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BMT/6/11
ORIGINAL: English
DATE: February 15, 2000

UPOV

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

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

Sixth Session
Angers, France, March 1 to 3, 2000

MICROSATELLITE MARKERS OF PYRUS SPP.: IDENTIFICATION OF PEAR ACCESSIONS BY APPLE SSRS AND SIMILARITY BETWEEN PEAR AND APPLE

prepared by experts from Japan

MICROSATELLITE MARKERS OF *PYRUS* SPP.: IDENTIFICATION OF PEAR ACCESSIONS BY APPLE SSRS AND SIMILARITY BETWEEN PEAR AND APPLE.

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1. Introduction

The cultivar registration is based on its distinctness, uniformity and stability (DUS) which were evaluated for a long time by comparison with standard or existing cultivars under the same growing condition. Because the characterization of cultivars requires a large set of phenotypic data. In fact, plant phenotypes are usually influenced by the environmental and growing conditions and difficult to assess exactly.

Recently, DNA fingerprint techniques have been well developed and enable for us to detect the polymorphism efficiently. So far, DNA profiling methods (RAPD, RFLP etc) have been applied to evaluate variety discriminations and DUS tests. However, either RFLP or RAPD has its drawback. RFLP assay is labor- and time-consuming. Analysis of RAPD is unreliable regarding reproducibility. As a method to recover their drawbacks, the analysis of using microsatellite marker is attentioned. Among the available molecular genetic markers, microsatellite marker is reliable i.e., codominant, easy to use, specific in species as well as variety and desirable for DUS test.

In this time, we have tried to confirm whether the microsatellite marker in apple is adapted to the identification of the pear cultivars or not. Because it is considered that genetic background of pear is similar to that of apple, because both pear and apple belong to the Rosaceae family.

2. Materials and Methods

Plant material and DNA extraction

Thirty-six pear accessions and 3 apple cultivars were used in this study. Pears include 19 Japanese pear (*Pyrus pyrifolia*), 6 Chinese pear (*P. bretschneideri*, *P. ussuriensis*), 5 European pear (*P. communis*), 2 hybrids between *P. pyrifolia* and *P. communis*, and 4 wild relatives (*P. aromatica, P. betulaefolia, P. calleryana*) (table 1). Genomic DNA was isolated from young leaves by a CTAB-based extraction method.

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Microsatellite PCR amplification

A total of 9 SSR primer sets were tested: 3 developed by Guilford *et al* (Theor Appl Genet 94: 249-254, 1997) and 6 reported by Gianfranceschi *et al* (Theor Appl Genet 96: 1069-1076, 1998). All the conditions of reaction and amplification, except that forward primers were labeled with fluorescent chemicals (FAM or TET or HEX) as the same as those described. Microsatellite alleles were analyzed by electrophoresis of PCR products on 6% denaturing sequence gel in 1xTBE buffer using 377 DNA sequencer (PE Applied Biosystems). Fragments were analyzed by using GeneScan software (PE Applied Biosystems).

Southern blot analysis and sequencing of amplified fragments

The existence of SSR in amplified products was confirmed by southern blot with nine SSR primer sets from pear cultivar 'Kousui' and apple cultivar 'Golden Delicious' biotin - labeled oligonucleotide (AG)15 was used as the probe and the blot was incubated in hybridization buffer (5xSSC, 0.1% sodium N-lauroyl sarcosinate, 0.02%SDS, 1% blocking reagent) at 50 °C for 16 hours. The hybridized membrane was washed twice with wash solution (2xSSC, 0.1%SDS) at room temperature. The fragments were detected using streptavidin-horseradish peroxidase conjugate and visualized using luminol and hydrogen peroxidase.

Furthermore, the resulting bands by 9 SSR primer sets from 8 pear cultivars 'Housui', 'Kousui', 'Choujyu', 'Choujyuurou', 'Okusankichi', 'Hongli', 'Bartlett', 'Iwateyamanashi' and 2 apple cultivars 'Cox's Orange Pippin', 'Golden Delicious' were cloned into pCR2.1 vector (TA cloning kit Invitrogen). Preparated plasmid DNA was sequenced with the 377 automated DNA sequencer (PE Applied Biosystems) using the BigDye terminator Cycle Sequencing kit (PE Applied Biosystems).

3. Results and Discussion

Microsatellite PCR amplification and Southern blot analysis

At first, we examined that amplified fragments obtained from pear varieties with 9 SSR primers derived from apple. All SSR primers (02b1, 05g8, 28f4, CH01B12, CH01E12, CH01F12, CH01H01, CH01H10, CH02B12) did produce discrete reproducible bands for pear varieties. These primers also produced specific bands for 3 apple cultivars. Fragment obtained from apple, which showed the almost same size reported, were different from those of pear.

Southern blot analysis was conducted in order to reveal that SSRs existed within the amplified fragments of pear varieties. Japanese pear cultivar 'Kousui' and apple cultivar 'Golden Delicious' were subjected for analysis on their fragments obtained from 9 SSR primer sets. When the blot was probed with the oligonucleitide of biotin-labeled (AG)15, it was revealed that all the bands from apple produced positive signals and that positive signals could be observed for all the fragments except for the CH01B12 fragment of 'Kousui' (Fig 1). Then, PCR products of the CH01H01 from 36 pear and 3 apple varieties were also analyzed using a probe with AG-repeats. Positive signals could be found for 32 pear and 3 apple varieties, however, 4 pear varieties possessing the 77 bp fragment did not produce any signals.

These results suggested that, in most cases, AG/TC repeats could be detected within DNA fragments of pears amplified with apple SSR primers.

Sequence of Pear Fragments

Nucleotide sequencing of several amplified bands of pear from 9 SSR primers was conducted, and then their sequences were compared with those of apples. Nucleotide repeats of AG were observed for all the bands from apples. Similarly, AG repeats were found on pear fragments with a few exceptions. PCR products from 'Kousui' and/or 'Housui' with 8 sets of primers (02b1, 05g8, 28f4, CH01E12, CH01F12, CH01H01, CH01H10, CH02B12) included 10 to 25 repeats of AG unit. It was revealed that the 77 bp fragment of 'Choujuurou' etc.. had small number of repeat (4 repeats) and that positive signal could not be detected for the 77 bp fragment of 4 pear varieties. On the whole, the sequences of pear on flanking region around SSRs were highly homologous to those of apple with more than 90 % of identity. We concluded that amplified fragments of pear by 8 primers included SSRs and that nucleotide sequence was highly conserved between pear and apple. It is interesting to reveal that these SSRs of pear and apple were diversed from the same origin or not.

Cultivar identification and parentage test of pear by apple SSRs

Eight SSRs (02b1, 05g8, 28f4, CH01E12, CH01F12, CH01H01, CH01H10, CH02B12) were examined for cultivar identification using 36 pear varieties. Amplified fragments were detected by using 8 SSR primer sets. The eight primers exhibited a total of 99 genotypes of amplified fragments. Variation of genotype was observed within species as well as among species. All pear accessions except for mutant cultivars were differentiated by these SSR primer sets. It was concluded that 8 SSRs used in this study were highly effective for cultivar identification on pear.

The inheritance of SSR bands was investigated using several pairs of parents and offspring. The hybrid accession 282-12 had one of each fragment generated by 8 SSR primers from parent cultivar 'Housui' and 'La France'. Similarly, parents' genotypes of 8 SSRs were inherited to their offspring for the other pairs. It was suggested that SSRs were very available to confirm the parentage test for pear.

Table 1. Plant Materials

No.	Variety Name	Species
1	Housui	*Pyrus pyrifolia* (Burm.) Nak.
2	Shinsei	*Pyrus pyrifolia* (Burm.) Nak.
3	Kousui	*Pyrus pyrifolia* (Burm.) Nak.
4	Chouju	*Pyrus pyrifolia* (Burm.) Nak.
5	Gold Nijisseiki	*Pyrus pyrifolia* (Burm.) Nak.
6	Choujuurou	*Pyrus pyrifolia* (Burm.) Nak.
7	Okusankichi	*Pyrus pyrifolia* (Burm.) Nak.
8	Nanseichabo	*Pyrus pyrifolia* (Burm.) Nak.
9	Nijisseiki	*Pyrus pyrifolia* (Burm.) Nak.
10	Osagold	*Pyrus pyrifolia* (Burm.) Nak.
11	Niitaka	*Pyrus pyrifolia* (Burm.) Nak.
12	Atago	*Pyrus pyrifolia* (Burm.) Nak.
13	Akiduki	*Pyrus pyrifolia* (Burm.) Nak.
14	Chikusui	*Pyrus pyrifolia* (Burm.) Nak.
15	Yasato	*Pyrus pyrifolia* (Burm.) Nak.
16	Hougestu	*Pyrus pyrifolia* (Burm.) Nak.
17	Syuugyoku	*Pyrus pyrifolia* (Burm.) Nak.
18	Shinsui	*Pyrus pyrifolia* (Burm.) Nak.
19	Kinchaku	*Pyrus pyrifolia* (Burm.) Nak.
20	Yali	*Pyrus bretschneideri* REHD.
21	Laiyangcili	*Pyrus bretschneideri* REHD.
22	Mili	*Pyrus bretschneideri* REHD.
23	Hongli	*Pyrus bretschneideri* REHD.
24	Beijin Baili	*Pyrus ussuriensis* Maxim.
25	Balixiang	*Pyrus ussuriensis* Maxim.
26	La France	*Pyrus communis* L.
27	Bartlett	*Pyrus communis* L.
28	Max Red Bartlett	*Pyrus communis* L.
29	Le Lectier	*Pyrus communis* L.
30	Silver Bell	*Pyrus communis* L.
31	282-12 (Housui x La France)	*P. communis* x *pyrifolia*
32	290-36 (Bartlett x Housui)	*P. communis* x *pyrifolia*
33	Iwateyamanashi	*Pyrus aromatica* Kikuchi et. Nakai
34	Betulaefolia	*Pyrus betulaefolia* Bunge
35	Mamenashi 6	*Pyrus calleryana* Decne.
36	Mamenashi 14	*Pyrus calleryana* Decne.
A1	Cox's Orange Pippin	*Malus pumila* MILL. var. *domestica* SCHNEID.
A2	Golden Delicious	*Malus pumila* MILL. var. *domestica* SCHNEID.
A3	Redfree	*Malus pumila* MILL. var. *domestica* SCHNEID.

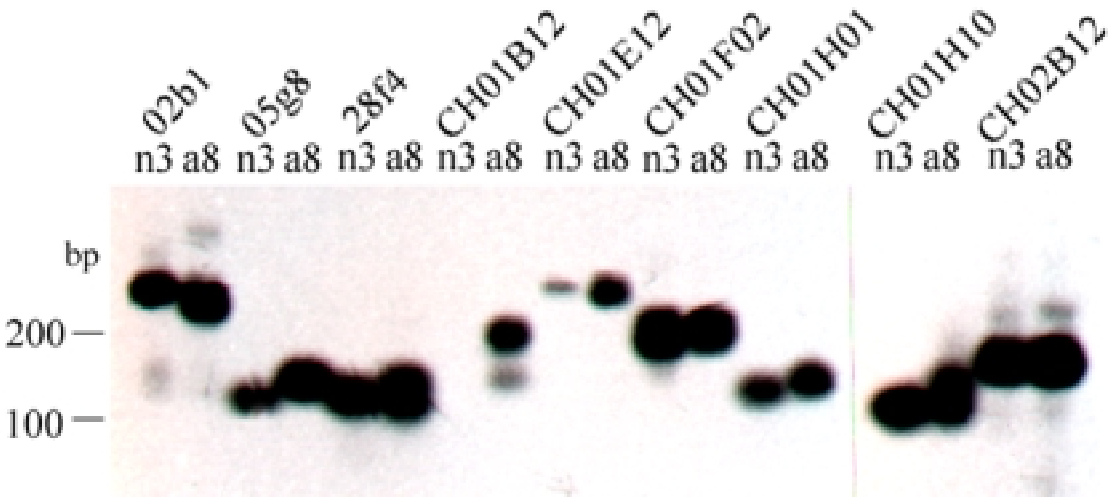

Fig. 1 Southern blots of SSR fragments amplified from Kousui (n3) and Golden Delicious (a8) probed with 5' -biotin- (AG)15

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