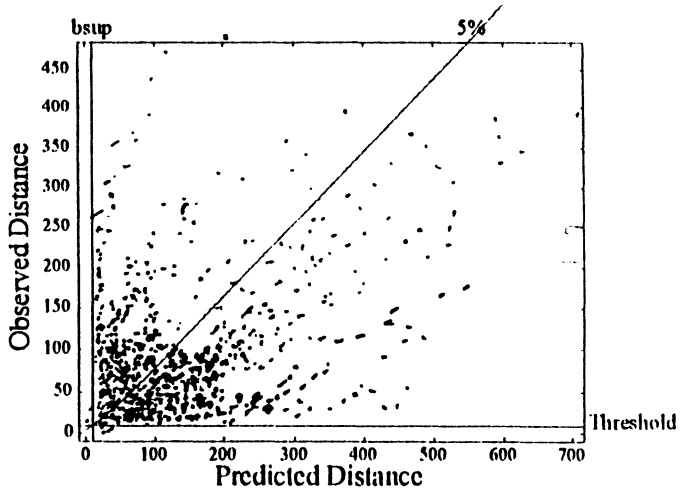
**Decisive Error «GEVES»** : to declare distinct a candidate line which is not distinct.

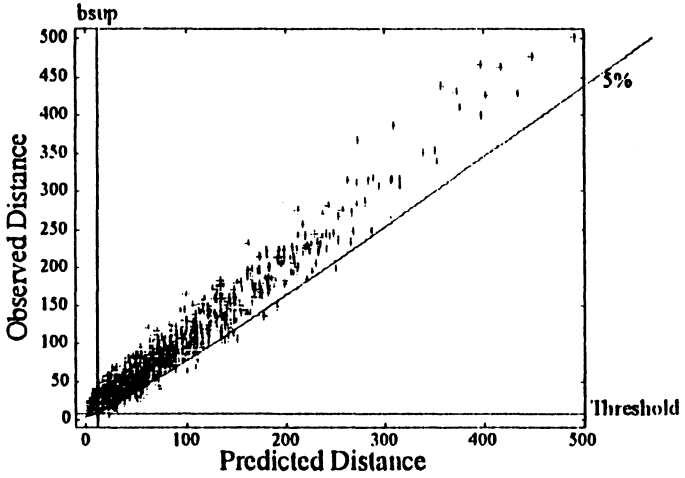


# III - Using these Predictions in the Distinction procedure :

## Results obtained with « genetic map» parameters

| 43.5 % | gain (lines of reference collection) | Errors GEVES | Decisive errors |
|---|---|---|---|
| min | 54.0 % | 1.6 % | 10 |
| mean | 63.9 % | 2.0 % | 11.4 |
| max | 80.0 % | 2.4 % | 13 |



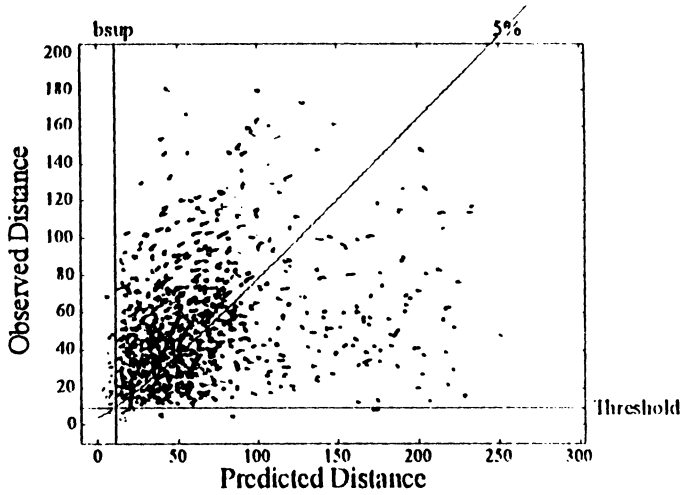



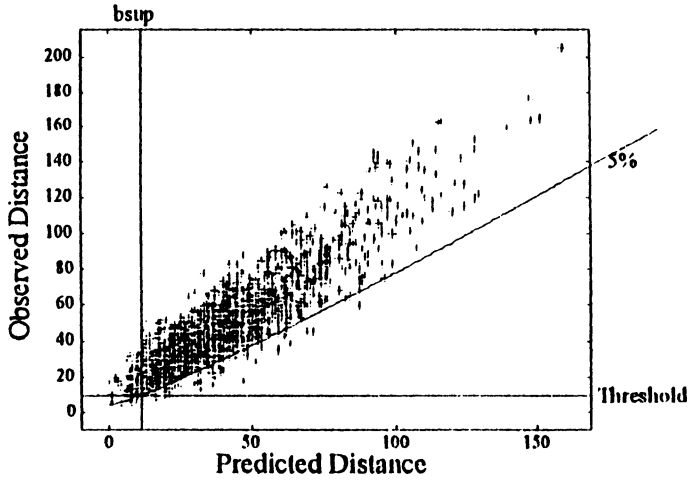

| 92.3 % | gain (lines of the reference collection) | Errors GEVES | Decisive errors |
|---|---|---|---|
| min | 33.8 % | 0.3 % | 0 |
| mean | 42.1 % | 0.6 % | 2.2 |
| max | 47.5 % | 0.9 % | 6 |

# III - Using these Predictions in the Distinction procedure :

## Results with «bootstrap » parameters

| 65.9 % | gain (lines of the reference collection) | Errors GEVES | Decisive errors |
|---|---|---|---|
| min | 51.0 % | 1.0 % | 6 |
| mean | 66.5 % | 1.5 % | 9.1 |
| max | 76.0 % | 2.0 % | 13 |

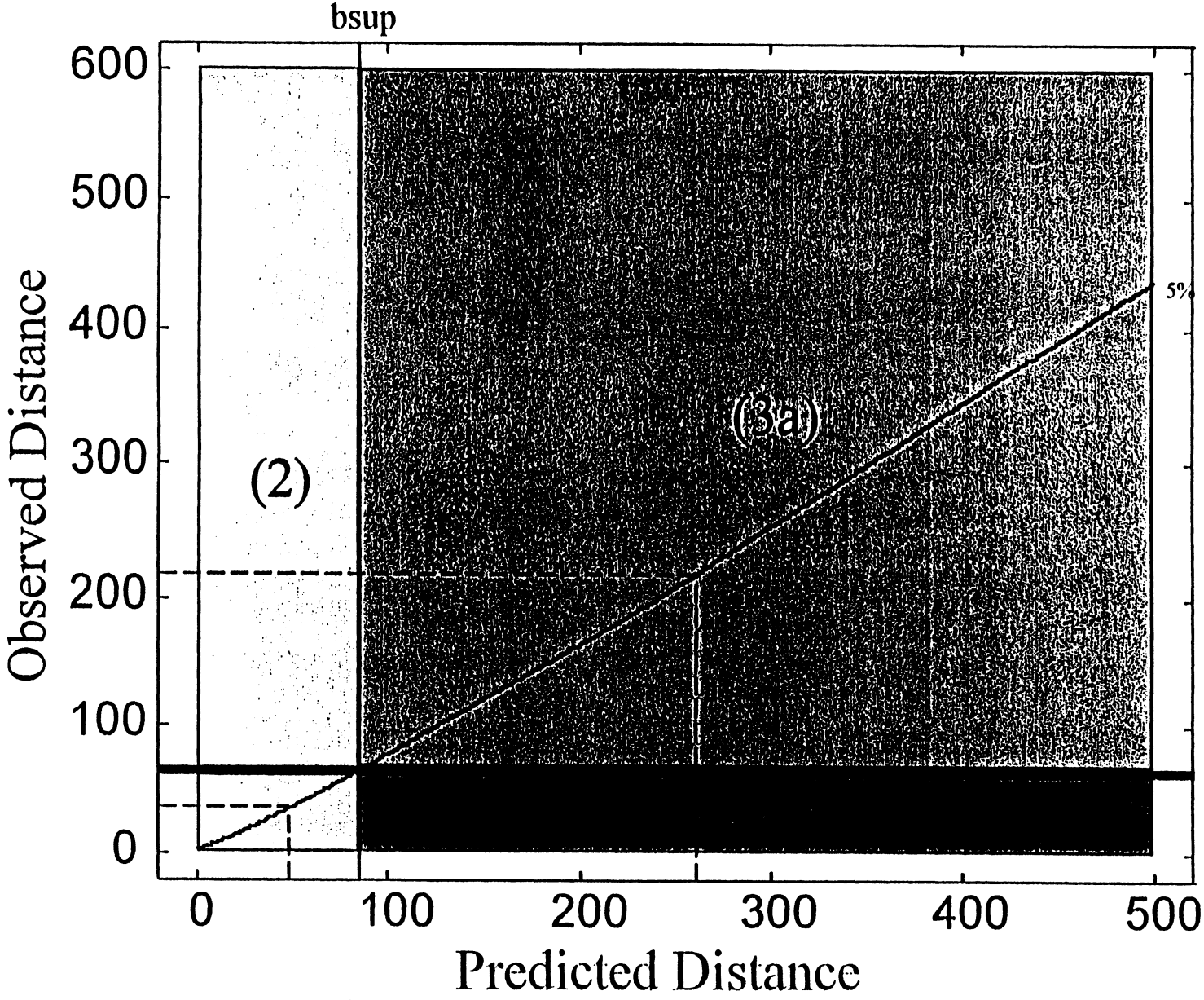



| 93.5 % | gain (lines of the reference collection) | Errors GEVES | Decisive errors |
|---|---|---|---|
| min | 51.3 % | 0.3 % | 1 |
| mean | 59.3 % | 0.7 % | 3.5 |
| max | 67.5 % | 1.2 % | 9 |

# III - Using these Predictions in the Distinction procedure :

## Choose a threshold




Threshold

# Conclusion

- Linear model for phenotypical quantitative variables
- predictions of phenotypical distances throught molecular data
- Using these predictions in the distinction procedure allows gain in terms of lines of the reference collection experimented with risks

# Prospects

- model taking into account quantitative plus qualitative variables
- best estimation of parameters and best choice of efficient markers
- validation based on more data from GEVES

[Annex IV follows]

ANNEX IV

**BMT**

**Washington - September 98**

# DEFINITION OF VARIETY

## (item 7 of the BMT agenda)

## Recalls on Convention 91

### Article 1, vi : Definitions

« **Variety** » means a plant grouping ... can be :

- Defined by **the expression of the characteristics resulting from a given genotype or combination of genotypes**,
- ...
- ...

### Article 7 : Distinctness

The variety shall be deemed **to be distinct if it is clearly distinguishable from any other variety**.

# Article 14-5 : Scope of the Breeder's Right

(a) The provisions of paragraphs (1) to (4) shall also apply in relation to :

**(i) varieties which are essentially derived from the protected variety ...**

(b) ..., a variety shall be deemed to be essentially derived from another variety (initial variety) when :

(i) ...

(ii) ...

(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**.

## Working group of February 1998

→ Proposals for the interpretation of articles 1, vi and 7 in connection with a possible use of characteristics based on molecular analyses.

➡ **4 options have been studied.**

## Option 1 :

Strict interpretation of article 1, vi with the varietal description only based on phenotypic characteristics.

No use of molecular characteristics except if they are strictly linked with phenotypic ones (markers)

Clear distinctness only based on phenotypic characteristics.

→ Actual position of BMT and ASSINSEL.

- Difficult position regarding the quick development of DNA engineering in plant breeding and diversity assessment.

# Option 2 :

Strict interpretation of article 1, vi with the varietal description only based on phenotypic characteristics.

Open interpretation of article 7 with two sets of characteristics :

- phenotypic characteristics on which a variety can be defined
- genotypic characteristics which can be used as an help to confirm a clear phenotypic difference not easily observable (set of small differences, phenotypic characteristics difficult to assess)

→ Keeping of basic notions phenotype/genotype.

→ Opening to use molecular characteristics with all the advantages as far as the phenotypic background of the difference between two varieties exists.

→ Limited risk of small minimum distances as far as the phenotypic differences are sufficient.

- Necessity to define more precisely the conditions of application of such an approach.
- Uniformity and stability criteria ?

# Option 3 :

Wide interpretation of article 1, vi in which DNA polymorphism would be considered as a result of the expression of genotype or combination of genotypes.

Definition of a clear difference based on any characteristic for the application of article 7.

→ Full opening to the use of molecular characteristics for description of varieties.

→ Increasing of the discriminative power for distinctness.

→ Coherence for the application of EDV concept.

- Loss of basic notions phenotype/genotype.
- Difficulty to determine a clear difference between varieties, possibility to use any difference as a clear difference.
- Uniformity and Stability criteria ?

# Option 4 :

Wide interpretation of article 1, vi (as for option 3) with no restriction to establish distinctness on the basis of molecular characteristics.

→ Same advantages as for option 3.

- Same limits as for option 3.

  \+ drastic reduction of minimum distances between varieties.

# CONCLUSION

- → Large majority in favor of option 2.
- → Remaining question about application of EDV concept.
- → Case of two varieties with the same phenotype resulting from two genotypes with only one gene difference.

**CAJ / TC : Summary of the draft report.**

[End of document]