E

BMT-TWA/Sugarcane/2/2
ORIGINAL: English
DATE: June 16, 2004

UPOV

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

AD HOC CROP SUBGROUP ON MOLECULAR TECHNIQUES FOR SUGARCANE

Second Session
Poznań, Poland, June 28, 2004

DRAFT GUIDELINES FOR HARMONIZING PROTOCOLS ON THE DEVELOPMENT OF MOLECULAR MARKERS FOR USE IN DUS TESTING WITH A SPECIFIC EMPHASIS ON SUGARCANE

Document prepared by experts from Australia

Introduction

1. Morphological, physiological and phenological characteristics are currently used to determine distinctness, uniformity, and stability (DUS) in the granting of Plant Breeders Rights. Recently however, some proposed uses of molecular markers in facilitating DUS testing have gained acceptance, based on certain conditions and assumptions, "within the terms of the UPOV Convention" (document TWA/31/15). Importantly it was also determined that these proposed uses of molecular markers "would not undermine the effectiveness of protection offered under the UPOV system".

2. This is an important progression for the use of DNA markers in DUS testing and may contribute to the development of improved methods for variety examination, particularly in the management of reference collections. One essential requirement before DNA markers can be implemented to facilitate DUS testing is that the procedure for generating and testing DNA markers is harmonized at an international level and demonstrates their effectiveness and repeatability in this context. The purpose of this document is to propose a basis for developing guidelines to harmonize protocols on DNA marker development for use in DUS testing procedures. The proposed model for these guidelines is outlined in Appendix I.

Assumptions

3. The following assumptions for this model are made:

a) Type of marker to be used
Sequence-tagged microsatellite markers (SSR, microsatellites) are used throughout these guidelines. There are many other marker types available however most of these involve techniques covered by patents and therefore may not necessarily be available to all testing authorities.

b) the model is focussed on vegetatively propagated varieties derived from controlled cross-pollination, using sugarcane as the example. Different models may need to be developed for outcrossing species, and varieties derived from mutation breeding.

4. The model consists of the following 10 steps:

(i) Coordination

5. The BMT recommends a leading expert as overall coordinator of the work. The coordinator may, for example, be identified as the one with most prior experience in implementing DNA markers for that particular crop or plant species.

6. Under the auspices of the BMT, the leading expert identifies other experts willing to participate. It is important that all experts and laboratories with an interest in developing DNA markers for a particular crop or plant species are provided an opportunity to contribute.

7. Harmonizing DNA testing procedures for a species or taxa requires a collaborative effort between a number of laboratories (minimum of three).

(ii) Selecting which varieties to test

8. After consultation with all interested experts including relevant breeders, a selection of varieties or genotypes is chosen to represent a range of genetic diversity so that as many alleles as possible can be revealed with the markers. If no prior information is available on the range of genetic diversity available, an initial study must be conducted to identify suitable germplasm for testing. Some closely related or morphologically similar genotypes are included to determine the discriminative power of the chosen technique.

(iii) Tissue sampling and DNA extractions

9. The type of material to be sampled and the procedure for sampling the material for DNA extraction will, to a large extent, depend on the crop or plant species in question. For example, in some plant species seed will be used as the source of DNA and in other cases DNA will be extracted from leaf material. In the first instance, to minimize the risk of mislabelled varieties, and differences in DNA quality affecting results, DNA extractions from the selected genotypes are conducted in a single laboratory (the primary laboratory) using documented or published procedures for each plant species. DNA quantity and quality will also be estimated by this laboratory. The DNA samples are then diluted to a standard

concentration of, for example, 20 ng/µl. An initial PCR test should be conducted, using the standardised protocols, prior to distribution of DNA aliquots to all other participating laboratories. This will identify any problems before the harmonizing experiments are begun.

(iv) Selection of primers

10. The initial selection of primers should be based on prior knowledge and experience and will take into consideration a number of criteria including:

Primers 'freely' available
Must be highly reproducible within a laboratory
Have a high power of discrimination particularly between closely related genotypes
Generate markers that are easy to score and interpret (i.e. simple profiles with little or no stuttering effect)
Are known to be uniform and stable
Generate markers distributed throughout the genome
If known, markers linked to a morphological characteristic also should be included.

11. If no prior information is available on primer suitability with respect to these criteria, preliminary experiments must be conducted to limit the number of primers to be tested. The aim is to have between 20 and 30 suitable primers for testing in all laboratories; this number is based on the results of Bredemeijer *et al*., (2002) for tomato, and Röder *et al*., (2002) for wheat, and also on the author's experience for sugarcane. The final number of primers selected will be dependent on the plant species being tested.

12. The primary laboratory will synthesise the selected primers from a single supplier to avoid the possibility of different primer quality affecting DNA profiles. Once resuspended, the primers are diluted to a standard concentration and aliquots of all primers are then distributed to the participating laboratories.

(v) PCR Components

13. A standardised set of PCR conditions should be developed based on the experience of the participants. The amount of each PCR component (PCR buffer, $MgCl_2$, dNTP, Primer, Taq polymerase, DNA template) as well as the final reaction volume must be specified. Different conditions also need to be specified for the different detection systems in place in each of the participating laboratories. For example, detection systems can be based on fluorescent or radioactive labelling, both of which require different sets of conditions. The coordinator should develop the specific protocols (including PCR components, cycling conditions, and conditions for fragment separation), after consultation and input from all other participating laboratories.

(vi) PCR Cycling conditions

14. The variables related to PCR cycling conditions include:

Length and temperature of initial denaturation
Number of cycles
Length and temperature of denaturation, annealing*, and extension
Length and temperature of final extension

*Note: Annealing temperature must be specified for each primer pair used in the study.

15. These variables should all be specified and adhered to by all participating laboratories.

(vii) Separation of markers

16. In the first instance PCR products should be loaded onto the detection system in the same sequence in all laboratories, for ease of comparison and interpretation between laboratories.

(viii) Documentation and interpretation

17. The results generated within each laboratory are sent to the coordinator for documentation and interpretation (comparison??). Where radioactive detection is used, it is preferable that the autoradiography film is sent to the coordinator rather than scanned images, as the quality of scanned images can be variable and sometimes difficult to read. For each primer pair, a minimum and maximum size range for scoring markers should be established. In the case where a gel-based system is used for revelation of markers, a suitable size ladder should be used to simplify interpretation of results between laboratories.

18. The results are scored by two experienced people independently. Any discrepancies in the data or in the interpretation of the data should be rechecked, and if any differences between laboratories are identified, the tests should be repeated in those laboratories.

19. The final selection of markers is made based on their reproducibility between laboratories and detection systems, and ease of interpretation. With respect to the latter, results are much clearer when primers generate fewer, well-separated markers with little or no stuttering effects; primers that generate many markers, particularly when there is stuttering, and overlap between markers and stuttering are often difficult to interpret. As a minimum, before a set of DNA markers are accepted, they must have a discriminative power not less than the existing methods of examination.

(ix) Data storage

20. The marker data should be entered in a database to allow for secure storage and retrieval of information. The database should be expanded with marker data of the most common varieties for that species.

(x) Further tests

21. A blind ring test should be performed to validate the effectiveness of the system in identifying varieties in different laboratories. A set of varieties for each selected primer should be established such that all alleles are revealed for each primer. These varieties can then be run as “example varieties” when testing is conducted in different laboratories independently of this harmonization procedure. This will function as a control measure for refining and improving the procedure if necessary.

22. The outcome of the blind ring test should be that the same decisions are made in all laboratories. If the same decisions are not made in all laboratories, some primers may need to be excluded, and the tests repeated in all laboratories using a different set of primers. A report

is then submitted through the BMT to the TC seeking adoption for the use of the selected markers.

23. Consideration must also be given to developing the relationship between the “distances” measured based on morphological and molecular characteristics. For example the TC could be invited to consider proposals for an Option 1 (i.e. for makers directly linked to a trait) or an Option 2 (i.e. for markers used in managing reference collections) approach for introducing the molecular technique in DUS testing.

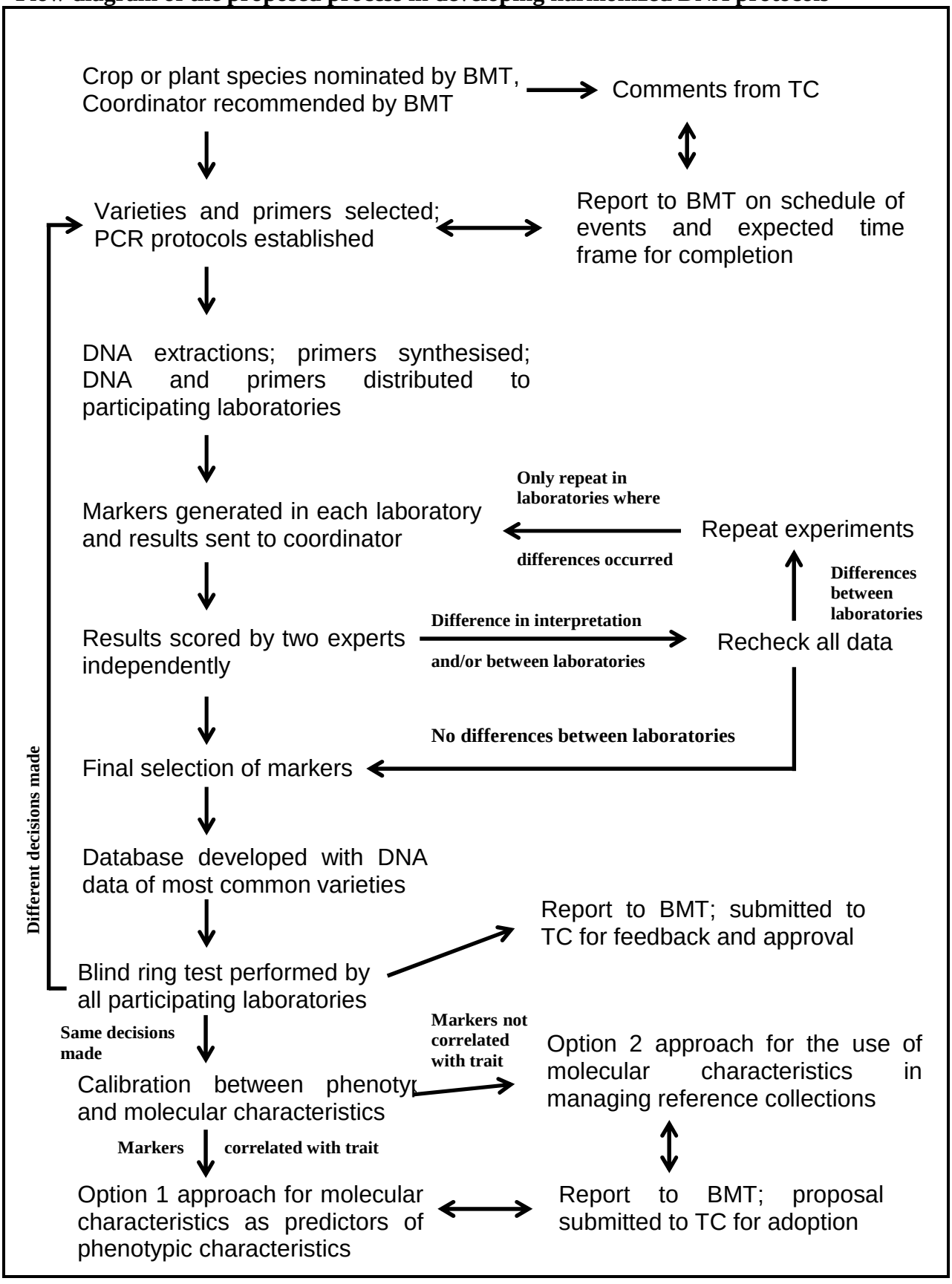
24. As new varieties are developed, these too can be tested, added to the database and the information used to identify the most similar varieties for comparative testing. Detailed protocols on database development, data entry and analytical procedures will also need to be developed, in close association with the Technical Working Party on Automation and Computer Programs.

References:

G. Bredemeijer, R. Cooke, M. Ganal, R. Peeters, P. Isaac, Y. Noordijk, S. Rendell, J. Jackson, M. Röder, K. Wendehake, M. Dijcks, M. Amelaine, V. Wickaert, L. Bertrand, B. Vosman. (2002) Construction and testing of a microsatellite database containing more than 500 tomato varieties. Theor. Appl. Genet. 105:1019-1026.

M. Röder, K. Wendehake, V. Korzun, G. Bredemeijer, D. Laborie, L. Bertrand, P. Isaac, S. Rendell, J. Jackson, R. Cooke, B. Vosman, M. Ganal. (2002) Construction and analysis of a microsatellite-based database of European wheat varieties. Theor. Appl. Genet. 106:67-73.

Flow diagram of the proposed process in developing harmonized DNA protocols



[End of document]