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INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

TECHNICAL COMMITTEE

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MOLECULAR TECHNIQUES

Document prepared by the Office of the Union

1. At its thirty-ninth session held in Geneva from April 7 to 9, 2003, the Technical Committee (TC) discussed a proposal from the Technical Working Party for Fruit Crops (TWF) for the preparation of a document on the possible use of molecular markers in the DUS examination. It was agreed that the Office of the Union, in conjunction with the Chairmen of the TC and the Working Group on Biochemical and Molecular Techniques, and DNA-Profiling in Particular (BMT), would use existing documents and, in particular document TC/38/14 Add.-CAJ/45/5 Add., to develop a summary of the current position, which would be considered by the TC at its fortieth session to be held in spring 2004. The TC would then consider whether to invite the Administrative and Legal Committee (CAJ) to examine the document.

2. The Annex to this document contains a draft document on the possible use of molecular markers in the DUS examination, which has been prepared in accordance with the request of the TC.

3. The TC is invited to:

(a) comment on the Annex to this document;

(b) consider whether to invite the CAJ to examine the document; and

(c) agree whether the document can be used as a summary of the current UPOV position.

[Annex follows]

ANNEX

Situation in UPOV Concerning
the Possible Use of Molecular Markers in DUS Examination

1. INTRODUCTION

The purpose of this document is to explain the situation in UPOV with regard to the possibility of using molecular markers in the examination of distinctness, uniformity and stability (DUS). The document starts by explaining the requirements for the DUS examination, followed by a brief overview of relevant molecular techniques and concludes by explaining the current position within UPOV concerning the possible use of molecular markers in the DUS examination.

2. THE DUS EXAMINATION

2.1 General Introduction

2.1.1 According to the UPOV Convention, protection can only be granted in respect of a new plant variety after examination of the variety has shown that it complies with the requirements for protection laid down in the Convention and, in particular, that the variety is distinct (D) from any other variety whose existence is a matter of common knowledge at the time of the filing of the application (hereinafter referred to as a "variety of common knowledge") and that it is sufficiently uniform (U) and stable (S), or "DUS" in short. The examination, or "DUS Test," is based mainly on growing tests, carried out by the authority competent for granting plant breeders' rights or by separate institutions, such as public research institutes, acting on behalf of that authority or, in some cases, on the basis of growing tests carried out by the breeder[1]. The examination generates a description of the variety, using its relevant characteristics (e.g. plant height, leaf shape, time of flowering), by which it can be defined as a variety in terms of Article 1(vi) of the 1991 Act of the Convention.

2.1.2 The General Introduction (document TG/1/3), and the associated series of documents specifying Test Guidelines' Procedures (the TGP documents) set out the principles which are used in the examination of DUS. The identification of those principles ensures that examination of new plant varieties is conducted in a harmonized way throughout the members of the Union[2]. This harmonization is important because it facilitates cooperation in DUS testing and also helps to provide effective protection through the development of harmonized, internationally recognized descriptions of protected varieties.

2.1.3 In addition, UPOV has developed "Guidelines for the Conduct of Tests for Distinctness, Uniformity and Stability," or "Test Guidelines," for many individual species or other variety

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[1] Reference in this document to the term "breeder" should be understood as defined in Article 1(iv) of the 1991 Act of the UPOV Convention, i.e.

"– the person who bred, or discovered and developed, a variety,
– the person who is the employer of the aforementioned person or who has commissioned the latter's work, where the laws of the relevant Contracting Party so provide, or
– the successor in title of the first or second aforementioned person, as the case may be"

[2] The term "member of the Union" means a State party to the Act of 1961/1972 or the Act of 1978, or a Contracting Party to the 1991 Act.

groupings. The purpose of these Test Guidelines is to elaborate certain of the principles contained in the General Introduction, and the associated TGP documents, into detailed practical guidance for the harmonized examination of DUS and, in particular, to identify appropriate characteristics for the examination of DUS and production of harmonized variety descriptions.

2.2 Characteristics as the Basis for Examination of DUS

2.2.1 For any variety to be capable of protection it must first be clearly defined. Only after a variety has been defined can it be finally examined for fulfillment of the DUS criteria required for protection. All Acts of the UPOV Convention have established that a variety is defined by its characteristics and that those characteristics are therefore the basis on which a variety can be examined for DUS.

2.2.2 The 1991 Act of the UPOV Convention makes this clear by stating in Article 1(vi) that a variety is a plant grouping that can be "defined by the expression of the characteristics resulting from a given genotype or combination of genotypes" and can be "distinguished from any other plant grouping by the expression of at least one of the said characteristics."

2.2.3 In addition to their use in defining a variety, characteristics are the basis for examining distinctness, uniformity and stability.

2.2.4 The basic requirements that a characteristic should fulfill before it is used for DUS testing or producing a variety description are that its expression:

(a) results from a given genotype or combination of genotypes;

(b) is sufficiently consistent and repeatable in a particular environment;

(c) exhibits sufficient variation between varieties to be able to establish distinctness;

(d) is capable of precise definition and recognition;

(e) allows uniformity requirements to be fulfilled;

(f) allows stability requirements to be fulfilled, meaning that it produces consistent and repeatable results after repeated propagation or, where appropriate, at the end of each cycle of propagation.

2.2.5 It should be noted that there is *no* requirement for a characteristic to have any intrinsic commercial value or merit. However, if a characteristic that is of commercial value or merit satisfies all the criteria for inclusion it may be considered in the normal way.

2.2.6 For inclusion in the Test Guidelines, further criteria are set out in the General Introduction section 4.8, "Functional Categorization of Characteristics" and in document TGP/7, "Development of Test Guidelines." The characteristics included in the individual Test Guidelines are not necessarily exhaustive and may be expanded with additional characteristics if that proves to be useful and the characteristics meet the conditions set out above.

3. MOLECULAR TECHNIQUES

3.1 The Plant Genome

3.1.1 Plant DNA is located in the nucleus and the organelles (the chloroplast and the mitochondria). The nucleus comprises around 10^9 base pairs (bp), compared to only around 150 kb (150,000 bp) for the chloroplast and 220-2,500kb for the mitochondria. The chloroplast and mitochondrial DNA are highly conserved and code for a relatively small number of genes.

3.1.2 The average gene accounts for around 4 kb. However, less than 2% of the DNA in the nucleus is in the form of genes coding for cell products and the average number of such "coding" genes is around 15,000 to 50,000. The remaining 98% of the DNA is in the form of non-coding DNA sequences. This non-coding DNA may be in the form of repetitive DNA sequences, either as tandem repeats (repeated sequences, one after the other) or dispersed repeats (repeated sequences dispersed throughout the genome). The tandem repeats of non-coding DNA are known as "satellite" DNA.

3.1.3 Most genes are present only once in the genome and are known as "single copy genes." In the case of diploid plants, chromosomes are present as homologous pairs with each chromosome containing its version of the gene, known as an "allele." If the two versions of the gene, i.e. alleles, are the same the plant is "homozygous" for that gene, but if the alleles are different, the plant is "heterozygous" for that gene.

3.2 Polymorphism

3.2.1 Variations in DNA (polymorphisms) can be observed by cutting, or "digesting" the DNA with restriction enzymes. Restriction enzymes are able to recognize particular sequences of 4 to 6 nucleotides (restriction sites) and cut the DNA inside or near these particular sequences. Any mutation occurring at these restriction sites will render the enzyme unable to recognize and, therefore, to cut the sequence. Thus, the restriction sites will differ and different plants can yield restriction fragments (fragments of DNA obtained after the action of the restriction enzyme) with different sizes.

3.2.2 Electrophoresis can be used to separate restriction fragments on a gel according to their sizes and thereby give patterns specific to each different DNA. However, nuclear DNA yields hundreds of thousands of bands of every size obtained after digestion producing a smear on the gel. In the RFLP (Restriction Fragment Length Polymorphism) method, the fact that complementary DNA strands spontaneously associate with each other, is exploited. A "probe," consisting of a particular sequence of DNA, is added to the gel and left to associate (hybridize) with the matching sequence in the smear. If the probe has been radioactively or biochemically labeled prior to the hybridization, it will be possible to locate the particular sequence of DNA in the gel. The probes can be of different types: genomic DNA (gDNA), complementary DNA (cDNA) or synthetic DNA sequences. The probes can be used to investigate a single locus (site of a gene on a chromosome) or many loci.

3.2.3 The polymorphism observed with monolocus probes arises mostly from mutations in the restriction sites leading to differences in the length of the restriction fragments. Conversely, multilocus probes, which are generally satellite DNA, reveal another type of variability which is due to differences in the number of repetitions of the particular sequence of DNA investigated. Compared to monolocus probes, multilocus probes yield more complex patterns. They provide

more information per gel, are codominant (i.e. all alleles are expressed) and are inherited in a Mendelian way.

3.2.4 Another approach involves a technique known as Polymerase Chain Reaction (PCR) whereby specific portions of the genome are amplified by the polymerase enzyme and can then be visualized on gels. This technique requires, firstly, that the DNA sequence of the two extremities of the specific portion of DNA are known and, secondly, in order that the polymerase can amplify the portion of DNA, two complementary sequences (primers) for the two extremities must be developed – an "on" switch and an "off" switch.

3.2.5 Polymorphism in the amplification products may arise either from a mutation in the sequence hybridizing with the primer or from a mutation between the two primers.

3.2.6 Some PCR-based methods (e.g. Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP)) require no preliminary information about the DNA to be amplified. Two random sequences of 10 to 20 bases are used as primers. If complementary sequences exist in the genome and if they are not too far from each other, the stretch of DNA between the primers is amplified. Sometimes numerous complementary sequences are found so that electrophoresis of the amplified fragments yields a "fingerprint" which can be highly polymorphic.

3.2.7 PCR technology is also used for investigating the polymorphism of "microsatellites" by using "microsatellite markers." Microsatellites are short sequences of 2-5 bases repeated multiple times and flanked by unique DNA. Polymorphism is generally detected as a length difference in the amplified sequence. The length difference may be very small, for example two base pairs.

3.2.8 The most common type of genetic variations are single nucleotide polymorphisms (SNPs), which are mutations producing a change at a single base in a DNA molecule. For example, a SNP might change the DNA sequence ATCTG to ACCTG. SNPs can be detected using high throughput screening methods such as "gene chips" or microarrays. In such methods, several different DNA sequences are placed on a matrix (e.g. glass) and exposed to samples of plant DNA. Complementary DNA sequences, if present in the plant DNA, will hybridize to a particular sequence and can be observed by, for example, fluorescence.

3.2.9 The merits of the techniques mentioned above are influenced by the context in which the techniques are to be used. The following section considers the use of molecular techniques in the DUS examination.

4. CONSIDERATION OF THE USE OF MOLECULAR TECHNIQUES IN THE DUS EXAMINATION

4.1 Issues to be Considered

4.1.1 UPOV has followed the developments in molecular techniques and has considered what role, if any, such techniques could have in the DUS examination. As a starting point in its consideration, it was recognized that any new methods would need to be consistent with the UPOV Convention. Furthermore, it was recognized that the current methods of DUS examination were very effective and ensured that the protection offered by the UPOV system was of high value. Therefore, it did not wish to introduce new methods which would have a negative impact on the strength of protection compared to that provided by current examination methods and thereby undermine the effectiveness of protection offered under the UPOV system. In this respect it was noted that molecular techniques could detect very small differences at the DNA level which may not be reflected in Test Guidelines characteristics. Thus, it was noted that varieties which would be found not to be distinct using existing Test Guidelines characteristics might be considered distinct if molecular techniques were used.

4.1.2 Nevertheless, UPOV recognized that, as with any new techniques, it was important to consider possible advantages in using such techniques in the DUS examination if the concerns could be addressed in a satisfactory way. In particular, it was apparent that the techniques were very rapid and were, perhaps, less influenced by the growing environment. At the same time as considering the possibilities for the techniques, it was also recognized that, as with all DUS examination methods, it was essential to examine the reliability of the techniques at the technical level and to ensure that any methods would be developed in a harmonized way.

4.1.3 Thus, it is apparent that there are issues to be considered at both the technical and administrative and legal level. The following section explains how UPOV addresses these different issues.

4.2 Procedure for Considering Molecular Techniques

4.2.1 As a first step in the consideration of the various issues concerned with molecular techniques, UPOV established the Working Group on Biochemical and Molecular Techniques, and DNA-Profiling in Particular (BMT). The BMT is a group open to DUS experts, biochemical and molecular specialists and plant breeders, and has the role of considering technical aspects of molecular techniques as set out in Appendix 1. In addition, *Ad hoc* Crop Subgroups (Crop Subgroups) have been developed to consider developments at the crop specific level. The role of the crop subgroups is to prepare documents and formulate proposals which could be a basis for further discussions in the BMT, the Technical Working Parties and the TC.

4.2.2 In order to ensure that any developments concerning the possible use of molecular techniques are considered with regard to their wider implications for the UPOV system of plant variety protection, UPOV has established the *Ad Hoc* Subgroup of Technical and Legal Experts on Biochemical and Molecular Techniques ("BMT Review Group"). The role of the BMT Review Group is to assess possible application models proposed by the TC, on the basis of the work of the BMT and Crop Subgroups, for the utilization of biochemical and molecular techniques in the examination of Distinctness, Uniformity and Stability in relation to the following:

(a) conformity with the UPOV Convention, and

(b) potential impact on the strength of protection compared to that provided by current examination methods and advise if this could undermine the effectiveness of protection offered under the UPOV system.

4.2.3 The BMT Review Group reports its assessment, as set out above, to the Administrative and Legal Committee and the TC, but this assessment is not binding for the position of those Committees.

4.3 Current Status of Molecular Techniques

4.3.1 Proposals considered by the BMT Review Group

At the request of the TC, the following options, developed by the Crop Subgroups and the BMT, have been considered by the BMT Review Group on the basis of detailed proposals presented by the relevant member of the Union as presented in Appendix 2 to this document:

Option 1: Molecular characteristics as a predictor of traditional characteristics

(a) Use of molecular characteristics which are directly linked to traditional characteristics (gene specific markers)

Option 2: Calibration of threshold levels for molecular characteristics against the minimum distance in traditional characteristics

Option 3: Development of a new system

4.3.2 Recommendations of the BMT Review Group

4.3.2.1 The BMT Review Group concluded as follows:

The proposal under Option 1(a) (Gene specific marker of a phenotypic characteristic) was, on the basis of the assumptions in the proposal, acceptable within the terms of the UPOV Convention and would not undermine the effectiveness of protection offered under the UPOV system.

The proposal under Option 2 (Calibration of threshold levels for molecular characteristics against the minimum distance in traditional characteristics for Oilseed Rape, Maize and Rose, respectively), where used for the management of reference collections was, on the basis of the assumptions in the proposal, acceptable within the terms of the UPOV Convention and would not undermine the effectiveness of protection offered under the UPOV system.

Regarding the proposals under Option 3 for Rose and for Wheat, it noted there was no consensus on the acceptability of these proposals within the terms of the UPOV Convention and no consensus on whether they would undermine the effectiveness of protection offered under the UPOV system. Concerns were raised that, in these proposals, using this approach, it might be possible to use a limitless number of markers to find differences between varieties. The concern was also raised that differences would be found at the genetic level which were not reflected in morphological characteristics.

4.3.2.2 The following general remarks were also made. Firstly, concern was raised regarding the accessibility of techniques covered by patents. Secondly, the BMT Review Group emphasized the importance of considering if there were cost benefits arising from any new approaches. Thirdly, the importance of the relationship between phenotypic characteristics and molecular techniques was discussed. Finally, the importance of examining uniformity and stability on the same characteristics as used for distinctness was emphasized.

4.3.3 Opinion of the TC and the CAJ regarding the recommendations of the BMT Review Group

4.3.3.1 The TC considered the conclusions of the BMT Review Group and agreed with those conclusions, namely that proposals under Options 1(a) and 2 could be pursued on the basis of the assumptions, whilst recognizing the need for further work to examine these assumptions and, in the case of the Option 2 proposal, to improve the relationship between morphological and molecular distances. It also noted the divergence of views which had been expressed regarding the proposals under Option 3.

4.3.3.2 The CAJ agreed with the conclusions of the BMT Review Group and endorsed the opinion of the TC.

4.4 Ongoing Developments

4.4.1 Section 4.3 sets out the current position within UPOV concerning molecular techniques. However, the situation is under continual review in the light of ongoing developments concerning molecular techniques and the need to develop suitable molecular techniques within the current position. In particular, the ongoing work can be summarized as follows:

(a) Development of advanced proposals under Option 1 (a), in which the assumptions have been evaluated and issues of cost, accessibility and uniformity and stability have been addressed. Such advanced proposals to be considered by the relevant Crop Subgroup, the BMT Review Group, the TC and the CAJ;

(b) Development of advanced proposals under Option 2, in which the assumptions have been evaluated, issues of cost, accessibility and uniformity and stability have been addressed and the relationship between morphological and molecular distances has been improved. Such advanced proposals to be considered by the relevant Crop Subgroup, the BMT Review Group, the TC and the CAJ;

(c) Consideration of new proposals under Option 1, Option 2, or Option 3, by the BMT, the relevant Crop Subgroup, the BMT Review Group, the TC and the CAJ;

(d) The Crop Subgroups to continue to consider developments at the crop specific level, with the establishment of new Crop Subgroups according to need; and

(e) The BMT to continue to monitor developments in molecular techniques and to develop guidelines and facilitate harmonization concerning the use of molecular techniques.

4.4.2 This document will be updated to reflect any substantial developments.

[Appendix 1 follows]

APPENDIX 1

Working Group on Biochemical and Molecular Techniques, and DNA-Profiling in Particular (BMT)

The BMT is a group open to DUS experts, biochemical and molecular specialists and plant breeders, whose role is to:

(i) Review general developments in biochemical and molecular techniques;

(ii) Maintain an awareness of relevant applications of biochemical and molecular techniques in plant breeding;

(iii) Consider the possible application of biochemical and molecular techniques in DUS testing and report its considerations to theTC ;

(iv) If appropriate, establish guidelines for biochemical and molecular methodologies and their harmonization and, in particular, contribute to the preparation of document TGP/15, "New Types of Characteristics." These guidelines to be developed in conjunction with the Technical Working Parties;

(v) Consider initiatives from TWPs, for the establishment of crop specific subgroups, taking into account available information and the need for biochemical and molecular methods;

(vi) Develop guidelines regarding the management and harmonization of databases of biochemical and molecular information, in conjunction with theTWC ;

(vii) Receive reports from Crop Subgroups and the BMT Review Group;

(viii) Provide a forum for discussion on the use of biochemical and molecular techniques in the consideration of essential derivation and variety identification.

[Appendix 2 follows]

APPENDIX 2

Option 1: Molecular characteristics as a predictor of traditional characteristics

(a) Use of molecular characteristics which are directly linked to traditional characteristics (gene specific markers)

PROPOSAL

prepared by experts from France

Gene Specific Marker for Herbicide Tolerance Introduced by Genetic Modification

Proposal

1. A variety is genetically modified by the insertion of a gene for tolerance to herbicide "Formula X." Varieties containing this gene are not harmed when sprayed with Formula X; however, varieties without this gene are always killed if sprayed with this particular herbicide. Tolerance of Formula X, examined in field trials by spraying of plots, is an accepted DUS characteristic, and it can be used to establish distinctness between varieties.

2. It is proposed that, rather than spraying varieties in the field (this is difficult to organize in the standard DUS trial), the characteristic "tolerance of Formula X" is examined by conducting a test for the presence of a molecular marker *linked* to the gene. This marker is located on a part of the gene "construct." The gene "construct" comprises all the elements which are inserted into the plant during the genetic modification and, in addition to the gene itself, contains additional elements for regulating the gene when in the plant. The marker may be located within the gene, partly on the gene or outside the gene itself.

Assumptions to be made in the Proposal

3. The following assumptions are made:

(a) The DUS Examination

It is assumed that the test for the marker would be conducted to the same extent as for the field test, i.e. the same number of individual plants, over the same number of years and with the same criteria for distinctness, uniformity and stability.

(b) Reliability of the Linkage

It is assumed that the link between the marker and the gene would be checked to ensure that the marker is a reliable predictor of tolerance to Formula X. This check would be necessary to ensure, for example, that the marker does not become separated from the gene and that the presence of the gene is still resulting in tolerance to Formula X.

(c) Development of Different Molecular Markers for the Same Gene

It would be possible to develop different gene constructs containing Formula X tolerance and to identify separate molecular markers for these individual gene constructs, all of which would be linked to exactly the same gene for Formula X tolerance. If all the different markers for the same gene were accepted as different methods for examining the *same existing phenotypic characteristic*, the consideration of the approach would be the same. Under Option 1, "Molecular characteristics as a predictor of traditional characteristics," it is necessary to work on the basis that the markers correspond to a traditional, i.e. existing, approved characteristic. Therefore, it is assumed that different markers for the same gene would be treated as different methods for examining the same characteristic, i.e. tolerance to Formula X.

(d) Different Genes Producing Tolerance to the Same Herbicide

It might be possible to develop different genes which confer tolerance to Formula X. In the simplest case, this could be considered in the same way as different markers for the same gene, i.e. the different genes, with their respective markers, would be considered as different methods for examining the same characteristic, i.e. tolerance to Formula X. However, the different genes are likely to have a different chemical mechanism to produce the tolerance to Formula X. Thus, the chemicals produced from the different genes will be different and, these different chemicals might be a basis for establishing distinctness in some circumstances. Nevertheless, under Option 1, it would first be necessary to approve the chemical components as UPOV characteristics, before accepting molecular markers linked to these potential characteristics. This in turn would be a separate proposal. Therefore, it is assumed that different genes would be treated as different methods for examining the same characteristic, i.e. tolerance to Formula X.

(e) Different Gene Constructs Producing the Same Herbicide Tolerance but With Different Control of the Expression

It is also possible that different gene constructs could be developed which contain the same gene for tolerance to Formula X, but which had different regulatory control. For example, the regulatory elements may result in the Formula X tolerance only being switched on at certain stages of development. For simplicity, in considering this proposal, it is assumed that the different markers linked to different regulatory elements for the same gene would all be treated as different methods for examining the same characteristic of tolerance to Formula X. However, it is also assumed that further consideration would be given to this matter at a later stage.

Potential Impact

4. In the basic proposal and on the basis of the assumptions made in section 3(a) to (e), it would appear that the potential impact on the strength of protection compared to that provided by the "current" examination method (i.e. the field test for tolerance to Formula X) should be nil, because the results of the DUS examination would always be the same regardless of whether the field test or test for the marker was used.

Option 2: Calibration of threshold levels for molecular characteristics against the minimum distance in traditional characteristics

PROPOSAL

prepared by experts from France

("OPTION 2" for Maize, Oilseed Rape and Rose)

Proposal

1. Option 2 is based on a calibration of threshold levels for molecular characteristics against threshold levels in traditional characteristics, principally based on information obtained in France on Maize, Oilseed Rape and Rose. In this particular proposal, the threshold levels in the traditional characteristics are based on an overall distance assessment, rather than a characteristic-by-characteristic approach and the application of the proposal is in the "management of reference collections." In this context, the term "management of reference collections" encompasses, in particular, the selection of varieties of common knowledge that can be excluded from the growing trial used for examination of distinctness, on the basis of comparing harmonized descriptions. A key feature of the process of eliminating varieties of common knowledge prior to the growing trial is that the threshold for deciding which varieties can be safely excluded (i.e. are distinct on the basis of descriptions), can be set with a suitable margin of safety, because those varieties which are not eliminated, but which are actually distinct, will be discovered in the growing trial. This threshold, with a safety margin, is termed the "Distinctness plus" threshold in this paper. In this proposal, the aim is to develop a Distinctness plus threshold for molecular characteristics.

Measuring distance in traditional characteristics

2. The first step is to consider how to measure the distance between varieties using traditional characteristics. This proposal is based on the use of an approach, using the GAÏA computer software, developed by France. This approach works by estimating the phenotypical difference between two varieties, based on the addition of the differences observed for the different characteristics. Each difference observed is weighted by the crop expert according to the value of the difference and to the reliability of each characteristic.

Measuring differences in molecular characteristics

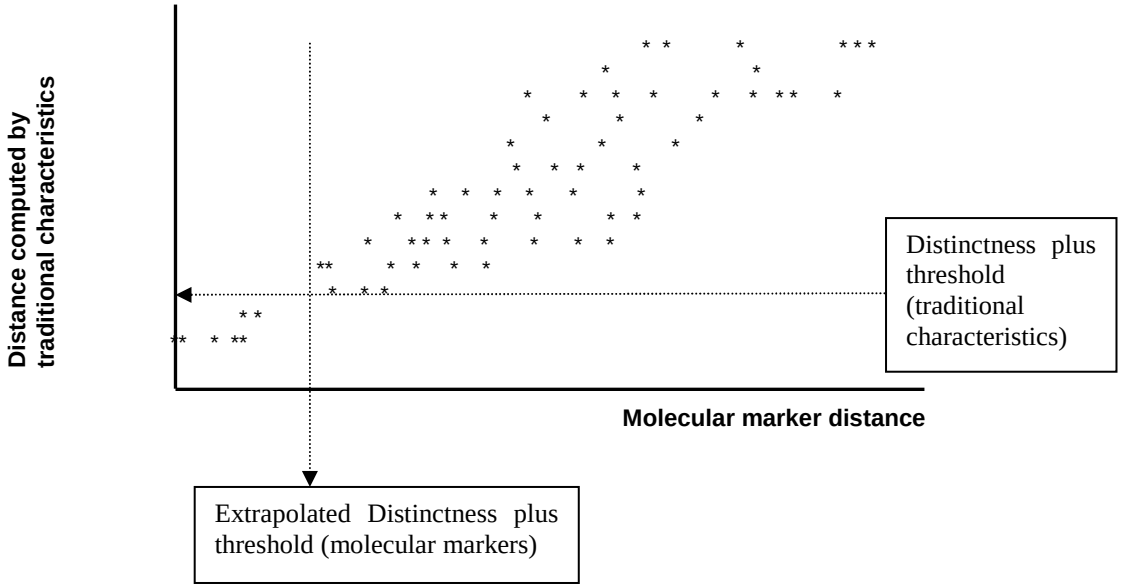
3. The difference between varieties on the basis of information from molecular markers is calculated, in this option, by the use of Rogers'[3] distances.

Calibrating threshold levels for molecular characteristics against the minimum distance in traditional characteristics

[3] Rogers, J.S., 1972: Measures of similarity and genetic distance. Stud. Genet. VII. University of Texas Publications, 7213: 145-153

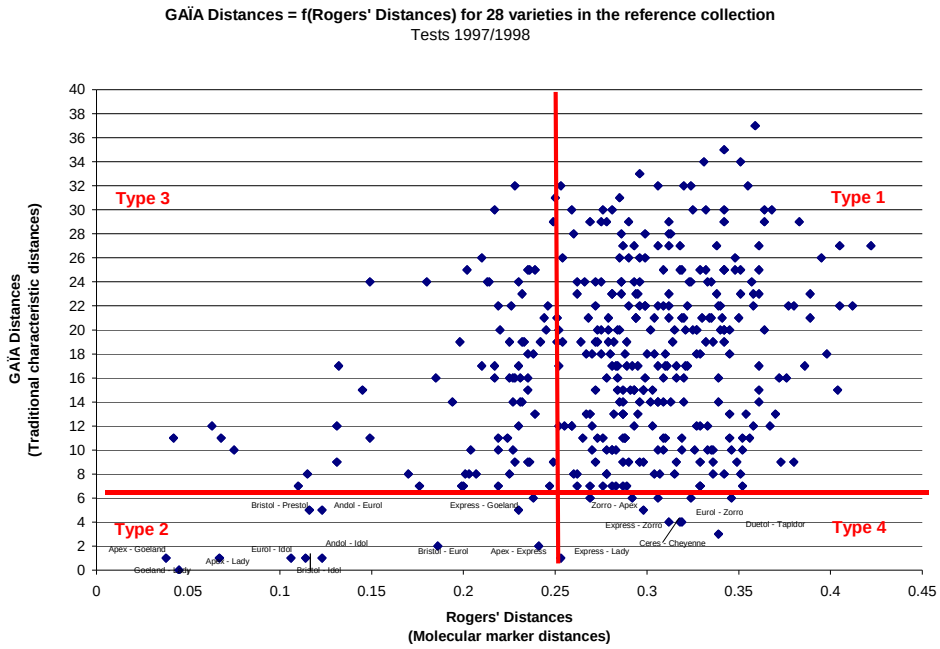
4. The calibration of threshold levels for differences in molecular characteristics against differences in traditional characteristics would be straightforward if there was a strong correlation between these two ways of measuring the differences between varieties. In such a situation, a graph of the different methods would look like figure 1. The threshold for Distinctness plus in molecular markers could be extrapolated from the Distinctness plus threshold in traditional characteristics in such a way that the same decisions would be made, regardless of which method of assessing variety differences was used.

FIGURE 1



5. However, in the case of Oilseed Rape, the correlation is less good, as illustrated in figure 2. It can be seen that, wherever the Distinctness plus threshold is set for the molecular markers, there would be some varieties with different decisions according to the method used for calculating the differences. The implications of this situation are explored in the section "Potential Impact."

FIGURE 2



Assumptions to be Made in the Proposal

6. The following assumptions are made:

(a) Uniformity and Stability

The uniformity and stability requirements for the molecular markers have not been developed in this proposal. However, the available information suggests that variability for molecular characteristics within varieties seems to be higher than that observed in traditional characteristics. It is assumed that the differences calculated between varieties on the basis of molecular markers fully take into account the variation within varieties. Furthermore, it is assumed that suitable uniformity standards could be developed for molecular markers without requiring varieties, in general, to be more uniform. This assumption is on the basis that molecular markers would be used for the establishment of a "Distinctness plus" threshold, based on genetic distance, in the management of reference collections and not for the judgement of distinctness on a characteristic by characteristic approach.

(b) Application of the Proposal

As explained in the Introduction, this proposal is made on the basis that it would only be used for the establishment of a "Distinctness plus" threshold in the management of reference collections.

(c) Reliability of the Techniques

It is assumed that the techniques would meet all the normal requirements for any characteristic to be used in the DUS examination and, in particular, would be checked to ensure they are sufficiently consistent and repeatable.

Potential Impact

7. The graph provided in figure 2 highlights the possible ways in which this proposal could have an impact on the strength of protection. In summary, the situation can be represented as follows:

	Distinctness plus (Traditional characteristics)	Distinctness plus (Molecular markers)
Type 1	Yes	Yes
Type 2	No	No
Type 3	Yes	No
Type 4	No	Yes

8. Types 1 and 2 outcomes would have no impact on the strength of protection because the result is the same for both methods used.

9. Type 3 outcomes would also have no impact on the strength of protection because the varieties would be discovered to be distinct using traditional characteristics in the growing trial.

10. Type 4 outcomes could have an impact on the strength of protection because they could result in varieties being considered to be distinct which would not have previously been considered to be distinct. Determining whether type 4 outcomes could undermine the effectiveness of protection offered under the UPOV system would require an analysis of such cases.

11. At present, type 4 cases are known in oilseed rape (examples can be provided). However, these cases only relate to pairs of varieties which were found to be distinct in a growing trial. The situation in which different decisions on distinctness would result can only be investigated where varieties are rejected for distinctness in the growing trial. This would require analysis of pairs of varieties rejected for distinctness in the past or, if such material is unavailable, a system of “parallel running” of the two systems in real time on candidate varieties. It would then be possible to discover if any such cases would occur and if these would undermine the effectiveness of protection. If it was considered that these cases would undermine the effectiveness of protection it could then be decided if a sufficiently high threshold could be set to eliminate these cases without losing the benefit of the approach for the management of reference collections.

12. It should be recognized that the case studies, envisaged in paragraphs 10 and 11, may not provide a complete assessment of the potential impact, since breeders would be operating under the existing system of DUS examination. Consideration should also be given, for example, to whether it would be easier under the proposed new system, if accepted, for new varieties to be selected from entirely within existing protected varieties. If this was the case, it could encourage “breeders” to try to select new varieties in this way, whereas, under the existing system there would be no incentive to do so because the varieties would not be considered distinct. This situation might be more likely to occur if the uniformity criteria for molecular markers was lower than for traditional characteristics.

Option 3: Development of a New System

PROPOSAL

prepared by experts from the Netherlands

("OPTION 3" for Rose)

Proposal

1. The basis of this proposal is that a set of molecular characteristics would be used in the same way as existing non-molecular characteristics.

2. A study of 76 rose varieties has shown that all these varieties, except for mutant variety pairs, could be distinguished using a limited number of molecular markers. Furthermore, when the individual plants of a number of varieties were examined they were all found to be uniform. The STMS ("sequence tagged micro-satellite") markers concerned seek certain repeat sequences in the plant DNA. At these marker sites, the plant DNA is amplified and the resultant fragments are run on a gel, which produces a set of bands or peaks corresponding to each fragment. Different banding or peak patterns resulting from the same markers indicate differences in the marker sites. It should be noted that it is unlikely that these sequences are linked with any existing Test Guidelines' characteristics and should be thought of as indicators of structural differences in the plant DNA.

3. The uniformity of the banding pattern for all the plants within a variety means that it would be possible to distinguish varieties on the basis of a single band difference. However, such a difference could result from a single mutation**,** i.e. by chance. For this reason, it is proposed that varieties would be considered to be clearly distinguishable only if there were three band/peak differences between varieties.

4. The following scheme is proposed:

Step 1: Use a fixed set of seven STMS markers (Set 1) to examine two plants of the candidate variety to see if it is clearly distinguishable from all other varieties.

If the candidate variety has at least 3 band/peak differences from all other varieties, using this first set of markers, it would be considered to be distinct. It would then be grown in a field trial to examine uniformity and stability for the relevant non molecular characteristics. In other cases, or where there are missing values, it would proceed to step 2.

Step 2: If the candidate variety is not considered distinct using the Set 1 markers, it is tested with a second, different set of seven STMS markers (Set 2).

If the candidate variety has at least 3 band/peak differences from all other varieties, using both sets of markers combined, it would be considered to be distinct. It would then be grown in a field trial to examine uniformity and stability for the relevant non-molecular characteristics. In other cases, or where there are missing values for more than one marker set, it would proceed to step 3.

Step 3: If the candidate variety was not considered to be distinct using both sets of markers, it is likely that it would be an existing variety or genetically very similar to an existing variety, e.g. resulting from a mutation. Such candidate varieties would be included in the growing trial to examine distinctness, as well as uniformity and stability, using non-molecular characteristics.

Assumptions to be Made in the Proposal

5. The following assumptions are made:

(a) The DUS Examination

It is assumed that the field examination would be conducted on the same number of plants as now. Only two plants would be necessary for the STMS marker examination because any variant plants would be seen in the subsequent field examination. This can be assumed because the chance of a mutation occurring in a marker site and not being seen in the non molecular characteristics is extremely small.

(b) Reliability of the Techniques

It is assumed that the STMS markers would meet all the normal requirements for any characteristic to be used in the DUS examination and, in particular, would be checked to ensure they are sufficiently consistent and repeatable.

(c) Uniformity

It is assumed that the situation found in the initial study, regarding the uniformity of the existing varieties, would be consistent when examined throughout the entire variety collection, or that there would be only very occasional single band differences within the varieties.

Potential Impact

6. The way in which this proposal could have a potential impact on the strength of protection is if varieties, which would not have been considered distinct using existing Test Guidelines' characteristics, would be considered distinct by this approach. The initial study suggests that this is unlikely, because the most similar varieties considered distinct under the existing system (i.e. mutant variety pairs) are *not* considered distinct using the two sets of STMS markers.

7. It is noted above that the risk of mutation exists and that this could produce a "distinct" variety from an existing variety, if the mutation occurred at an STMS marker site. However, this risk is reduced within the proposal by the requirement for differences in three bands to be able to consider a variety distinct using STMS marker sets. This would require three separate mutations to occur, all within marker sites. If the rate of mutation is assumed to be 1 in 10,000, then the chance of finding a plant with three mutations is 1 in $10,000^{3}$ i.e. 1 in 1,000,000,000,000 and the need for these three mutations to occur in marker sites would make the possibility of screening for such variants uneconomic.

Option 3: Development of a New System

PROPOSAL

prepared by experts from the United Kingdom

("OPTION 3" for Wheat)

Proposal

1. The basis of this proposal is that a set of molecular markers would be used in wheat (i) to expand and organize the reference collection, and (ii) to screen candidates prior to field testing.

2. Currently there is considerable discrepancy in the constitution of reference collections in different countries, and it is considered that the existence of a database of DNA profiles of varieties, used as in this proposal, would improve this situation and strengthen the value of PBR.

3. Final decisions on distinctness of candidates could be made on the basis of the screening using molecular markers or, if this is not conclusive, on the basis of a reduced set of existing non-molecular characteristics recorded in field trials.

4. A study of 40 wheat varieties has shown that all of these varieties, except for one pair of sister lines, could be distinguished using 8 microsatellite (simple sequence repeat, SSR) markers. Microsatellites are highly polymorphic, tandemly repeated DNA sequences with a basic repeat unit (or core sequence) of 2-8 base pairs (e.g. GA, CTT and GATA). The polymorphism found in microsatellites is due to variations in the copy number of the basic repeat unit. In various crop species, multiple such variations ("alleles") have been shown to exist for many microsatellites in different varieties, arising from these differences in copy number. Microsatellites can be analyzed as sequence-tagged sites (STMS), which require the use of pairs of DNA primers (short sequences) that flank the microsatellite. The use of these primer pairs in a polymerase chain reaction (PCR) amplifies the microsatellite region. Different alleles of the microsatellite site ("locus") can then be separated and visualized by electrophoresis or other analytical techniques.

5. It should be noted that it is unlikely (but not impossible) that these microsatellite sequences are linked to existing UPOV characteristics. However, they can be mapped and their inheritance can be followed in crosses. The expression of the alleles, for instance as bands on a gel, is not affected by the environment or by the developmental stage of the plant.

6. The 8 SSRs are all known to map to different chromosomal locations in the wheat genome and can be reliably and repeatably examined.

7. The uniformity of the 40 varieties with respect to the 8 SSR loci has been studied. Preliminary analysis showed that the uniformity of the banding pattern for all the plants within a variety depended on the variety and the molecular marker. In 15 out of the 40 varieties, no variant banding patterns were found out of 48 plants, for any of the 8 SSRs. A further 8 varieties had only one variant in 48 plants, whilst 2 varieties had an individual plant with different alleles at 2 loci. This analysis has yet to be completed, but will ultimately

provide an indication of the uniformity of existing, protected varieties at these loci, i.e. what is achieved by wheat breeders currently with no specific effort to purify varieties for these characteristics.

8. The following scheme is proposed:

Step 1: A candidate variety is received by the testing office. It is then profiled using an agreed and fixed set of 8 SSR markers.

Step 2: The initial DNA profile information is used to determine if the candidate is clearly distinguishable from the varieties of common knowledge, and/or to determine from which varieties it is not clearly distinguishable (according to the agreed basis below).

Step 3: If the candidate variety can be clearly distinguished using this set of markers, it is considered distinct. One basis for distinctness might be the occurrence of a different allele at one marker locus for which the candidate and the reference variety are sufficiently uniform. However, it is possible that a more strict requirement (e.g. different alleles at more than one locus, i.e. differences in more than one marker) could be used ("Distinctness Plus"), although this would, of course, reduce the discriminating power of the markers.

Step 4: The uniformity standard will be based on that currently found in protected varieties (see 7 above), which, in turn, will determine the number of individuals to be analyzed. If a "Distinctness Plus" approach is taken, then the Uniformity criteria will have to be similarly adjusted. Plants for which the difference was less than that used to establish distinctness would not be regarded as variants for the purposes of assessing uniformity.

Step 5: Candidates which are not sufficiently uniform for any of the 8 markers will not undergo further testing and will not be protected.

Step 6: If the candidate cannot be clearly distinguished from all varieties of common knowledge, then the varieties from which it is not distinct (according to an agreed criterion) are selected for inclusion in the field trial.

Step 7: The process is repeated for all candidates, and the field trial is then planned so that similar varieties are grown close together, i.e. comparisons can be readily made between most similar groups of candidates/reference varieties. The planning could also utilize information supplied by the breeder on the TQ.

Step 8: All candidates are sown in field trials, to check uniformity and stability of the relevant, non molecular characteristics.

Step 9: The characteristics recorded in the field trials would comprise a reduced set of those currently recorded, based e.g., on an analysis of their discriminating power, or on their lack of environmental interaction, or on their usefulness for descriptive purposes (including certification).

Step 10: If the establishment of distinctness is still difficult, additional characters could be used, in a special test. Such characteristics would have to meet the same criteria as existing characteristics.

Step 11: The variety description would consist of both the DNA profile and the recorded field trial characteristics.

Assumptions to be Made in the Proposal

9. The following assumptions are made:

(a) The DUS Examination

It is assumed that the standards for the use of the SSR markers would be agreed (see 7 above, plus 8, steps 2-4). The uniformity and stability standards for the marker data would be determined as in 7 above, based on what is achievable currently. There is no need to examine marker data in more than one year. The same standards as now would apply to the field trials, with the currently used criteria for uniformity and stability.

(b) Reliability of the Techniques

It is assumed that the SSR markers would meet all the normal requirements for any characteristic to be used in the DUS examination (see "General Introduction"), including the need to be sufficiently consistent and repeatable.

(c) The Set of Markers

The set of 8 SSR markers used for creating the database and assessing candidates would be 'fixed.' However, should improved and/or additional markers become available over time, the original marker set might be augmented, or alternatively less useful markers replaced. Any such additional markers would have to be tested in the same way as the original set of eight.

(d) Uniformity

It is assumed that the situation found in the initial study on 40 varieties, particularly regarding the uniformity of existing varieties, would be broadly indicative of all existing, protected varieties.

(e) Database of DNA Profiles

It is assumed that a suitable database can be created and maintained, incorporating the DNA profiles of varieties of common knowledge, probably also partitioned, for example, according to the origin of the variety and/or agri-climatic regions.

Potential Impact

10. A significant positive impact on the strength and quality of protection would be the potential to screen a much more comprehensive reference collection. It is now well established that reference collections vary widely in their coverage of varieties of common

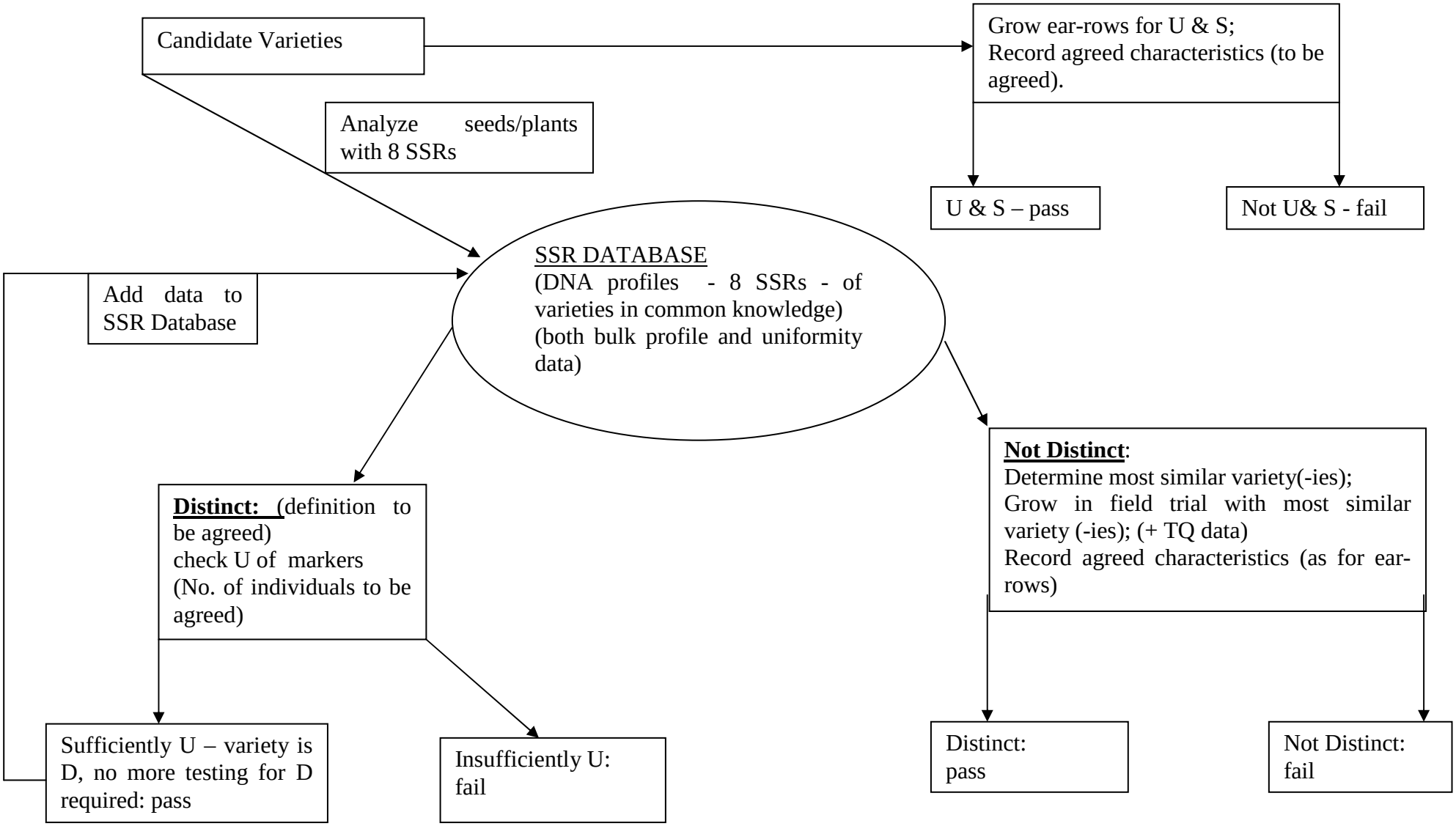
knowledge, and that environmental interactions with many morphological characters compromise the effectiveness of published descriptions (see document TWA/30/16). This proposal offers an opportunity to address both of these problems.

11. It is possible that the proposed system could allow varieties to be declared D, U and S in a single year of testing.

12. One way in which this proposal could have a potential negative impact on the strength of protection is if varieties, which would not have been considered distinct using traditional characteristics, would be considered distinct using this approach. This could be assessed by a parallel running exercise over an agreed number of years (or, where possible, could be done retrospectively).

13. If a breeder sought to produce a new variety by changing only the molecular marker profile, this could become apparent from the description of the variety (and could then presumably trigger an investigation of possible EDV status).

14. The risk of a new variety being produced by selection from an existing variety could be minimized by requiring differences at more than one SSR locus to be able to consider a variety distinct (see 8, steps 3 and 4 above). In any case, this risk is no greater with this proposal than that which currently exists. This proposal preserves the link between the size of differences required to establish clear distinctness and uniformity standards. Therefore, it would be futile to select and purify parts of a sufficiently uniform variety because such a collection of plants would not be clearly distinct from the original variety.



[End of Appendix 2 and of document]