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PREPARATION OF GUIDELINE FOR METHOD VALIDATION OF DNA IDENTIFICATION FOR THE ENFORCEMENT OF PLANT BREEDER'S RIGHTS IN JAPAN

Document prepared by experts from Japan

Toru Semba[1], Takeshi Sugisawa[2]

[1]Plant Variety Protection and Seed Division, Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan

[2]Plant Variety Protection Division, National Center for Seed and Seedlings (NCSS)

Use of DNA variety identification techniques for the enforcement of PBRs in Japan

1. Plant Breeder's Rights (PBRs) is one of the intellectual properties which can be granted to breeders who develop new plant varieties. The grant of rights, which allows breeders to monopolize the use of their varieties, motivates breeders to develop new plant varieties. In this regard, whether such a Plant Variety Protection (PVP) system achieves its purpose is highly dependent on whether PBRs have the substance of the rights, in other words, whether the right holders can enforce their rights.

2. In Japan, the National Center for Seed and Seedlings (NCSS) provides breeders with variety identification services for the enforcement of their rights as one of initiatives from public sector to strengthen the system. The services, which are conducted at the breeder's request, are intended to help breeders to identify any infringement material. There are three methodologies for the variety identification services available at NCSS, namely the Characteristics Test, the Growing Test and the DNA Identification Test. The Characteristics Test compares materials of a breeder's variety and corresponding materials of a suspicious variety in the initial form in which they are obtained. The Characteristics Test is very rapid, but correct results may not prevail because, in most cases, the materials have been grown in different circumstances. The Growing Test examines characteristics of two samples which have been obtained by growing together in the same circumstance in a similar manner to a DUS test where the candidate variety and example varieties are compared. Breeders can obtain precise results through the method; however, they have to wait for rather a longer period of time for the results.

3. The DNA Identification Test has advantages of both. The test, which uses DNA-fingerprint techniques, is rapid and precise where the selection of markers and examination are conducted appropriately. In addition, it is useful in particular cases. For example, breeders may appeal to the customs office to stop the import and export of infringement material, as the Japanese Customs Law provides. In this case, breeders have to take a risk that the stopped materials may be damaged, for example by spoiling during the examination and, if it turns out that the materials do not infringe their right, the breeders may be required to provide compensation for the damage to the owner of the materials. The DNA Identification Test is expected to solve these obstacles for breeders to enforce their rights.

4. However, species for which the technique is available are very few and expansion of the number is necessary. The government of Japan promotes the expansion by supporting developers of the techniques financially, and then introduces the outcomes in the DNA Identification Test.

Species to which DNA variety identification techniques are practically applied in Japan

1) Species which propagating, harvesting and processed materials are identifiable:
Rice (50 or more varieties), Azuki bean (9 varieties), Igusa (2 varieties)

2) Species which propagating and harvesting materials are identifiable:
Ingen bean (8 varieties), Strawberry (125 varieties), Cherry (100 varieties), Japanese pea (100 varieties), Sweet potato (103 varieties), Shiitake mushroom (140 varieties)

Species for which the techniques are in the process of development

5. Wheat, Barley, Egg plant, Cabbage, Chinese cabbage, Hot pepper, Water melon, Apple, Peach, Plum, Apricot, Loquat, Citrus, Grape, Chestnut, Devil's Tongue, Shiba, Peanuts, Chrysanthemum, Carnation, Gentian, Nori

Guideline for method validation of DNA identification

6. As DNA identification is being used, interest in the reliability of DNA-identification protocols in enforcing Plant Breeder's Rights (PBRs) has risen. Currently, various guidelines have been reported, including the Guidelines for DNA-Profiling: Molecular Marker Selection and Database Construction (BMT Guidelines) in UPOV, the Scientific Working Group on DNA Analysis Methods (SWGDAM; a Working Group of the Federal Bureau of Investigation), and the Official Methods of Analysis in AOAC INTERNATIONAL (an organization in the United States of America that establishes method validation). However, these guidelines do not seek to identify DNA in plants, and no reports have been published on method of validation of DNA identification in plants.

7. Therefore, we have prepared a guideline for a method validation of DNA identification using SSR analysis for the purpose of enforcing PBRs in Japan. The validated method was implemented for DNA identification of sweet cherries by SSR markers based on the guideline.

8. In the meantime, ISO/TC34/SC16 (horizontal methods for the detection of molecular biomarkers in: foods; seeds and propagules of food crops; commodity food crops; fruits; vegetables and derived foods) will be established as a new subcommittee in ISO in 2008, and DNA identification will be discussed there. We are interested in following future trends in this area.

Contents of the guideline

9. The method validation in our guideline consists primarily of two processes: confirmation of developed techniques; and collaborative study.

a) Confirmation of developed techniques

This process consists of confirmation procedures of developed techniques and protocols that laboratories should clearly describe for DNA identification, before collaborative study. Participating laboratories should clarify the following items: information of analyzed plant and SSR marker, DNA extraction, PCR conditions, SSR analysis and peer-reviewed publication.

b) Collaborative study

A collaborative study should be conducted among different laboratories to confirm the reproducibility and reliability of the method for DNA identification. If reproducible and reliable results cannot be obtained by following the protocol instructions, a modified and suitable method should be revalidated to confirm the new method. In addition, a preliminary study should be conducted before collaborative studies. The preliminary study is conducted for laboratories to acquire skill and experience in the procedures and to confirm whether the laboratory personnel are sufficiently skilled.

Validation of DNA identification method for sweet cherries

10. We validated the method of DNA identification for sweet cherries (*Prunus avium* L.) by SSR markers, according to the protocol's instruction that Yamagata General Agricultural Research Center (Sagae, Japan).

11. Sweet cherry belongs to the genus *Prunus*, and the family *Rosaceae*, the same as the sour cherry, peach, almond, apricot and plum. Because it was reported that the genome structure was conserved very well within *Prunus* (Dirlewanger et al., 2004), SSR markers can be applied within *Prunus* at a high rate. Therefore, SSR markers used for DNA identification on sweet cherry were chosen and evaluated, based on those for peach and sour cherry.

Confirmation of developed techniques for DNA identification of sweet cherry

1. Information on subject plant

1.1 Plant materials used for analysis

Leaves, peduncles and pericarps of sweet cherry varieties 'Satonishiki' and 'Benisyuhou' were used in this study. Standard trees of 'Satonishiki' and 'Benisyuhou' maintained at Yamagata General Agricultural Research Center (Sagae, Japan) were used.

1.2 Selection of varieties for reference alleles

We used varieties 'Satonishiki' and 'Banisyuho' for selecting reference alleles, based on document BMT Guidelines 2.2.1.4

2. SSR markers

2.1 Selection of SSR markers

Ten SSR markers were chosen because of their distribution in the *Prunus* reference genetic linkage maps and because of their independence. First, one marker was selected for each of eight linkage groups. Second, two markers from linkage groups 1 and 4 were added for analysis, after confirmation that the distance between the two markers chosen from linkage groups 1 and 4 exceeded 50cM.

2.2 Confirmation of null allele

The 10 SSR markers confirmed that null alleles exhibited almost negligible values of H_E (expected heterogezygosity), H_O (observed heterogezygosity) and null allele frequency, calculated by CERVUS ver. 2.0 software (Marshall *et al.,* 1998).

3. DNA extraction

3.1 Tissues used for extraction

Peduncles and pericarps of sweet cherry fruits were used for DNA extraction because these tissues are included in circulating fruits. Leaves were also chosen as samples for DNA extraction.

3.2 Condition of samples for DNA extraction

Sampled peduncles and pericarps were kept at -80 °C for six weeks after harvesting.

4. PCR condition

Suitable PCR conditions and dilution of PCR products were established for each marker.

5. Peer-reviewed publication

A paper on DNA identification of sweet cherry was published by the Japanese Society for DNA Polymorphism Research:
Tadashi Takashina, Makoto Ishiguro, Koichi Nishimura and Toshiya Yamamoto (2007) Genetic identification of imported and domestic sweet cherry varieties using SSR markers.
DNA Polymorphism.vol15: 101-104

Collaborative study for DNA identification of sweet cherry

12. A collaborative study was conducted to confirm the reproducibility of the method of DNA extraction and performance of SSR analysis with 11 laboratories. Before the collaborative study, preliminary experiments were conducted to confirm the accuracy of scoring molecular data by participating laboratories.

1. Reproducibility of DNA extraction method

Each laboratory extracted DNA according to the method of identifying sweet cherry DNA. There was a total of 18 samples in each laboratory, including leaves, peduncles and pericarps of two varieties with three replications. The concentration and purity of extracted DNA were confirmed by agarose gel electrophoresis. Thereafter, SSR-PCR products were used to confirm sufficient quality for PCR reaction. Extracted DNA from leaves and peduncles confirmed on agarose gel and PCR products were evaluated for almost all samples of all laboratories. Extracted DNA from pericarps was confirmed to have sufficient volume and length for 10 laboratories. Only one laboratory was unable to extract DNA either on agarose gel or PCR product, because the extracted DNA might have included a lot of impurities. We did not think that the result influenced the reproducibility of the DNA extraction method because the failure was caused by the laboratory not paying sufficient attention to prevention of mixing of the many sarcocarp. This confirmed the reproducibility of the method for extracting DNA from leaves, peduncles and pericarps.

2. Reproducibility of SSR marker performance

SSR marker performance was defined by the degree that SSR primers annealed to specific genomic regions. If PCR conditions were suitable for each marker and primers clearly annealed to specific genomic region, specific fragments must be stably detected. All laboratories performed PCR and SSR analysis using capillary electrophoresis (DNA sequencer) according to the method of identifying sweet cherry DNA. Ten SSR markers, pchcms4, UDP98-022, MA007a, BPPCT039, BPPCT005, PS12A02, BPPCT037, MA027a, MA020a and BPPCT012 (Cipriani et al., 1999, Dirlewanger et al., 2002, Sosinski et al., 2000, Yamamoto et al., 2002), and two DNA samples, Satonishiki and Benishuhou, were used in each laboratory. All laboratories repeated the combinations of SSR markers and DNA samples six times. It was not necessary to confirm whether SSR markers were applicable to other varieties because these SSR markers confirmed that the null allele frequency was zero or very low. This suggested that two DNA samples were sufficient to obtain accurate results.

The allele size was distributed within 1 bp in the analyses of all laboratories for eight markers. For the remaining two markers, the deviations of the same alleles were less than 1 bp in all analyses of 10 laboratories, except for one remaining laboratory that had deviations of more than 1 bp. We believe that the deviations found in one laboratory were caused by analyzing the DNA of two varieties on separate runs (days). In contrast, the deviations of the data in two varieties were less than 1 bp, and the deviations of the other data using the same markers on the same run in the laboratory were less than 1 bp (Table.1). This suggested that this deviation did not influence reproducibility of performance of SSR markers because the protocol of DNA identification stated that all samples should be analyzed in the same run and that the results obtained for different varieties should be compared. In conclusion, reproducibility and stability of SSR analysis for DNA identification was confirmed. The confirmation of developed techniques and collaborative study validated the method of identifying the DNA variety of sweet cherry.

Table 1. Example of deviation caused to perform SSR analysis at separate runs (days) and same run.

Separate runs(days)

Labo	Marker	Variety	Size of fragment(bp)	Maximum deviation each in variety(bp)	Maximum deviation between varieties(bp)
F	BPPCT005	Satonishiki	169.37 / 202.91	0.13 / 0.18	0.67 / 1.14
			169.37 / 202.99		
			169.30 / 202.88		
			169.43 / 202.89		
			169.30 / 202.81		
			169.30 / 202.83		
		Benisyuhou	169.85 / 203.83	0.14 / 0.14	
			169.94 / 203.85		
			169.89 / 203.85		
			169.83 / 203.87		
			169.97 / 203.81		
			169.96 / 203.95		

Same run

Labo	Marker	Variety	Size of fragment(bp)	Maximum deviation each in variety(bp)	Maximum deviation between varieties(bp)
F	BPPCT005	Satonishiki	169.78 / 203.79	0.18 / 0.15	0.19 / 0.16
			169.90 / 203.85		
			169.83 / 203.86		
			169.90 / 203.94		
			169.96 / 203.87		
		Benisyuhou	169.85 / 203.83	0.14 / 0.14	
			169.94 / 203.85		
			169.89 / 203.85		
			169.83 / 203.87		
			169.97 / 203.81		
			169.96 / 203.95		

* Firstly laboratory F performed SSR analysis at same run. But one of 6 repeatability of "Satonishiki" was failed to analyze. Then laboratory F tried again about "Satonishiki" only.

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