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PLANT GENETICS

Using IRAP Markers for Analysis of Genetic Variability in Populations of Resource and Rare Species of Plants

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Abstract—Species-specific LTR retrotransposons were first cloned in five rare relic species of drug plants located in the Perm' region. Sequences of LTR retrotransposons were used for PCR analysis based on amplification of repeated sequences from LTR or other sites of retrotransposons (IRAP). Genetic diversity was studied in six populations of rare relic species of plants *Adonis vernalis* L. by means of the IRAP method; 125 polymorphic IRAP-markers were analyzed. Parameters for DNA polymorphism and genetic diversity of *A. vernalis* populations were determined.

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INTRODUCTION

Mobile genetic elements are obligatory components of eukaryotic genome. Barbara McClintok established that the genome of maize contains many transposable elements [1]. DNA of mobile elements in *Drosophila* was isolated and cloned by teams of Russian researchers headed by G.P. Georgiev and V.A. Gvozdev in the Soviet Union and by D. Hogness in the United States [2]. Currently, many researchers have been extensively studying mobile elements of different types, including retrotransposons, mechanisms of their transposition, their role in the genome, and systems of genetic instability [3–5].

As an intermediate stage of the life cycle, retrotransposons copy their RNA by reverse transcription to obtain a DNA copy that integrates in host DNA with the aid of integrase [6, 7]. Sequences of retrotransposons carry regulatory sites (promoters) recognized by nuclear factors of transcription initiation for RNA synthesis with polymerases II and III. Most sequences in retrotransposons are inactivated due to mutations and transcribed only in part. Specific retrotransposons in various species may be completely inactive, rarely active, or permanently active [4]. In some cases, the number of retrotransposon copies constitutes up to 90% of the nuclear genome [6].

Sequences of LTR retrotransposons are employed to identify polymorphism of analyzed forms belonging to a single species by PCR-fingerprinting technique by IRAP, REMAP, and SSAP methods [8–10]. In Alu-PCR (SINE-PCR), the regions located between short retrotransposons without LTR—SINEs (Short Interspersed Elements)—are used [11, 12].

IRAP (Inter-Retrotransposon Amplified Polymorphism) is a method for amplification of genomic DNA between closely located sequences of retrotransposons [8, 9, 13]. The product of PCR amplification of genomic DNA is a stable genetic IRAP-marker. In this case, polymorphism is caused by a mutation in the site of binding with primer or by unique biological process (retrotransposition resulting from insertion of retrotransposon into a novel site of genomic DNA without a loss of the original site).

The aim of the present work was developing the IRAP method for five rare relic species of plants from the Perm' region by cloning sites of genomic DNA containing LTR retrotransposons and subsequent screening for the appropriate primers, using primers from retrotransposons for PCR-fingerprinting, and conducting genetic analysis of polymorphism in six populations of rare relic species *A. vernalis*.

MATERIALS AND METHODS

Five rare relic species of decorative and medicinal plants located in Perm' region became the object of this study: *Adonis vernalis* L. and *Adonis sibirica* Patrin ex Ledeb. from the family Ranunculaceae, *Paeonia anomala* L. from the family Paeoniacea, *Adenophora lilifolia* (L.)A.DC. from the family Campanulaceae, *Digitalis grandiflora* Mill. from the family Scrophulariaceae, with the category of threatened state 3 (R), a rare species [14]. For analysis of DNA molecular–genetic polymorphism, leaves were collected from 30 randomly chosen plants of each species located 30–50 m apart. DNA was isolated using the method of A.M. Torres et al. [15] with minor modifications. An

examination of population structure and molecular–genetic analysis of five rare plant species were conducted from 1994 to 2009 in the molecular–genetic laboratory of Department of Plant Botany and Genetics, Perm' State University. Cloning, analysis of DNA sequences, choosing LTR primers, and assessment of their effectiveness were made in the Plant Genomics Laboratory at the Institute of Biotechnology, University of Helsinki. Sequences of retrotransposons were amplified from genomic DNA by the method of retrotransposon universal primers [16]. A DNA fragment was extracted from agarose gel according to the protocol of QIAGEN, and DNA fragments were ligated to the pGEM-T (Promega) plasmid T-vector. Plasmid DNA was transformed into *Escherichia coli* cells of strain JM109. Cells with the plasmid carrying an insertion of foreign DNA fragment were detected through white-blue selection on a medium with ampicillin, X-Gal, and IPTG. Positive colonies were tested for the presence of cloned PCR products in the PCR reaction with universal pUC primers (forward and reverse M13 primers). DNA sequences were analyzed in a capillar ABI3700 sequencer (Applied Biosystems). Cloning of the entire retrotransposon sequence was conducted in inverted PCR with extension synthesis using closely located LTR-primers with divergent orientation. The amplification product contains both LTR sequences and the whole core sequence of the retrotransposon. Amplification was run at high temperature of annealing, in double-step PCR (denaturation at 95°C for 30 s; primer annealing and synthesis simultaneously at 68°C for 4 min) during 15 cycles using Phusion DNA Polymerase (Finnzymes). Cloning of extended amplification products was conducted, as in the case of short PCR products, in the pGEM-T vector, the product of amplification being previously prepared for ligation by adding to blunt ends the dTs (each at the 3' end) with *Taq* polymerase. In accordance with the conserved regions of oppositely directed LTR retrotransposons, primers were developed by means of the FastPCR program [17]. For IRAP analysis of five rare species in Ural, 70 primers were synthesized in MWG Biotech AG.

For the approbation of identified IRAP-markers, a rare species *A. vernalis* was chosen, the most promising for the treatment of coronary heart diseases, which contains heart glycosides that have no cumulative effect and do not accumulate in the heart muscle [18]. We studied six *A. vernalis* populations located in the island part of Cungur forest-steppe at least 20 km apart. The most northern population (the second *Av2*) is located on the Spass mountain in the Cungur district 45 km from the nearest population. The first (*Av1*) and third (*Av3*) populations are located in the northern part of Cungur forest-steppe within the Ordin oblast'. The fourth (*Av4*), fifth (*Av5*) and sixth (*Av6*) populations are encountered in the central region of the island territory of Cungur forest-steppe within the Oktyabr' oblast'.

The reaction mixture (25 μl) used for PCR, which was conducted by the IRAP method, contained 25 ng of DNA, 1× PCR buffer (20 mM Tris–HCl, pH 8.8, 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$), 0.2 μM primer, 0.2 mM dNTP, 1 unit of *Taq* polymerase FirePol (Solis BioDyne), 0.04 U of *pfu* polymerase (Fermentas), and 5 μl of genomic DNA. Amplification was conducted using a PTC-100 thermal cycler (BioRad) or Master Cycler (Eppendorf) and 0.2-ml tubes or 96-well plates. The DNA amplification program consisted of the following cycles: preliminary denaturation at 95°C for 3 min; 32 cycles at 95°C for 20 s; annealing at 60°C for 1 min; and at 68°C for 1 min. The terminating cycle of elongation lasted 5 min at 68°C. The annealing temperature varied from 55 to 68°C, depending on G/C composition of primers. Amplification products of PCR were fractionated by electrophoresis in a 1.6% agarose gel (RESolute Wide Range, BIOZym) and visualized using ethidium bromide. Gels were scanned at University of Helsinki in a FLA-5100 (Fuji) scanner or at Perm' State University in a system Gel-DocXRW (Bio-Rad). In order to determine lengths of DNA fragments, molecular marker 100 bp + 1.5 + 3 Kb DNA ladder (Sibenzim-M, Moscow) was used. Lengths of fragments were determined by means of the Quantity One computer program in the system Gel Doc XR (Bio-Rad). The effectivity of detecting DNA polymorphism was estimated for five examined species according to a 1 : 5 scale: from low (1) to high (5). To describe the genetic structure of population subgroups, the following parameters were used: an expected proportion of heterozygous genotypes (H_T) in the entire population as a measure of general gene diversity; the expected proportion of heterozygous genotypes H_S in subpopulations as a measure of its intrapopulation diversity a proportion of interpopulation genetic diversity in the general diversity, or an indication of subgroups in populations (G_{ST}) [19]. Computer-aided analysis of these data was conducted by means of a POPGENE1.31 software package and by specific macros GenA1Ex6 for MS-Excel.

RESULTS

In this work, for amplification of retrotransposon sequences from genomic DNA, we employed (Fig. 1a) the original method of retrotransposon universal primers [16] including amplification of tRNA primer-binding sites (PBS) identified in the core portion of all retrotransposons. The sequence of PBS region is positioned immediately following several nucleotides after the first LTR. We identified putative LTR regions and, using the method of DNA sequence alignment, revealed conserved regions on which screening for conserved LTR primers had been conducted. The complete sequence of LTR retrotransposon was determined through inverted amplification with primers specific for the detected LTR region. Some nucleotide

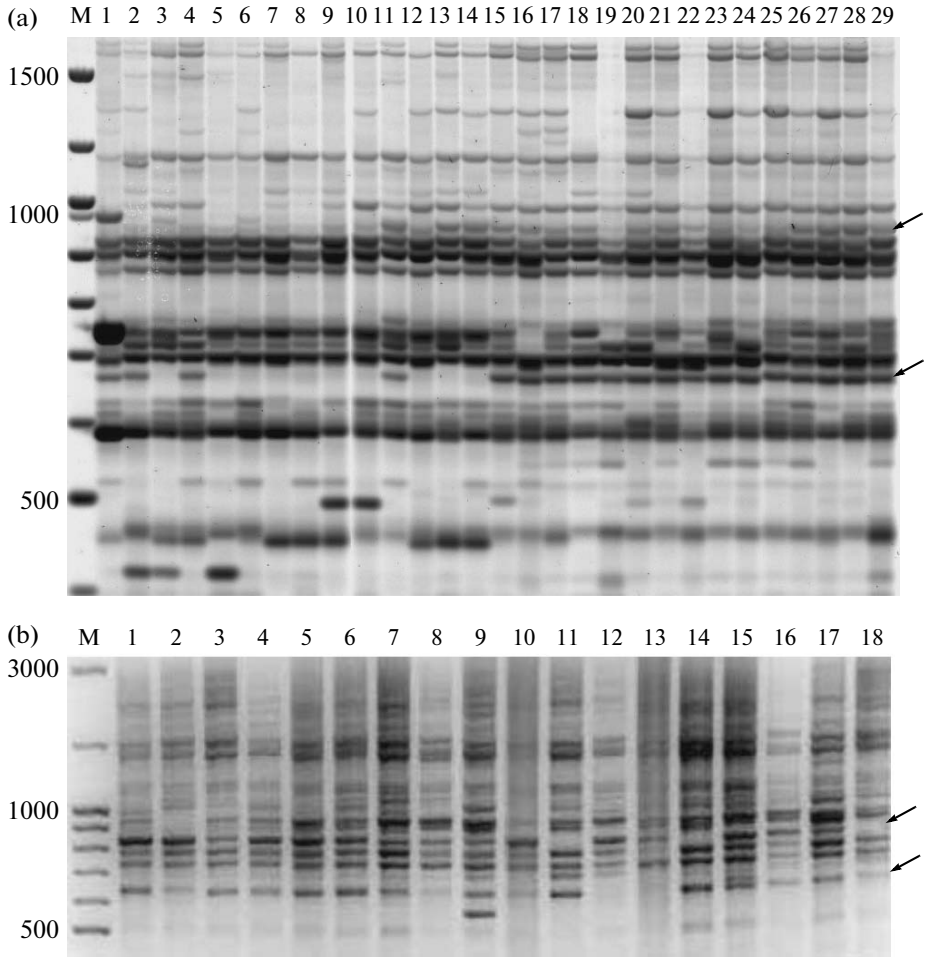

Fig. 1. IRAP spectra of *A. vernalis*: (a), *Av5* populations with primer 2079 (5'-AGGTGGGCGCCA-3') as a result of using the method of retrotransposon universal primers; (b), *Av6* populations with primer 2204. Ordinals indicate numbers of samples; M, molecular marker; arrows mark several polymorphic DNA fragments. Some of the spectra are presented.

sequences of fragments of LTR retrotransposons for the examined plant species located in the territory of Perm' were registered in the database NCBI and assigned the accession nos. EF191000–F191012 (http://www.ncbi.nlm.nih.gov/).

For IRAP analysis of five rare relic plant species, 70 LTR primers [20] for putative LTR sequences were chosen. Each primer was analyzed separately in PCR (IRAP) with genomic DNA of examined species. As a result, effective primers were detected (Table 1), and their species affiliation was determined.

Molecular–genetic analysis of six *A. vernalis* populations was conducted with five most informative LTR primers (Table 2). Analyzing PCR-amplified DNA fragments with five LTR-primers in six studied *A. vernalis* populations, we found 127 DNA fragments, 118 of which were polymorphic. The number of amplified DNA fragments varied in the total plant sample from 19 (2202) to 31 (2197), depending on the primer. In IRAP analysis, one primer, on average, is responsible for initiating the synthesis of 25 DNA fragments *A. vernalis*. The number of polymorphic fragments varied in the total *A. vernalis* plant sample from 17 to 30, their sizes amounting to 190–2500 bp (Fig. 1b; Table 2). Results obtained in analysis of DNA polymorphism, which was conducted in two geographically distant laboratories, are identical.

A proportion of polymorphic loci (P_{95}) in the total sample varied from 86 to 96%, depending on the LTR primer and amounted to 93% per sample (Table 2). The polymorphism level of amplified fragments of *A. vernalis* DNA obtained in PCR with five LTR primers varied from 54% in *Av4* to 77% in *Av5* and *Av6*.

The expected heterozygosity at loci (H_E) in the total sample of *A. vernalis* amounted to 0.291. *Av5* exhibits the highest parameter ($H_E = 0.270$), whereas the lowest value was observed in *Av1* ($H_E = 0.177$) (Fig. 2). The absolute number of alleles per locus (n_a) (in our case, per DNA fragment) in the total sample of *A. vernalis* constituted 1.992, and the effective number of alleles per locus (n_e) amounted to 1.497. Both

Table 1. Primers from LTR sequences of retrotransposons in examined species

No.	Sequences of LTR primers (5' ⟶ 3')	Source of LTR	*Adonis vernalis*, *A. sibirica*	*Digitalis grandiflora*	*Paeonia anomala*	*Adenophora lilifolia*
2156	ACAAGTTGTCCAAGGGCTTTCCTC	*Adenophora lilifolia*	**2**	**1**		**5**
2157	AGGTGGGCGCCAAACTGTTTTGG	*Adenophora lilifolia*	**2**			**4**
2194	CTACTGATCATGATGCCGCTG	*Adenophora lilifolia*	**1**			**2**
2196	CCGGCGAGTTCAGCATGTCG	*Adenophora lilifolia*	**1**			**4**
2175	TTAGACCCGGAACCGCCGTG	*Adonis sibirica*	**4**	**1**	**2**	
2209	AATTGGTCAAGAGTGGAGAGGAC	*Adonis sibirica*	**2**			**1**
2211	GTTGGAGTGTATAGTCCCACATCG	*Adonis sibirica*	**3**			
2149	GTAGTTTCGGGTTCGGAATTGCA	*Adonis vernalis*	**2**	**1**		
2197	GAAGTACCGATTTACTTCCGTGTA	*Adonis vernalis*	**4**			
2198	ATCCTTCGCGTAGATCAAGCGCCA	*Adonis vernalis*	**4**			
2200	ATGTGACAGTCGACTAACCAC	*Adonis vernalis*	**3**			
2201	CCTAGGTGGTTAGTCGACTGTCAC	*Adonis vernalis*	**5**			
2202	TGGCGCTTGATCTACGCGAAGGA	*Adonis vernalis*	**5**			
2203	ATCCCACAACTTGGACGTTTGCTG	*Adonis vernalis*	**3**			**1**
2204	AACTTGATCCAGATCATCTCC	*Adonis vernalis*	**4**			
2155	AGCTTGATATCCCGCCCCGGTCAA	*Digitalis grandiflora*		**5**		**1**
2158	CCATCGGGTCCGGGCAATATCG	*Digitalis grandiflora*		**4**		**1**
2159	AGCGAATCAACAGGGGCTGCCCGA	*Digitalis grandiflora*	**2**	**3**		**3**
2183	TTGCAAATACCAGTGGCGGGTCGT	*Digitalis grandiflora*	**2**	**2**		
2185	AATTCCACAACCGCTAGTGGCG	*Digitalis grandiflora*		**4**		**1**
2186	CGGTTTAGAACGCCACAAATGG	*Digitalis grandiflora*		**4**		
2152	AGTGAGCATGGGAGCGGACAAGC	*Paeonia anomala*	**2**		**4**	**4**
2153	ATCTTTTGAGACCAAGCTTCCGTC	*Paeonia anomala*			**2**	**1**
2164	GTGTCTCCCAGTCAAAGCGGACAA	*Paeonia anomala*		**3**		
2165	GTTCTCCTTACTAGCCGATGTGGGA	*Paeonia anomala*		**1**		**2**
2187	TGATTCCTAAGCATGGTACAAC	*Paeonia anomala*				**3**
2216	TACTATGTGAACGGGTCTGGGCTG	*Paeonia anomala*		**1**	**4**	**3**

Note: Primers with low efficiency are not presented (only 1 or 2).

parameters were maximal in *Av5* (n_a = 1.859, n_e = 1.445) and minimal in *Av1* (n_a = 1.617, n_e = 1.281).

The expected proportion of heterozygous genotypes per subdivided population of *A. vernalis* (H_T) inferred from polymorphism of PCR fragments in the overall population is equal to 0.305, being 0.225 in subpopulations (H_S). Thus, an expected proportion of heterozygous genotypes in *A. vernalis* subpopulations is lower than that in the overall population. The population subdivision coefficient (G_{ST}) indicates that the interpopulation component of genetic diversity in *A. vernalis*. is equal to 26%. The examined populations of rare relic species of *A. vernalis* plants are strongly differentiated.

DISCUSSION

The sequences of retrotransposons in eukaryotic genomes are interesting from the viewpoint of evolution. In some cases, the total number of all retrotransposons (with and without LTR) can amount to 90% of the nuclear genome of plants and animals [6, 7]. Retrotransposons were successful as applied to genetic analysis with the employment of various fingerprinting techniques: hybridization on a membranes or in PCR [8, 10]. When there are sequenced retrotransposons, it is possible to select primers for their most conserved sites, which can be used for PCR fingerprinting. In this connection, a search for retrotransposon sequences is necessary and perspective for new species. Sequences

Table 2. Analysis of DNA polymorphsm in *A. vernalis* L. populations by the IRAP method

IRAP primers	Nucleotide sequence (5' → 3')	Sizes of DNA fragments, bp	Number (frequency) of polymorphic fragments in populations						Number of registered DNA fragments	Number (frequency) of polymorphic DNA fragments
			Av1	*Av2*	*Av3*	*Av4*	*Av5*	*Av6*		
2175	TTAGACCCG-GAACCGCCGTG	190–2400	16 0.695	13 0.542	18 0.783	18 0.783	19 0.792	22 0.917	24	23 0.958
2198	ATCCTTCGCGTA-GATCAAGCGCCA	310–2470	18 0.600	16 0.533	18 0.600	22 0.733	18 0.600	21 0.700	30	26 0.867
2202	TGGCGCTTGATC-TACGCGAAGGA	300–1650	11 0.579	11 0.579	11 0.579	10 0.526	14 0.737	16 0.842	19	17 0.895
2200	ATGTGACAGTC-GACTAACCAC	360–2500	16 0.696	14 0.609	13 0.565	7 0.304	16 0.696	18 0.783	23	22 0.956
2197	GAAGTACCGATT-TACTTCCGTGTA	340–2430	13 0.419	28 0.983	20 0.645	12 0.387	31 0.969	21 0.677	31	30 0.68
Total			74 0.583	82 0.646	80 0.630	69 0.543	98 0.772	98 0.772	127	118 0.929

Note: Designations of populations are given in the text.

of retrotransposons can be obtained with various approaches; for instance, by amplification of reverse transcriptase gene and subsequent cloning of the closely located LTR site. Another method is based on amplification of genomic DNA with a single primer (or combined with the other primer) complementary to PBS region. PCR fragments amplified by this method contain inverted PBS sequences immediately followed by putative LTR sequences [16]. The sequences of various retrotransposons in the chromosomal DNA undergo clustering and interact with each other; therefore, the probability that LTR fragment may be found upon amplification with primers complementary to PBS region is rather high [16].

Analysis of 70 developed LTR primers revealed that they are species-specific primers, i.e., fingerprinting is observed only for the specific primer, from which the retrotransposon was isolated. In related species,

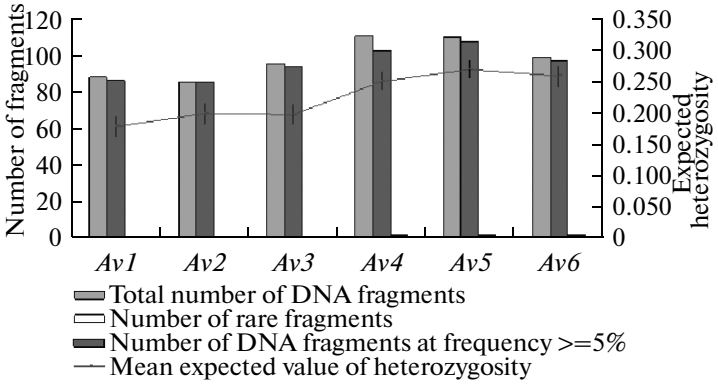

Fig. 2. Parameters of genetic diversity in six populations of *A. vernalis*.

sequences of identical LTR retrotransposons differ in accordance with the degree of relatedness among analyzed species [16]. In this respect, LTR primers developed in related species for one of them can be used to amplify DNA of other species. Many more retrotransposons have been detected in genomes of plants and animals, and therefore, the IRAP method has high potential for providing information (the number of loci and their polymorphism) [21, 22]. New retrotranspositions in the host genome may, depending on their location, lead to changes in gene activity, induce chromosomal alterations, dynamically affect chromosome sizes, and also promote recombination of chromosomes upon crossing over [4].

Molecular–genetic analysis of DNA conducted by means of the IRAP method revealrd that the first (*Av1*) population of *A. vernalis* located in the Ordin oblast' exhibits the lowest values of genetic diversity (P_{95} = 58%; H_E = 0.177; n_e = 1.281), whereas the highest parameters were observed in *Av5* located in the Oktyabr' oblast' (P_{95} = 77%; H_E = 0.270; n_e = 1.445).

PCR method, in which primers from highly repeated elements (such as retrotransposons) are used, allows effective detection of intraspecific polymorphism. With the employment of IRAP technique, molecular–genetic analysis was conducted, genetic diversity of rare relic plant species [23–25] as well as widely distributed resource species [26] was studied, and a method of molecular–genetic identification and classification of gene pools in rare and defence-requiring plant species was designed [24].

Thus, the use of DNA-fingerprinting technique developed on the basis of sequences of retrotransposons proved successful for the evaluation of intra- and interpopulation genetic diversity of rare plant species and for characterization of the state of their gene pools.

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