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**WORKING GROUP ON BIOCHEMICAL AND MOLECULAR TECHNIQUES
AND DNA-PROFILING IN PARTICULAR****Fourteenth Session
Seoul, Republic of Korea, November 10 to 13, 2014****POTENTIAL USES OF MOLECULAR MARKERS IN MANAGEMENT OF ROSE VARIETIES FOR THE PVP
SYSTEM***Document prepared by experts from China**Disclaimer: this document does not represent UPOV policies or guidance***INTRODUCTION**

1. Rose is one of the largest ornamental crops, and more than 25,000 varieties of modern roses across the world have been described. Such a large number of rose varieties would impose difficulties in choosing the most similar varieties from the collection of reference varieties for DUS (Distinctness, Uniformity and Stability) testing.
2. In China the number of applications for PBR is the largest for roses compared to other woody plants. Breeders often complain the DUS test cycle is too long, and the proficient are very busy to do many DUS test and to take good care of the large number of common knowledge varieties.
3. Molecular markers are considered a useful tool for identifying the most similar variety to be included in the growing trials for DUS examination. Moreover, molecular markers have great potential in quickly providing evidences for law suits in infringement cases. With large quantity of genetic studies of rose varieties using molecular markers, sufficient information has been accumulated on the genetic background of the rose varieties, providing possibilities for direct DUS testing with use of molecular markers.
4. Several first-generation molecular marker techniques have been used for DNA profiling of rose varieties, but these marker systems have some major drawbacks for variety identification, such as lack of high levels of polymorphisms, being dominant markers, difficult to reproduce, complicated to operate, high cost or time consuming, complex patterns undesirable for database development. In contrast, SSR markers are highly polymorphic, co-dominant, easy to operate, stably repeatable, simple banding patterns, especially suitable for automated and objective analysis, and therefore easy to store in a database. New varieties or new markers can easily be added to an existing database. SSR was successfully demonstrated in developing databases for many species.
5. The main objectives of this study are to develop a database of DNA profiling using a set of selected SSR markers. The database will be used as a supplementary tool for DUS testing, especially for assisting screening of similar varieties, and potentially directly identifying rose varieties in the future. Molecular evidence is useful for dealing with infringement cases, and they can be obtained much more quickly than morphological evidence from growing trials.

MATERIAL AND METHODS

Plant materials

6. In 2012, a total of 152 rose (common knowledge) varieties were sampled, of which 104 varieties from Beijing Botanical Garden and 48 varieties from Rose Test Station in Kunming. From 2013 to 2014, about 700 rose (common knowledge) varieties were sampled from the Rose Test Station. The samples were composed of variety types of 'China', 'Climber', 'Floribunda', 'Hybrid Tea', 'Miniature', 'Polyantha' and 'Shrub'.

7. Theoretically all plants of a rose variety are the same genotype, therefore only 1 sample plant was randomly selected from each variety, and the second and third leaves from the top of sample plants were collected and exsiccated with silica gel and stored at -80°C after complete desiccation.

DNA profiling

8. DNA was isolated from the dry frozen leaf tissue using the DNA secure Plant Kit (TianGen, CN) following the manufacturer's instructions. DNA quality was tested via electrophoresis on 1.2% (percent) agarose gel in 1x (times) TAE buffer and observed under UV light, the main band should be uniform, clear and no-tail. Quantitative determination via UV-Vis Spectrophotometer (Nanodrop8000 Thermo Fisher, US), the ratio of DNA intermixture of A260 to A280 should be approximately 1.8.

9. Information on SSR primer pairs was collected from published research papers and public databases, or developed through enrichment, sequencing and other methods if no information is available on primers. 8 DNA samples were randomly selected as templates to optimize the conditions of PCR and test the polymorphism of the amplified successful primers. Polymorphic primers were identified and used for the analysis of all samples. PCR products were tested by fluorescence capillary electrophoresis, using the automatic genetic analysis system (CEQ 8000, Beckmen). DNA fingerprint data was manipulated with Excel software.

Data analyses

10. According to the observed presence and sizes of the amplified fragments, a 2-way matrix was established and the number of alleles and the number of allelic phenotypes were recorded for each of the SSR loci. The ability of detecting a variety (D) at a locus was calculated with the formula:

$$D_i = 1 - C_i = 1 - \sum_{i=1}^l c_i = 1 - \sum_{i=1}^l p_i \frac{(NP_i - 1)}{N - 1}$$

11. Where D_i is the ability of detecting a variety at locus i , C_i is the extent of confusion of genotypes at locus i . P_i is the frequency of allelic genotypes at locus i , and N is the size of total samples.

12. The polymorphic information content (PIC) were calculated with $PIC = 1 - \sum p_i^2$, where p_i is the allelic frequency at locus i .

13. The effective number of alleles (N_e) was expressed as $N_e = 1 / \sum p_i^2$, where p_i^2 is the allele frequencies of locus i .

Database construction

14. The results of DNA fingerprints were recorded in an Excel file arranged in a form of two-way table, respectively varieties in rows and loci in columns as presented in the simplified form (Table 1)

Testing the distinctness and screening of similar varieties

Search for similar varieties for DUS testing

15. It is easy to find whether the variety under test exists in the database by a typical search function of the Excel.

Hybrid and ploidy detection

16. Detection of hybrid varieties and ploidy of a variety can be done by an analysis of the number of alleles at each locus.

Genetic similarity (S)

17. The formula used to calculate the genetic similarity between two varieties was: $S=1-b/a$, where S is the genetic similarity, a is the average number of bands and b is the number of different bands. The greater the value of S, the closer the genetic relationship.

Number of Different Loci (DL)

18. DL was used to denote the number of different loci. The larger the value of DL, the greater the differences between the two varieties. If $DL \leq 2$, the two varieties were the same variety or essentially derived variety (EDV). If $DL > 2$, the two varieties are considered distinct from each other. The variety under test were compared with all varieties of the database to selected the most similar variety to the candidate variety.

RESULTS AND DISCUSSION

Selection of core primer pairs

19. A total of 130 microsatellite markers were selected from public papers, 8 DNA samples were randomly chosen as templates of pre-PCR, after tests with varying the annealing temperatures, 41 primer pairs were successful in amplifying rose DNA, 40 loci showed polymorphism and were used in this study (Table 1).

Table1: Information of the SSR markers selected for genetic analysis of Rosa varieties

No.	No. allele	No.allelic	No. unique allelic	Ne	D _j	PIC
		phenotype	phenotype			
1	15	86	52	8.286	0.988	0.879
2	17	91	59	7.387	0.988	0.864
3	16	92	67	7.192	0.986	0.86
4	17	81	52	7.03	0.985	0.857
5	14	80	45	7.513	0.984	0.866
6	19	71	44	6.673	0.977	0.85
7	17	69	41	6.745	0.975	0.851
8	9	42	19	5.357	0.975	0.813
9	18	76	48	6.797	0.973	0.852
10	14	60	33	6.356	0.963	0.842
11	9	55	25	6.496	0.958	0.846
12	10	46	20	5.3	0.957	0.811
13	10	41	17	5.121	0.956	0.804
14	19	45	21	5.513	0.954	0.818
15	11	52	26	5.395	0.949	0.814
16	14	50	27	5.028	0.945	0.808
17	12	44	24	4.851	0.942	0.793
18	8	33	12	4.374	0.933	0.771
19	12	45	24	4.886	0.932	0.795
20	14	35	16	4.356	0.927	0.77
21	11	32	15	4.379	0.918	0.77
22	8	22	7	4.004	0.914	0.75
23	11	36	18	4.999	0.912	0.799
24	13	43	26	3.997	0.905	0.749
25	8	32	16	4.132	0.893	0.763
26	14	41	25	4.001	0.889	0.75
27	6	20	5	3.623	0.885	0.724
28	11	32	20	3.561	0.873	0.719
29	8	25	10	3.634	0.871	0.724
30	5	16	2	3.702	0.87	0.729
31	16	37	21	4.267	0.864	0.765
32	8	21	7	3.254	0.831	0.692
33	4	12	2	3.401	0.826	0.706
34	18	39	25	3.399	0.821	0.705
35	7	17	7	3.164	0.808	0.684
36	9	24	13	3.09	0.805	0.676
37	6	15	2	2.886	0.772	0.653
38	7	22	9	3.588	0.771	0.721
39	5	10	2	1.551	0.746	0.644
40	8	18	6	2.745	0.677	0.635

Function of the database

20. The results indicated that the divergence of DNA profiling among hybrid varieties was obvious. It is obvious that all varieties are hybrid and tetraploid according information revealed by the 40 loci (the data were too large to be shown here).

Screening of similar varieties and variety identification

Table 2, Parts of DNA fingerprinting of 4 varieties

Variety	Locus 1				Locus 2				Locus 3				Locus 4				..
	A1	A2	A3	A4	A1	A2	A3	A4	A1	A2	A3	A4	A1	A2	A3	A4	..
1	182	197	203		235	241			248	253	276		289	298	301		..
2	182	203			235	241			248	253	276		289	298	301		..
3	182	189	197	201	241	247	256		238	276			287	289	291	301	..
4	182	189	201		241	247	256		238	276			287	289	291		..
.....																	..

21. It is clear that variety 1 and variety 2 were most similar to each other (S=0.94), and they may be the same one or EDV (DL=1); Variety 3 and variety 4 were most similar to each other (S=0.88), and they may be the same one or EDV (DL=2). Variety 1 and variety 3 were not similar to each other (S=0.31), and they were different varieties (DL=4).

22. But in fact, when the loci were added to 40 and the number of varieties were added to 152 or more, it is difficult to count the value of S and DL of every two varieties and compare them by manual, so in the future, it is imperative to develop a functional model of automatic statistics. Otherwise, it is necessary to confirm standard varieties and standard DNA to check the size of band to make the data as stable and credible as possible.

23. Continue study to develop more powerful markers which should be more polymorphic and stable, Use the least markers to identify the most varieties to save time and cost. Standardizing for construction of Plants Varieties DNA Fingerprinting Database, which could make the labs all over the world share the results of every research.

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