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Effectiveness of AFLP and SSR molecular markers in determination of genetic relationships among pear (*Pyrus* spp.) genotypes

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A comparative study of discriminating capacity of AFLP and SSR markers regarding their effectiveness in establishing genetic relationships among 94 pear genotypes was performed by calculating confusion probability, discriminating power and effective number of patterns per assay unit. The AFLPs analyses produced more polymorphic markers than SSRs. The average discriminating power was very high for AFLPs and a bit lower for SSRs. Both molecular marker techniques proved their reliability to assess genetic relationships among pear genotypes.

Key words: AFLP, SSR, molecular markers, pear, *Pyrus*, discriminating capacity

INTRODUCTION

Pears (*Pyrus* spp.) belong to the family Rosaceae, subfamily Maloideae and genus *Pyrus* L. As fruit trees they originated in prehistoric times. Their breeding has a long history and the results are numerous cultivars that are grown in all temperate and some subtropical regions of the world. Cultivars and rootstocks used for commercial production are maintained through vegetative propagation. Pear is an allogamous perennial, and consequences are high levels of heterozigosity and high allelic diversity within the genus.

Early efforts to identify cultivars by means of phenotypic data (Westwood 1982) proved useful only for a limited number of cultivars in certain conditions. The phenotypic variability seen amongst accessions grown on areas with slightly different environments and production practices demonstrates a number of problems with this approach (Hokanson et al. 1998). Due to high variability of morphological, anatomical and physiological characters, the conclusions about relatedness between genotypes based on phenotypic markers are not always reliable. More appropriate are molecular markers, which appear to be more stable. An efficient molecular research requires the selection of highly informative markers among the available marker systems. In recent years, randomly amplified polymorphic DNA (RAPD) markers have been used in several studies of genetic variability of pears (Botta et al. 1998, Monte-Corvo et al. 2000, Schiliro et al. 2001, Teng et al. 2002, Stark-Urnau 2002, Lee et al. 2004). The amplified fragment length polymorphism (AFLP) markers, developed by Vos et al. (1995), and simple sequence repeat (SSR) have also been applied to study the genetic relationships among pears (Monte-Corvo et al. 2000, Monte-Corvo et al. 2002, Shengua and Chengquan 2002, Yamamoto et al. 2001, Yamamoto et al. 2002a, Yamamoto et al. 2002b, Kimura et al. 2002, Hemmat et al. 2003, Tartarini and Sansavini 2003, Pierantoni et al. 2004, Van Dyk et al. 2005, Bassil et al. 2005, Ghosh et al. 2006).

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In the study presented in this paper a comparison between two molecular markers (AFLP and SSR) used for measuring genetic diversity among pears was investigated. The objective of this paper is to compare the discriminating and informative capacities of AFLP and SSR molecular markers for genotype identification and genetic diversity analyses in pears. The discriminating power D_L can be considered as a good estimator of the efficiency of a primer or a locus. It allows one to compare different types of molecular markers and can be used to predict the efficiency of primers taken in combination, and risks of confusion associated with the combination used. This parameter can be of great interest for the varietal identification by molecular techniques, especially to evaluate the costs in terms of amplifications (Tessier et al. 1999).

MATERIALS AND METHODS

Plant material and DNA isolation

The study included ninety-four pear genotypes (including four commercial cultivars of *P. communis*), which were collected in different parts of Slovenia. The total genomic DNA was extracted from fresh and young leaf material as described by Kump et al. (1992).

Marker analysis

AFLPs

The total genomic DNA (500ng) was restricted using two restriction enzymes (*Eco*RI and *Mse*I) and ligated to *Eco*RI and *Mse*I adapters. Preamplifications were performed in 50 µl volumes of 1×PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 75 ng *Eco*RI and *Mse*I primers, 1.5 U Taq polymerase and 5 µl of ligated DNA, using 20 cycles of 94 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s. Two µl aliquots of the diluted (1:10) pre-amplification product were used as templates for the selective amplifications with five primer combinations: E-AGC + M-CTG, E-AGC + M-CAT, E-AGC + M-CTC, E-AGC + M-CTT, E-AGC + M-CTA. *Eco*-

RI primers were labelled with Cy5 at the 5' end for selective amplification, allowing fluorescence detection. The selective amplification was carried out in a 10 µl volume using the following temperature profile: 13 cycles of 94 °C for 30 s, 65 °C for 30 s, with a decrease of anneling temperature of 0.7 °C per cycle, and 72 °C for 60 s, followed by 23 cycles at the annealing temperature of 56 °C. To PCR products, equal volumes of formamide loading dye were added and denaturated at 94 °C for 4 min. The samples were separated on a 7.5 % polyacrilamide denaturing gel in an automated ALFexpressII sequencer (Amersham Biosciences). A fluorescent-labelled size marker (Cy5 Sizer 50-500; Amersham Biosciences) was used as a molecular weight reference.

SSRs

Seven microsatellite loci developed earlier (Yamamoto et al. 2002a; Gianfranceschi et al. 1998; Guilford et al. 1997) were selected: KB16, KU10, BGA35, CH01H10, CH01F02, O2B1 and 28f4. Ten µl of PCR mixture contained 20 ng DNA, 0.25 U Taq DNA polymeraze (Promega), reaction buffer (Promega; 50 mM KCl, 1.5 mM MgCl2 10 mM Tris-Hcl), 0.5 µl of each primer and 200 µM of each dNTP. PCR condition consisted of an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, primer annealing for 30 s at 47 °C - 60 °C and DNA extension for 30 s at 72 °C. Polymerase chain reaction (PCR) was performed using Gene AMP 9700 thermocycler.

Data analysis

For the comparison of marker system (AFLP and SSR) efficiency in assessment of genotype diversity we estimated the following parameters, according to Tessier et al. (1999) and Belaj et al. (2003):

(1) Number of assay units (U) (assay unit is the product of PCR amplification obtained with one set of primers);
(2) Number of polymorphic bands (n_p);
(3) Number of monomorphic bands (n_{np});
(4) Number of loci (L);
(5) Number of polymorphic bands/assay unit (n_p/U);
(6) Number of loci/assay unit (nu = L/U);
(7) Average number of patterns/assay unit (I);
(8) Average confusion probability of the j-th assay unit:

$$Cj = \sum_{i=1}^{I} p_i \frac{(Np_i - 1)}{(N-1)} \quad ,$$

where pi is the frequency of the i-th pattern; N- sample size; I- total number of patterns generated by the j-th assay unit;
(9) Average discriminating power ($D_j = 1 - C_j$);
(10) Average limit of discriminating power ($D_L = \lim (D_j)$;
(11) Effective number of patterns/assay unit

$$(P = \frac{1}{1 - D_L});$$

(12) Average number of alleles per locus (n_{av});
(13) Fraction of polymorphic loci

$$(\beta = \frac{n_p}{n_p + n_{np}});$$

(14) Effective multiplex ratio ($E = n_u * \beta$)

RESULTS AND DISCUSSION

Table 1 shows calculated parameters associated with discriminating capacity for both markers. There were 141 loci for AFLP and 7 for SSR. We obtained two times more polymorphic bands per each assay with AFLP units. The total number of polymorphic bands was 93 in AFLP and 64 in SSR. To compare the efficiency of both marker systems in genotype identification, we estimated the discriminating power (D_j). The average confusion probability (C_j) is the probability that two randomly chosen individuals from the sample of 94 pears have identical banding patterns and the discriminating power ($D_j = 1 - C_j$) represents the probability that two randomly chosen individuals have different patterns, and therefore are distinguishable from one another. In our case, the confusion probability was low for both marker systems, especially for the AFLPs (0.029284). The discriminating power (Dj), which was negatively correlated with the confusion probability, showed high value for AFLPs (0.970716) and a bit lower for SSRs (0.77114). The average limit of discriminating power estimated for both marker systems were very close to the actual discriminating power of each of them, respectively.

Table 1: Comparison of discriminating capacity of AFLP and SSR marker systems

Parameter	AFLP	SSR
Number of assay units (U)	5	7
Number of polymorphic bands (n_p)	93	64
Number of monomorphic bands (n_{np})	48	0
Number of loci (L)	141	7
Number of polymorphic bands/assay unit	18.60	9.14
Number of loci/assay unit (n_u)	28.20	1
Average number of patterns/assay unit I	302	113
Average confusion probability (C_j)	0.029284	0.22886
Average discriminating power (D_j)	0.970716	0.77114
Average limit of discriminating power (D_L)	0.960389	0.76429
Effective number of patterns/assay unit (P)	38.80388	6.472
Average number of alleles per locus (n_{av})	2	9.14
Fraction of polymorphic loci (β)	0.66	1
Effective multiplex ratio (E)	18.61	1

Table 2: Characteristics of discriminating power for the AFLP assay units (number of observed AFLP profiles, confusion probability (Cj), discriminating power (D_j), estimate of the discriminating power (D_L) and effective number of AFLP profiles per primer combination (P))

AFLP primer combination	Number of observed AFLP profiles	Confusion probability (C_j)	Discriminating power (D_j)	Estimate of the discriminating power (D_L)	Effective number of AFLP profiles per primer combination (P)
E-AGC + M-CTG	74	0.0066346	0.9933654	0.9827976	58.13158
E-AGC + M-CAT	87	0.0022878	0.9977122	0.9870982	77.50877
E-AGC + M-CTC	39	0.0375200	0.9624800	0.9522408	20.93839
E-AGC + M-CTT	37	0.0702356	0.9297644	0.9198732	12.48023
E-AGC + M-CTA	65	0.0297415	0.9702585	0.9599366	24.96045
	302	0.029284*	0.970716*	0.960389*	38.80388*

*Average value

Table 3: Characteristics of discriminating capacity for the SSR assay unit (confusion probability (C_j), discriminating power (D_j), estimate of the discriminating power (D_L) and effective number of SSR profiles per assay unit (P))

Locus	Confusion probability (C_j)	Discriminating power (D_j)	Estimate of the discriminating power (D_L)	Effective number of patterns per assay unit (P)
KB16	0.62938	0.37062	0.36668	1.579
KU10	0.09128	0.90872	0.89905	9.906
BGA35	0.35164	0.64836	0.64147	2.789
CH01H10	0.14505	0.85495	0.84586	6.488
CH01F02	0.18760	0.81240	0.80376	5.096
O2B1	0.08625	0.91375	0.90403	10.420
28f4	0.11080	0.88920	0.88920	9.026
	0.22886*	0.77114*	0.76429*	6.472*

*Average value

The effective number of patterns indicates the size of an ideal population in which, given the frequencies of the patterns obtained with marker system, all studied individuals can be distinguished (Belaj, 2003). In our case this means that with one AFLP primer combination up to 39 varieties, in average, can be differentiated, and with one SSR primer set about six genotypes can be distinguished.

Different primer sets used in AFLP analysis have different characteristics, as shown in Table 2. The highest discriminating power was obtained with E-AGC + M-CAT primer set (0.9977122) with confusion probability as low as 0.0022878. Effective number of patterns (P) was more than 77 for this primer combination. The lowest discriminating power (0.9297644) we calculated for E-AGC + M-CTT primer set, which is still very high.

In SSR analysis (Table 3), the highest discriminating power was observed on locus 02B1 (0.91375). KB16 locus shows very low discriminating power (0.37062), which has a big influence on average discriminating power of SSR. Without this locus, the average discriminatig power would be 0.83056 instead of 0.7714, and the effective number of patterns 0.83056.

According to our results, AFLP marker system shows a little higher capacity for quantifying the genetic diversity, but both markers proved to be highly effective in discriminating studied pear genotypes. If we keep in mind that AFLPs are much more time consuming method and that the evaluation of bands obtained on gels can be sometimes a hard work (where mistakes can easily occur), the SSR marker system could be better solution for obtaining genetic diversity in pears. But we should also keep in mind that the use of different marker systems simultaneously, could exploit much more sources of polymorphism.

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