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INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

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

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ISSUE PAPER FOR THE *AD HOC* CROP SUBGROUPS
ON MOLECULAR TECHNIQUES

Document prepared by the Office of the Union
in cooperation with the chairpersons of the Subgroups

Introduction – Objectives of the Subgroups

1. The aim of the Subgroups is to discuss technical issues regarding the possible application of molecular techniques, e.g., DUS assessment, the management of reference collections, and the assessment of essentially derived varieties, with reference to existing empirical data of the crop in question. Discussion in the Subgroups will be held on the manner in which molecular techniques might be applied.

2. It should be noted that the Subgroups will not discuss the wider policy questions, e.g., phenotype vs. genotype and the concept of minimum distance. The Crop Subgroups should be open to all possible options and will discuss these from a technical viewpoint without prejudice to the final conclusion in the BMT, CAJ or TC.

3. The following outcomes at the Subgroups are anticipated as a report to the BMT/TC:

(a) to construct possible application models (options)
(b) to identify outstanding technical problems for their applications
(c) to assess the possible impact of their applications on protection

This issue paper is designed to identify important issues for certain possible applications of molecular techniques in plant variety protection and to serve as the basis of discussion in the Subgroups.

Important Issues

A. General questions

4. For any type of application, molecular markers should be able to produce consistent, reproducible results across different laboratories at an acceptable cost.

5. Consistency and Reproducibility: It has been reported that reproducibility varies according to molecular techniques, choice of molecular markers and observed bands, preparation of samples, equipment, conditions for amplification, detection systems and experience in individual molecular experts. The required levels of consistency and reproducibility depend on the purpose of application. On the one hand, for the assessment of distinctness, molecular markers must at least produce consistent differences between varieties in a particular set of condition. It might not necessarily require the reproducibility of the same band patterns across different laboratories. On the other hand, absolute reproducibility of molecular band patterns across different laboratories is required for the most effective management of reference collection through the harmonization of the molecular characterization (see Paragraph 30).

Considerations for participants

- ✓ *How do different factors (e.g., the quality of chosen molecular bands, the preparation of samples, equipment…) influence consistency or reproducibility?*
- ✓ *How far can consistency within the same laboratory or reproducibility between different laboratories be currently achieved?*
- ✓ *To what extent is reproducibility considered necessary for the purpose of DUS examination or the management of reference collections ?*

- ✓ *Is the reproducibility currently achieved acceptable for these purposes? For example, can DNA profiles obtained in different laboratories be directly comparable?*
- ✓ *What kinds of standardization are required to achieve an acceptable level of reproducibility?*

6. Cost/Accessibility: High cost of molecular assessments could be a potential obstacle for their application. In addition, the protection of some molecular markers by patent might limit their availability to breeders and national offices.

Necessary information

- ✓ *Costs for equipment and materials (initial costs and running costs)*
- ✓ *Charge by service companies per assessment*
- ✓ *Patent protection of molecular markers or methods*

B. Examination of Distinctness, Uniformity and Stability

B-1. Use of gene specific markers linked to traditional characteristics

7. One approach is the use of presence or absence of (a) certain molecular band(s) linked to a (probably qualitative) traditional characteristic, for example, disease resistance gene.

8. Availability and usefulness of gene specific markers: One of the questions is the availability of such gene specific markers that are clearly linked to traditional characteristics. Gene specific markers have already been developed for other purposes, such as marker assisted breeding. However, only a few useful examples have been proposed with respect to possible application for plant variety protection. For example, absence or presence of one allele by the markers directed to amplify granule-bound starch synthase gene in wheat has proved useful for discriminating the suitability of varieties for a special end-use (Udon noodle) in wheat[1].

Considerations for participants

- ✓ *Availability of gene specific markers linked to traditional characteristics*
- ✓ *Usefulness of such markers as characteristics in current DUS tests*

9. Assessment of distinctness, uniformity and stability: A molecular characteristic might be treated as a truly qualitative characteristic with states: absence (1) or presence (9) of a certain band. The question is

Considerations for participants

- ✓ *How can distinctness be assessed? Can they be treated in the same way as traditional qualitative characteristics?*

10. Nature of linkage with traditional (morphological or physiological) characteristics: There might be only a few molecular bands which show clear direct linkage (e.g. complete cosegregation) with the expression of traditional characteristics. The level of the linkage to

[1] L.R. Preston, et al (1999) Plant cultivar identification using DNA analysis Plant Varieties and Seeds (1999) 12, 191-205

the expression of traditional characteristics might need to be examined for the application of this category.

11. Association with traditional quantitative characteristics: In the case of traditional characteristics controlled by many scattered genes (especially quantitative characteristics), the application of such genetic information (e.g., QTL loci) for DUS assessment might not be straightforward and probably could not be treated in the same way as traditional qualitative characteristics.

B-2 Use of DNA profiles regardless of their linkage with traditional characteristics

12. This second category is the use of a set of molecular bands (DNA profile) which provide information on a slice of the whole genome structure without interpretation of expression. One main question is how to set the **method** and **threshold level** for **judging distinctness, uniformity and stability**. Another question is **how these different approaches might affect the strength of variety protection**.

B-2-1. Distinctness

13. Treatment as traditional characteristics: One option is that molecular information will be treated in the same way as traditional characteristics. What is the difference in molecular data required to discriminate varieties? Inevitably it will vary according to the choice of molecular markers, the bands selected for observation and the species. In particular, the threshold level for distinctness is highly dependent on the level of variability within varieties. For example,

(a) if molecular bands were absolutely uniform within varieties, i.e., all individuals had the same band pattern, one band difference could discriminate between two varieties. In this case, individual molecular bands might be treated as qualitative characteristics with expression observed as absent or present.

(b) If molecular band patterns exhibit a degree of variability within uniform varieties, one option is to treat a set of molecular bands as a characteristic and to assess differences between varieties as a quantitative characteristic[2] by the totality of differences in several molecular bands (e.g., genetic distance or similarity measurement). Larger variation than the variability observed within varieties will be required to discriminate between varieties. Another option is to treat individual molecular bands as independent characteristics and to consider the frequency of individual molecular bands (e.g., the percentage of the presence of each molecular band observed in a variety).

Considerations for participants

- ✓ *According to studies on variability within and between existing varieties, how big a difference is required to discriminate between varieties according to an objective/statistical analysis?*
  - *vegetatively propagated varieties, self-pollinated varieties, inbred lines*
  - *cross-pollinated and synthetic varieties*
  - *hybrid varieties*

[2] This option might be also considered as a multivariate analysis of a set of qualitative characteristics (individual bands with the expression of absent or present).

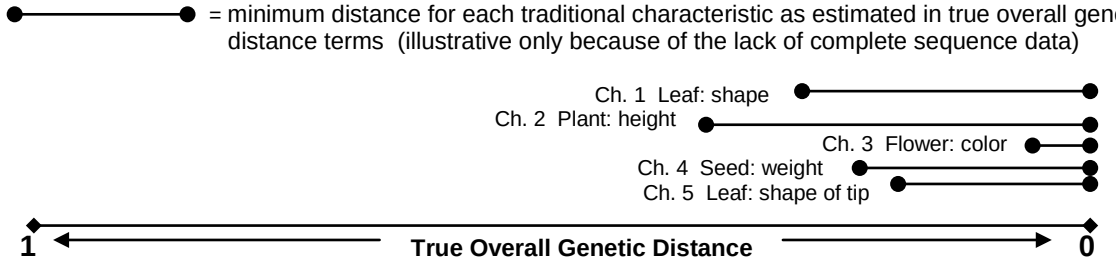
14. Regardless of any decision on the threshold level for distinctness, this information is useful basic information which indicates the discrimination power of molecular techniques (the degree of difference between varieties which can be distinguished by molecular techniques). The comparison between these data and differences observed between close pairs of protected varieties will produce some guidance on the possible impacts on the strength of protection from the introduction of molecular techniques.

15. Level of difference to ensure no, or acceptable changes, of minimum distance: One of the concerns of the introduction of molecular techniques into DUS examination is an erosion of the "minimum distance" (difference needed for distinctness) in a manner which might weaken the value of protection. Therefore, one option is to set a threshold of molecular distance corresponding to "minimum distance" in traditional characteristics. However, it should be noted that, since most molecular markers are not directly linked to traditional characteristics, direct correspondence between traditional characteristics and molecular marker distance[3] cannot be expected.

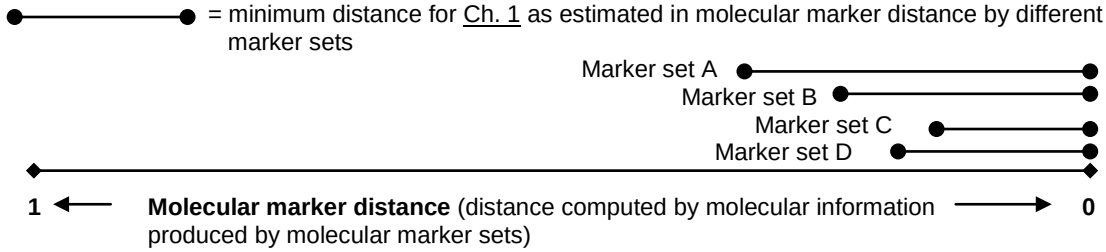
16. A conceptual diagram is shown in the BOX below. The true overall genetic distance corresponding to the "minimum distance" for traditional characteristics varies between species and also between characteristics. In addition, we cannot observe true overall genetic distance because this would require full DNA sequencing of all varieties, but we can observe "molecular marker distances" between existing varieties, which might be different in more than one characteristic even for the closest pairs.

BOX: Molecular marker distances corresponding to "minimum distance" for traditional characteristics

1. "Calibration" of minimum distances for traditional characteristics into true overall genetic distance



2. "Calibration" of minimum distance for traditional characteristics into molecular marker distance



No direct relationship between true overall genetic distance and molecular marker distances.

[3] Genetic distances computed by molecular information produced by molecular marker sets refer to hereinafter as "molecular marker distances".

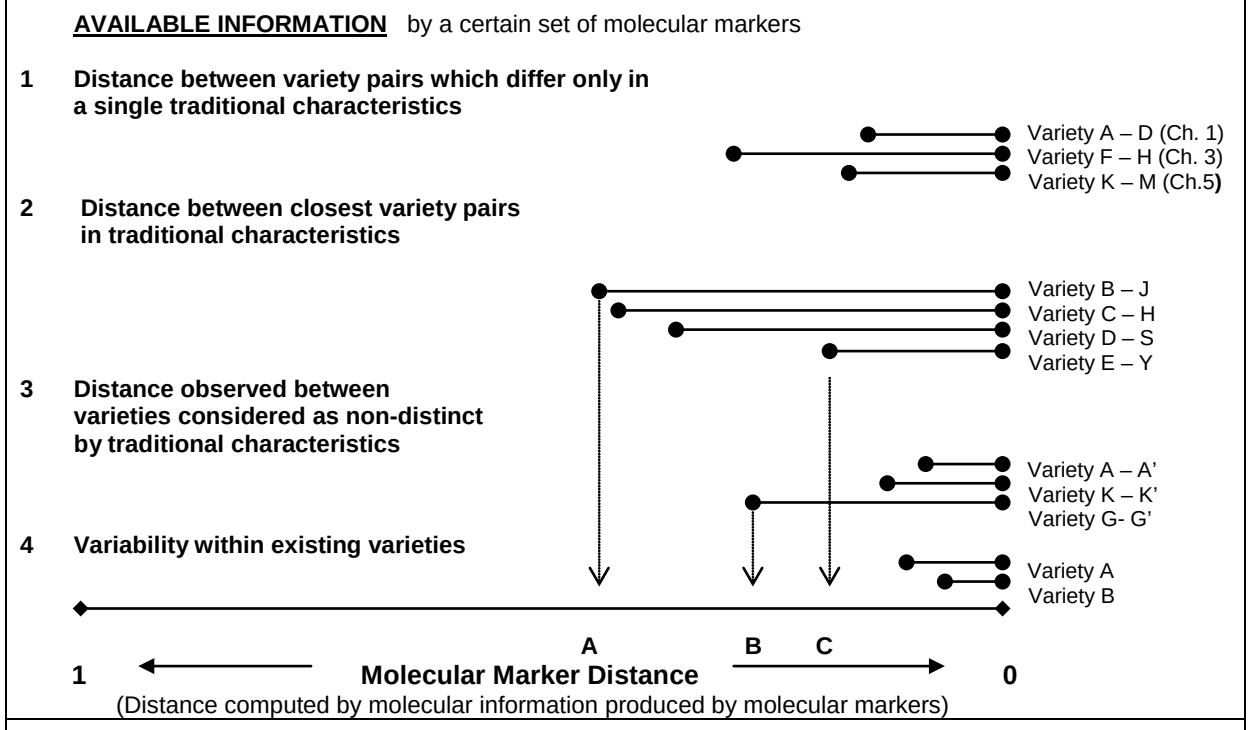
3. "Calibration" of minimum distance in traditional characteristics into molecular marker distances

Paragraph 1 illustrates the calibration of minimum distance in traditional characteristics into true overall genetic distance according to the choice of traditional characteristics. However, the actual overall genetic distance is not known because of lack of full DNA sequence information and it is therefore not possible to set a true overall genetic distance as established by historical use of traditional characteristics and then convert this into a corresponding distance for molecular marker distances. As illustrated in Paragraph 2, only the direct calibration of minimum distance into molecular marker distance could be possible, but there is no direct relationship between them. The calibration highly depends on the choice of molecular markers.

One solution is to seek to generally calibrate traditional characteristics and molecular information without knowing how they correspond to each other on an individual basis.

A practical approach would be to compare the size of difference in molecular information between pairs of varieties which differ only by a minimum distance in a single traditional characteristic. If such variety pairs are not available, molecular information of close variety pairs would be reviewed with information of their differences in traditional characteristics. Also, differences in molecular information between pairs of varieties considered as non-distinct by traditional characteristics shall also be reviewed.

Data from such work would provide some guidance on the size of difference for molecular information which would provide reasonable confidence that if this was used as a minimum distance there would be no fundamental erosion of the minimum distance which has been established by use of traditional characteristics.



Examples for distances between closest variety pairs (No. 2 in BOX)

- *AFLP analysis of 55 wheat varieties with 90 polymorphic AFLP bands produced by six primer pairs*[4]: Differences in at least four or more polymorphic AFLP bands were observed in any pairs of 55 varieties (4 band difference = Point C)
- *Most similar variety analysis of 35 maize varieties*[5]:
  Similarity indexes of most similar variety pairs: AFLP 0.441 (A) to 0.915 (C)
  SSR 0.706 (A) to 0.952 (C)

[4] J. R. Law et al. (1998): DNA profiling and plant variety registration III: The statistical assessment of distinctness in wheat using amplified fragment length polymorphisms, Euphytica 102: 335-342, 1998

[5] BMT/5/3

17. Differences within varieties and differences observed between varieties considered as non-distinct by traditional characteristics should also be taken into account for determining the threshold level of distinctness. In particular, information on differences between varieties which are currently considered as non-distinct should also be reviewed.

Example for distances observed between varieties considered as non-distinct by traditional characteristics (No. 3 in BOX)

- *AFLP analysis of Oilseed Rape Varieties*[6]*:*
  - Genetic distances between three varieties that show no morphological difference 0.036, 0.044, 0.065 (Nei &Li distance)
  - Genetic distances observed between some morphologically distinct variety pairs were less than those between non-distinct varieties (Point B).

18. The following points might be considered in the Subgroup:

Considerations for participants

- ✓ *Is the conceptual diagram an appropriate summary? Where can possible threshold levels be set for distinctness with no or only acceptable changes of minimum distances?*
- ✓ *The following data are required:*
  - ✓ *Differences between existing protected varieties as observed by molecular data, in particular, differences between most similar variety pairs, preferably those distinct by a single traditional characteristic*
  - ✓ *Differences between varieties which are not clearly distinguishable by traditional characteristics*
  - ✓ *Differences within existing protected varieties (see below, i.e. uniformity and stability)*

B-2-2. Uniformity

19. Data on variability within existing protected varieties: The Subgroups need data of variability observed within varieties for discussion on this subject.

Necessary data

- ✓ *Data of variability observed within existing protected varieties*

20. Vegetatively propagated or self-pollinated varieties: Document BMT/6/9 (AFLP analysis of Oilseed Rape inbred line varieties) indicated that AFLP markers could identify all the morphological off-types. Questions are

Considerations for participants

- ✓ *Can the same results be achieved for other species and varieties by an appropriate set of molecular markers?*
- ✓ *Can the standard tolerance level of off-types (determined in UPOV Test Guidelines or document TC/34/5) be applied?*
- ✓ *How can uniformity (off-types) be judged by molecular information?*

[6] BMT/5/5

If the current simple off-type system is not suitable, might the application of a relative tolerance approach as used for cross-pollinated and synthetic varieties be considered for vegetatively propagated or self-pollinated varieties?

21. Cross-pollinated varieties: In this category, uniformity is assessed by relative tolerance limits. If the same principle were used for molecular information, variability within a candidate should be compared with variability observed within existing protected varieties. There might be no methodological difficulties for this approach. One important question is

Considerations for participants

- ✓ *Is there a need for correlation between uniformity observed in traditional characteristics and uniformity observed by molecular data?*[7] *Can varieties judged as non-uniform in conventional characteristics be identified by molecular data?*

B-2-3. Stability

22. The introduction of new characteristics for DUS assessment is associated with new requirements for breeders and maintainers to maintain these characteristics over the protection period. The impact of molecular characteristics on the maintenance practices of breeders/maintainers may need to be considered.

23. Concerns about possible high mutation rates have sometimes been expressed in molecular markers. It has been suggested that, if molecular characteristics are introduced for DUS, stability for molecular characteristics would need to be judged taking into consideration such mutation rates. However, few empirical data are available.

Considerations for participants

- ✓ *Variability in the form of stability of molecular data from different years, seed generations and seed sources*

B-2-4. Choice of Molecular Marker Sets, Observed Bands and Statistical Techniques

24. The BMT has received reports on the influence of the number and choice of molecular markers and observed bands and the choice of statistical techniques. Document BMT/6/12 proposed several criteria of molecular markers for the purpose of variety identification: freely available, highly polymorphic, mapped[8], evenly distributed over the genome, suitable for multiplexing, and easily and reproducibly scored in different laboratories. For the purpose of the DUS assessment, other concerns should be considered. For example, document BMT/6/4 pointed out the significant influence of the choice of molecular markers to variability observed within varieties.

[7] There may be several explanations. For example, different degrees of intra-varietal variability between traditional and molecular characteristics may partially result from the lack of interest in molecular characteristics during the selection/breeding.

[8] for consideration of location with greater risk of mutation as well as for equal distribution over the gene

Considerations for participants

- ✓ *What are necessary criteria for molecular markers for the purpose of DUS assessment (especially in addition to those proposed in BMT/6/12)?*
  e.g., Molecular markers should be usefully polymorphic within the collection of existing protected varieties
  Variability within varieties observed by molecular markers should correspond with level of variability observed by traditional characteristics. For example, for self-pollinated varieties, morphological off-types should be identified by molecular data within varieties.

B-2-5. Variety Description by Molecular Information

25. Another consideration is the preparation of variety descriptions. If highly uniform DNA profiles are observed in a variety, the variety description for molecular characteristics might be made only by presence or absence of each molecular band. However, if this is not the case, variety descriptions for molecular characteristics are more complicated.

Considerations for participants

- ✓ *For example, how can existing protected varieties be described by molecular information?*

B-2-6. Others

26. Information on coding or non-coding portion of DNA: Another question is to which extent molecular bands are linked with functional genes or coding parts of DNA. This information might be useful not only for policy discussion, but also for improving the correlation between molecular information and traditional characteristics. Whilst the majority of microsatellites may appear in the non-coding portion of DNA, AFLP and ISTR for example might contain fragments from the coding portion. Another consideration is molecular bands which occur only in coding parts, but are not clearly linked to expression of traditional characteristics, for example, microsatellites on storage protein genes.

C. Management of Reference Collection

27. Genetic distances based on a set of molecular band information might be used to screen different varieties from the reference collection before conducting the field trial. If screening by molecular techniques is introduced and distinctness is only acceptable on the basis of traditional characteristics, the most important consideration is to **minimize the risk of discarding similar (=non-distinct) varieties, which should be included in the comparative field trial**. One potential solution is to use molecular information where molecular distances are sufficiently correlated with differences in traditional characteristics. The threshold level for screening should then be set at an acceptable risk and be transparent.

28. Correspondence between similar varieties identified by traditional characteristics and by molecular information: The overall conclusion from many studies appears to be that the extent of correlation between distances computed by traditional characteristics and by

molecular information produced by molecular markers is variable[9]. In addition, similar varieties identified by traditional characteristics have not necessarily corresponded well with those identified by molecular marker distances. The question remains:

Considerations for participants

- ✓ *Is it possible or necessary to develop a set of molecular markers which will be able to predict similar varieties (high correlation between genetic distances and distances computed by traditional characteristics)? And how?*

29. Threshold level of screening at an acceptable level of risk: If the threshold level of genetic distances for screening is high, only a limited number of varieties can be screened, but the risk of misjudgment is low. If the threshold level is low, many varieties can be screened, but the risk for screening similar varieties will be high. The usefulness of this approach depends on how many varieties can be screened by molecular data at no or an acceptable level of risk for screening non-distinct varieties. In order to reduce the risk of misjudgment, a system which uses molecular information together with other information (e.g., traditional characteristics) might be considered.

Considerations for participants

- ✓ *For the available molecular marker sets, what are the threshold levels of molecular distance for screening at an acceptable risk? How many varieties could be screened out in these cases?*

30. Reproducibility: Prerequisite for this application is that molecular information can be compared among different laboratories. One possible system is that all DNA profiles of varieties are stored in a computer database and that DNA profiles of a candidate variety will be compared with those in the computer database.

Considerations for participants

- ✓ *Can molecular information be sufficiently reproducible to meet the objective of comparing DNA profiles among different laboratories?*

D. Assessment of Essentially Derivation

31. The use of the analysis of genetic similarity for the assessment of essential derivation has been discussed in the BMT. In particular, **levels of genetic similarity** have been observed between known pairs of essentially derived varieties and non-essentially derived varieties, so that a threshold level of essential derivation could be considered. Because only a few essentially derived varieties have been studied for this purpose, the Subgroups need more data of genetic conformity for EDVs and non-EDVs.

[End of document]

[9]For example, BMT/6/2 reported correlation between morphology vs ISSR and morphology vs ISTR were 0.152 and 0.469 respectively and that the similar varieties chosen by molecular information did not correspond to those by morphology.