Sel'skokhozyaistvennaya Biologiya [Agricultural Biology], 2011, № 5, p. 45-50

UDC 635.649:577.29:57.2.08

POLYMORPHISM OF MICROSATELLITE LOCUS CAMS-336 IN PEPPER VARIETIES AND CLOSELY RELATED SPECIES

***E.A. Snigir*[1, 2], *O.N. Pyshnaya*[2], *E.Z. Kochieva*[1, 3], *N.N. Ryzhova*[1]**

[1] *Scientific center "Bioengineering", RAS, Moscow 117312, Russia*
e-mail: ekochieva@yandex.ru

[2] *All-Russia Research and Development Institute of Vegetable Crops Selection and Seed Growing, RAAS, Moscow province, Odintsovo region, Lesnoy Gorodok 143080, Russia*
e-mail: pishnaya_o@mail.ru

[3] *M.V. Lomonosov Moscow State University, Moscow 119899, Russia*

Received October 19, 2010

S u m m a r y

The polymorphism of microsatellite locus CAMS-336 was investigated in 45 pepper varieties of native and foreign selection, and also in variants of closely related cultivated species of the *Capsicum frutescens, C. chinense, C. baccatum*. The authors revealed 6 alleles of this locus, distinguished in length, and determined the frequency of its occurrence. The unique alleles for some varieties and species were isolated and the PIC quantities for complete set of variants were calculated. The allelic variability was confirmed by sequencing, which permitted to determine the accurate nucleotide sequence and the size of revealed alleles of microsatellite locus CAMS-336. In addition, the sequencing of this locus permits to determine the mechanism of appearance of new B and D alleles, notably point substitution of T for A. The obtained data suggests the possibility of the use of microsatellite locus CAMS-336 for issue passports to the native varieties of *C. annuum* L.

Keywords: SSR analysis, *Capsicum annuum*, cultivar fingerprinting.

Identification and certification of cultivated plant varieties is one of the main ways to protect intellectual property rights in plant breeding and seed production. Currently, this problem is being widely solved by molecular labeling of genomes, primarily using SSR-analysis (simple sequence repeats) of microsatellite loci - short (less than 6 nucleotides) tandemly repeated DNA sequences. Such repeats are found in both hetero- and euchromatic regions of plant genomes including exons, introns and intergenic sequences; as a rule, they are uniformly distributed over the genome (1). The number of repeats within a microsatellite locus as well as its length can differ even in closely related genotypes, while the flanking sequences in genotypes of the same species are similar (2, 3). Variable number of repeats within a microsatellite is the result of DNA slippage during replication or unequal crossing-over (4). The analysis of allelic polymorphism of microsatellite loci is a highly discriminative technique, that's why it is widely used for genotyping plant cultivars, during intervarietal and introgressive hybridization (3, 5-12).

It has been repeatedly confirmed the efficiency of SSR-markers for assessing the inter- and intravarietal polymorphism, as well as for genotyping varieties and strains of cultivated crops (3, 13-22). Solving these tasks requires determining the set of most informative microsatellite loci and primers, owing to high variability of source genetic material used for creation of modern cultivars in different countries. It's quite often that SSR-markers recommended for genotyping one particular set of varieties can be less efficient for identification of other samples (13, 16)

There are only few works on studying microsatellite loci in the genome of pepper (20-22). The most informative data are presented in the report about obtaining the genetic map of *Capsicum annuum* based on SSR-markers (22). The analysis of the microsatellite loci CAMS-336 in seven varieties of Japanese and American selection has revealed the allelic polymorphism with high levels of PIC (polymorphism information content) (22).

The purpose of this work was characterization of the microsatellite loci CAMS-336 and determining its diversity in cultivated varieties of pepper *Capsicum annuum* L. and in samples of closely related species, as well as assessing the possibility of using this locus for certification of domestic varieties of *C. annuum* L.

Technique. The object of study were 45 cultivars, hybrids and lines of pepper *C. annuum* L. of domestic and foreign selection obtained from the collection of the All-Russia Research and Development Institute of Selection and Seed Growing of Vegetable Crops, as well as the samples of closely related cultivated species - *C. frutescens, C. chinense, C. baccatum*.

Total plant DNA was isolated from 8-10-day-old seedlings using the method proposed by K. Edwards et al. (23), with additional deproteinization with phenol-chloroform mixture (1:1). This technique provides rapid isolation of total DNA of high quality ($OD_{260/280}$ 1,6-1,9) at the quantity more than 5 micrograms.

Polymerase chain reaction (PCR) was performed using a set of reagents ("Dialat", Russia). Amplification of microsatellite loci was performed in the reaction mixture of 15 ul volume containing 1× buffer from the set, 0,16 mM each dNTP, 0,3 uM primer, 0,3 units Taq-polymerase and 100 ng genomic DNA. The optimal concentration of $MgCl_2$ was selected. Amplification was performed in the thermocycler GeneAmp PCR System 2700 ("Applied Biosystems", USA) under the following regime: denaturation - 30 s at 94° C, annealing of primer – 45 s at 50 °C; DNA synthesis – 1 min at 72 °C with pre-denaturation for 5 min at 94°C (35 cycles); final elongation of PCR fragments - 10 min at 72 °C. Melting temperature of primers was found using the formula: $T_{m.} = 69{,}3 + 0{,}41$ (GC) – 650/L, where L - number of nucleotides in the primer sequence, GC - content of GC-bases in the primer,% (26). The initial temperature of annealing was calculated under the formula: $T_{ann.} = T_{m.} - 3$ °C. The reaction products were separated by electrophoresis in 1,7% agarose gel in 1× TBE buffer, stained with ethidium bromide and photographed. The molecular weight marker GeneRuler ™ 100 bp Plus DNA Ladder ("Fermentas", Lithuania) was used.

To reveal allelic polymorphism of microsatellite loci, the amplification products were separated in the denaturing 6% polyacrylamide gel (PAAG) and visualized by staining with silver nitrate from the kit SILVER SEQUENCE ™ DNA ("Promega", USA) according to manufacturer's recommendations. Allelic polymorphism of SSR-locus was assessed using PIC value: $PIC = 1 - \sum p_i^2$, where p_i - frequency of the i^{th} allele in a sample (24).

The primary sequences were determined on the ABI 310 capillary DNA Analyzer (USA). Nucleotide sequences were analyzed in the program Mega 3.0 (25).

Results. Earlier, the optimal conditions for amplification of CAMS-336 locus with primers S3F and S3R were found using 5 DNA samples: annealing temperature of primers – 56 °C, the concentration of $MgCl_2$ – 1,1 mM at other standard parameters (22).

Under these conditions, the fragments of expected lengths (150-200 bp) were obtained for all investigated samples (Fig. 1). The only one major fragment was detected in 1,7% agarose gel. The electrophoresis allowed identifying four alleles of the microsatellite in *C. annuum* cultivars and two other allelic variants in samples of *C. chinense* and *C. baccatum*.

The separation of the obtained PCR fragments in denaturing PAAG has resulted in revealing 6 alleles of CAMS-336 locus (A, B, C, D, E and F) (Fig. 2).

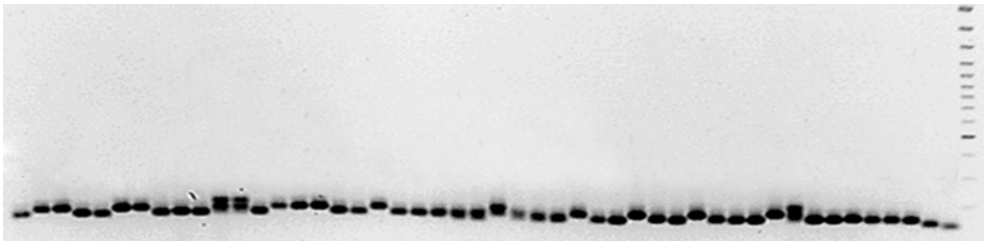

Fig. 1. Electrophoregram spectra in 1,7 % agarose gel showing the products of PCR-amplification of CAMS-336 locus in 45 cultivars, hybrids and lines of pepper *Capsicum annuum* L., and in closely related cultivated *Capsicum* species (left to right)**:** Kaliforniiskoe Chudo, Madonna, Spady, Rubin, Salad Festival, Marconi pepper, Chimes, F_1 Raisa, F_1 Purpurnaya Krasavitsa, Mazurka, Oranzhevoe Chudo, Pirati, Memphis, RS 87001, Hybrid 167, Bolero, Sharm, Shregeda, Benda, Medal', F_1 Ekaterina, Belosnezhka, Maria, Zheltyi Buket, Ezhik, Mayak, Kaskad, Zolotoi Dozhd', F_1 Khrizolit, F_1 Ocharovanie, Karlik, F_1 Sibiryak, Malysh, F_1 Ruza, F_1 Aria, F_1 Isabella, Zlatozar, Line 71, Siren', Agapovsky, Zdorov'e, Mavr, Rodnik, Nezhnost', Sirenevyi Tuman, *C. frutescens*, *C. chinense*, *C. baccatum;* rightmost – molecular weight marker GeneRuler™ 100 bp Plus DNA Ladder.

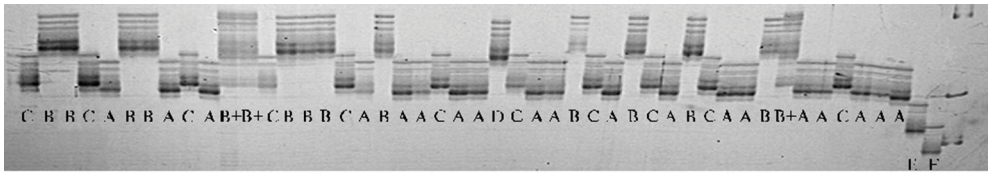

Fig. 2. Electrophoregram spectra in 6 % denaturing polyacrylamide gel showing the products of PCR-amplification of CAMS-336 locus in 45 cultivars, hybrids and lines of pepper *Capsicum annuum* L., and in closely related cultivated *Capsicum* species (left to right)**:** A, B, C, D, E and F — alleles of the locus ("+" indicates the assumed heterozygous state), rightmost – molecular weight marker GeneRuler™ 100 bp Plus DNA Ladder.

A allele was detected in cultivars Salad Festival, Mazurka, Shregada, Medal', Ekaterina, Maria, Zheltyi Buket, Kaskad, Zolotoi Dozhd', Karlik, Zlatozar, Zdorov'e, Mavr, Nezhnost, Sirenevyi Tuman, F1 Raisa, F1 Ruza, Line 71, and in the species *C. frutescens;* allele B – in cultivars Madonna, Spady, Marconi pepper, Chimes, Oranzhevoe Chudo, Pirati, RS 87 001, Bolero, Aria, Siren', Agapovsky, F1 Khrizolit, F1 Sibiryak, Hybrid 167; allele C – in cultivars Kaliforniiskoe Chudo, Rubin, Memphis, Sharm, Belosnezhka, Mayak, Malysh, Isabella, Rodnik, F1 Purpurnaya Krasavitsa, F1 Ocharovanie. The cultivar Ezhik was found to carry the variety-specific allele D. Specific alleles E and F were detected in samples of *C. chinense* and *C. baccatum*, respectively; the lengths of these alleles were significantly distinct from that of *C. annuum*. In cultivars Oranzhevoe Chudo, Agapovsky, Pirati, CAMS-336 locus was represented by two fragments of different lengths, which suggests its heterozygous state (B +).

The frequency of each allele of the microsatellite locus CAMS-336 was determined as the ratio of the number of varieties carrying this allele to the total number of analyzed cultivars. Thus, for A allele it amounted to 40, for B allele - 31, C allele – 23, for alleles D, E and F - 2%. So, A, B and C allelic variants were the most widespread and they provided allelic variability in 94% cultivars.

The value of PIC for a total set of samples was 0,69; for samples of *C. annuum* - 0,67; for cultivars of domestic selection – 0,62; for varieties of European selecton - 0.57. In earlier study of Y. Minamiyama et al. (22), 14 samples of pepper have been analyzed including seven samples of *C. frutescens, C. chinense, C. baccatum, C. shacoense* and *C. pubescens*, and 7 varieties of *C. annuum* obtained from different breeding centers. For a total set of samples there have been identified 6 alleles, and PIC value of 0,82. In 7 samples of *C. annuum,* four alleles and PIC value of 0,61 have been detected. Therefore, the PIC values of pepper varieties are generally comparable in both studies. Somewhat larger value of PIC in species can be explained by the fact that Japanese researchers analyzed representatives of *C. chacoense* and *C. pubescens*, which were not used in this work.

For explanation of the obtained data on the microsatellite alleles and using the microsatellite loci CAMS-336 in cultivar certification, the results of analysis in PAAG must be confirmed by definition of primary sequences of these allelic variants. This was done by sequencing all six types of alleles using forward and reverse primers.

```
B  TTCACCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCACTCTCAGTATTAG
D  TTCACCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC--ACTCTCAGTATTAG
C  TTCACCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC----------AGTATTAG
A  TTCACCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC----------AGTATTAG
E  TTCACCCTCTCTCTCTCTCTCTCTCTC----------AGTATTAG
F  TTCACCCTCTCTCTCTCTCTC----------AGTATTAG
```

Fig. 3. Nucleotide sequences of alleles A, B, C, D, E and F of CAMS-336 locus in 45 cultivars, hybrids and lines of pepper *Capsicum annuum* L., and in closely related cultivated species *C. frutescens*, *C. chinense* and *C. baccatum* identified in the program Mega 3.0.

Characteristics of alleles of CAMS-336 locus in 45 cultivars, hybrids and lines of pepper *Capsicum annuum* L., and in closely related cultivated species *C. frutescens*, *C. chinense* and *C. baccatum*

Allele	Allele length	Nucleotide composition of allele
A	157 bp	$(TC)_{16}$
B	171 bp	$(TC)_{20}AC(TC)_2$
C	159 bp	$(TC)_{17}$
D	169 bp	$(TC)_{19}AC(TC)_2$
E	147 bp	$(TC)_{11}$
F	141 bp	$(TC)_8$

Each of the resulting fragments contained $(TC)_n$ sequence of the analyzed microsatellite locus (Fig. 3), whose length varied in different samples depending on number of repeating units in the microsatellite sequence. The analysis of sequenced CAMS-336 has confirmed the data of PAAG electrophoresis: there were revealed 6 alleles ranging in size from 141 to 171 bp and differing by number of repeats of the microsatellite unit (Table). Alleles B and D were characterized by the transversion T → A in one of the repeated microsatellite units, which has resulted in a new microsatellite type instead of the common: $(TC)_n \rightarrow (TC)_nAC\ (TC)_n$.

Thus, polymorphism of the microsatellite locus CAMS-336 has been assessed in 45 varieties, hybrids and lines of pepper *Capsicum annuum* L. of domestic and foreign selection, as well as in samples of closely related cultivated species. Six allelic variants have been identified including A, B and C alleles found to be the most frequent in the studied set of samples. Allelic diversity has been confirmed by results of direct sequencing. The exact nucleotide composition of CAMS-336 locus and size of its allelic variants have been determined, as well as the origin of new alleles B and D. The possibility of using the microsatellite loci CAMS-336 for certification of domestic varieties of *C. annuum* L. has been suggested.

REFERENCES

1. Katti M.V., Ranjekar P.K. and Gupta V.S., Differential Distribution of Simple Sequence Repeats in Eukaryotic Genome Sequences, *Mol. Biol. Evol.,* 2001, vol. 18, no.7, pp. 1161-1167.
2. Toth G., Gaspari Z. and Jurka J. Microsatellites in Different Eukaryotic Genomes: Survey and Analysis, *Genome Res.,* 2000, no. 10, pp. 967-981.
3. Varshney R.K., Graner A. and Sorrells M.E., Genetic Microsatellite Markers in Plants: Features and Applications, *Trends Biotechnol.,* 2005, vol. 23, pp. 48-55.
4. Schlotterer C. and Tauz D., Slippage Synthesis of Simple Sequence DNA, *Nucl. Acids Res.,* 1992, vol. 20, pp. 211-215.
5. Kochieva E.Z., Using Methods Based on Polymerase Chain Reaction for Marking Plant Genomes, *S.-kh. biol.,* 1999, no. 1, pp. 1-19.
6. Kochieva E.Z. and Ryzhova N.N., Using PCR-Amplification Based on Satellite Sequences for Marking the Genomes of Different Pepper Species, *S.-kh. biol.,* 2001, no. 1, pp. 94-97.
7. Tommasini L., Batley J., Arnold G.M., Cooke R.J., Donini P., Lee D., Law J.R., Lowe C., Moule C., Trick M. and Edwards K.J., The Development of Multiplex Simple Sequence Repeat (SSR) Markers to Complement Distinctness, Uniformity and Stability Testing of Rape (*Brassica napus* L.) Varieties, *Theor. Appl. Genet*., 2003, vol. 106, pp. 1091-1101.
8. Spooner D.M., Nunez J., Trujillo G., Herrera M.R., Guzman F. and Ghislain M., Extensive Simple Sequence Repeat Genotyping of Potato Landraces Supports a Major Reevaluation of Their Gene Pool Structure and Classification, *PNAS USA*, 2007, vol. 104, no. 49, pp. 19398-19403.
9. Koopman W.J.M., Li Y., Coart E., Van deWeg E., Vosman B., Roldan-Ruiz I. and Smulders M.J.M., Linked vs. Unlinked Markers: Multilocus Microsatellite Haplotype-Sharing as a Tool to Estimate Gene Flow and Introgression, *Mol. Ecol.,* 2007, vol. 16, pp. 243-256.
10. He C., Poysa V. and Yu K., Development and Characterization of Simple Sequence Repeat (SSR) Markers and Their Use in Determining Relationships among *Lycopersicon esculentum* Cultivars, *Theor. Appl. Genet.,* 2003, vol. 106, pp. 363-373.
11. Macaulayet M., Ramsay L., Powell W. and Waugh R., A Representative, Highly Informative «Genetyping Set» of Barley SSRs, *Theor. Appl. Genet.,* 2001, vol. 102, pp. 801-809.
12. Ghislain M., Spooner D.M., Rodriguez F., Villamón F., Núñez J., Vásquez C., Waugh R. and Bonierbale M., Selection of Highly Informative and User-Friendly Microsatellites (SSRs) for Genotyping of Cultivated Potato, *Theor. Appl. Genet*., 2004, vol. 108, pp. 881-890.
13. Barandalla L., de Galarreta R.J.I., Rios D. and Ritter E., Molecular Analysis of Local Potato Cultivars from Tenerife Island Using Microsatellite Markers, *Euphytica*, 2006, vol. 152, pp. 283-291.
14. Moisan-Thiery M., Marhadour S., Kerlan M.C., Dessenne N., Perra-Mant M., Gokelaere T. and Hingrat Y.L., Potato Cultivar Identification Using Simple Sequence Repeats Markers (SSR), *Potato Res.,* 2005, vol. 48, pp. 191-200.
15. Giarocco L.E., Marassi M.A. and Salerno G.L., Assessment of the Genetic Diversity in Argentine Rice Cultivars with SSR Markers, *Crop Sci.,* 2007, vol. 47, pp. 853-858.
16. De Galarreta R.J.I., Barandalla L., Lorenzo R., Gonzales J., Rios D.J. and Ritter E., Microsatellite Variation in Potato Landraces from the Island of La Palma, *Spanish J. Agric. Res.,* 2007, vol. 5, no. 2, pp.186-192.
17. Manifesto M.M., Schlatter A.R., Hopp H.E., Suárez E.Y. and Dubcovsky J., Quantitative Evaluation of Genetic Diversity in Wheat Germplasm Using Molecular Markers, *Crop Sci.,* 2001, vol. 41, pp. 682-690.
18. Bracci T., Sebastiani L., Busconi M., Fogher C., Belaj A. and Trujillo I., SSR Markers Reveal the Uniqueness of Olive Cultivars from the Italian Region of Liguria, *Scientia Horticulturae*, 2009, vol. 122, no. 2, pp. 209-215.
19. Wang L., Guan R., Zhangxiong L., Chang R. and Qiu L., Genetic Diversity of Chinese Cultivated Soybean Revealed by SSR Markers, *Crop Sci.,* 2006, vol. 46, pp. 1032-1038.
20. Prince J.P., Lackney V.K., Angeles C., Blauth J.R. and Kyle M.M., A Survey of DNA Polymorphism Within the Genus *Capsicum* and the Fingerprinting of Pepper Cultivar, *Genome*, 1995, vol. 36, pp. 404-417.
21. Nagy I., Polley A. and Ganal M., Development and Characterization of Microsatellite Markers in Pepper, *Proc. Xth Meeting on Genetics and Breeding of Capsicum and Eggplant (Avignon, France, 7-11 September, 1998),* Paris: INRA, 1998, pp. 235-237.
22. Minamiyama Y., Tsuro M. and Hirai M., An SSR-Based Linkage Map of *Capsicum annuum, Mol. Breed.,* 2006, vol. 18, pp. 157-169.
23. Edwards K., Johnstone C. and Thompson C., A Simple and Rapid Method for the Preparation of Plant Genomic DNA for PCR Analysis, *Nucl. Acid Res*., 1991, vol. 19, no. 6, p. 1349.
24. Nei M., Analyses of Gene Diversity in Subdivided Populations, *PNAS USA*, 1973, vol. 70, pp. 3321-3323.
25. Kumar S., Tamura K. and Nei M., MEGA3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment, *Brief. Bioinform.,* 2004, vol. 5, pp. 150-163.