Allelic and epigenetic DNA variation in relation to F_1 heterosis manifestation in F_1 hybrids of *Capsicum annuum* L.

M.N. Shapturenko[1] ✉, S.V. Vakula[1], L.A. Tarutina[1], T.V. Nikitinskaya[1], T.V. Pechkovskaya[1], L.A. Mishin[2], L.V. Khotyleva[1]

[1] Institute of Genetics and Cytology of the National Academy of Sciences of Belarus, Minsk, Belarus
[2] Institute of Vegetable Growing, Samokhvalovichy, Minsk region, Belarus

Managing F_1 heterosis is one of the major objectives in hybrid crop breeding programs. The classical theory considers the heterozygosity in F_1 hybrids to be the main factor contributing to heterosis and therefore presumes a linear relationship between the value of genetic polymorphisms in parental lines and the heterotic response of their F_1 offspring. Therefore, the genetic diversity information is viewed as a tool for selection of promising cross-combinations, but results published by different researchers are inconsistent. In this work, we studied the contributions of structural and nonstructural DNA polymorphisms to F_1 heterosis manifestation. We used SSR and methyl-sensitive AFLP (MSAP with *Hpa*II and *Msp*I izoshisomers) protocols for obtaining specific patterns for heterotic and nonheterotic F_1 hybrids of sweet pepper (*Capsicum annuum* L.) from a Belarusian breeding program. We found out that a certain portion of heterosis for yield-related traits might be explained by the polymorphism revealed by SSR analysis. According to our data, the total number of polymorphic SSR loci and the ratio of polymorphic and nonpolymorphic loci demonstrate a significant predictive value and can serve as additional prognostic criteria for the selection of promising cross-combinations. From the MSAP assay, we found a relationship between heterosis and the numbers of methylated and nonmethylated DNA loci for yield traits. Our results indicate that cross-hybridization may favor epiallelic modifications in F_1 hybrids, presumably responsible for heterosis. Thus, epigenetic DNA variation may explain the absence of a linear relationship between the level of structural DNA divergence and F_1 heterosis, as well as the manifestation of heterosis in crosses of related (genetically similar) accessions.

Key words: *Capsicum annuum* L.; heterosis; F_1 performance; SSR allelic variation; DNA methylation.

Аллельная и эпигенетическая вариация ДНК в связи с проявлением гетерозиса в F_1 *Capsicum annuum* L.

М.Н. Шаптуренко[1] ✉, С.В. Вакула[1], Л.А. Тарутина[1], Т.В. Никитинская[1], Т.В. Печковская[1], Л.А. Мишин[2], Л.В. Хотылева[1]

[1] Институт генетики и цитологии Национальной академии наук Беларуси, Минск, Беларусь
[2] Институт овощеводства, Самохваловичи, Минская область, Беларусь

Одна из основных задач селекции – получение гибридов с выраженным эффектом гетерозиса. При этом информация о генетическом разнообразии селекционного материала рассматривается как инструмент отбора перспективных комбинаций скрещивания, так как, согласно классической теории, гетерозиготность является основным фактором, обусловливающим превосходство гибридов F_1 над родителями. В связи с этим предполагается наличие прямой зависимости между уровнем генетического полиморфизма исходных родительских форм и гетерозисом в поколении F_1 их гибридов. Опубликованные к настоящему времени данные, направленные на поиск критериев прогнозирования гетерозиса у растений, показали разноречивые результаты. В нашем исследовании мы изучили вклад структурного и неструктурного ДНК-полиморфизма в реализацию гетерозиса F_1 у перца сладкого. Были использованы SSR- и метилчувствительный AFLP-протокол (MSAP с использованием изошизомеров *Hpa*II и *Msp*I) для выявления специфичных аллельных вариантов и эпигенетических паттернов у гетерозисных и негетерозисных гибридов перца сладкого, включенных в белорусскую селекционную программу. При изучении структурного полиморфизма ДНК с использованием микросателлитных маркеров обнаружено, что часть вариации в проявлении гетерозиса может быть объяснена полиморфизмом, который выявляется при SSR-анализе. Согласно нашим результатам, общее число полиморфных локусов и коэффициент соотношения полиморфных и мономорфных локусов могут служить дополнительным критерием отбора перспективных комбинаций скрещивания наряду с классическими методами селекции. При изучении эпигенетических модификаций ДНК, возникающих при гибридизации, была обнаружена тесная связь между статусом метилирования ДНК и гетерозисом для основных показателей продуктивности гибридов перца сладкого. Полученные результаты подтвердили предположение о том, что гибридизация способствует возникновению эпиаллельной вариации ДНК у гибридов первого поколе-

✉ e-mail: marinashapturenko@gmail.com

ния, которая может обусловливать гетерозис в F₁. Таким образом, существование эпигенетической вариации ДНК способно объяснить отсутствие линейной связи между уровнем структурной ДНК-дивергенции и F₁ гетерозисом, так же как и проявление гетерозиса при гибридизации генетически сходного материала.

Ключевые слова: гетерозис; *Capsicum annuum* L.; аллельная SSR-вариация; ДНК-метилирование.

The phenomenon of heterosis, known as superior performance of F₁ hybrids over their parents, has been exploited by agricultural practices since the beginning of the 20th century. It became a milestone event in plant breeding. The first bushels of hybrid corn seeds were sold in 1924 (Crabb, 1947), and hybrid production has been in rapid progress since that. With the large body of experimental information obtained in the study of maize hybrids and mathematical calculations, several genetic concepts were put forward to explain heterosis by various types of gene action (Shull, 1908, 1952; Bruce, 1910; East, Hayes, 1912; Jones, 1917). These concepts underwent various modifications and interpretations with new methodological approaches and knowledge about molecular mechanisms (Charlesworth, Willis, 2009; Kaeppler, 2012). According to the types of gene action, all of them refer to single-locus or multi-loci models. The first one proposes dominance and overdominance, whereas the second is focused on epistasis or nonallelic interactions. In fact, this segregation is not obvious. To date, there is evidence for single-locus heterosis (Shpak et al., 2004; Krieger et al., 2010; Singh et al., 2013), but its usefulness is limited. Most of quantitative traits are polygenic, and their phenotypic expression is influenced by multiple factors with relatively low effects. Evidently, a heterotic phenotype comes out from crosstalk of two parental strains in a context-dependent manner rather than from interaction in a single specific locus. If so, it is reasonable to assume a close relationship between heterozygosity and heterosis F₁, i. e. the heterotic expression of phenotype should be correlated with genetic diversity (Melchinger, 1999; Springer, Stupar, 2007).

Earlier studies demonstrated that the relationships between molecular marker heterozygosity and hybrid performance were highly variable, depending on germplasm, mating design, type of used markers, and the architecture of the target trait (Perenzin et al., 1998; Gutierrez et al., 2002; Schrag et al., 2009; Usatov et al., 2014). In spite of considerable efforts, DNA markers promising for prediction of heterosis have not been well developed for hybrid breeding (Reif et al., 2012; Kawamura et al., 2016).

Some investigations suggest that the regulation of heterotic response in F₁ is mediated by epigenetic modifications of DNA, in particular, methylation, which alters differential gene expression (Groszmann et al., 2011; He et al., 2013; Ryder et al., 2014; Greaves et al., 2015). It has been found that hybrids F₁ have not only parental epialleles but hybrid-specific epialleles with altered frequencies (Shen et al., 2012). Some of these alterations in the F₁ epigenome may be the first in the set of events leading to the formation of a perfect

(heterotic) phenotype. This concept assumes the key role of regulatory genes under epigenetic modifications, so that even the expression of their small portion can cause the distribution of their effect at the level of regulatory networks involved in the formation of the mature phenotype (Becker, Weigel, 2012). Probably, the differences in gene activity caused by both differential methylation of parental forms and epigenetic modifications F₁ due to hybridization influence the formation of heterotic response.

In this work we evaluated SSR allelic variation and the DNA methylation status in sweet pepper with regard to heterosis manifestation to demonstrate thereby that a heterotic phenotype can be a product of both structural and nonstructural (epigenetic) variation.

Materials and methods

Plant materials. Eleven sweet pepper accessions from different geographic areas were taken as parents in breeding aimed at developing high-yielding long-fruited hybrids (Suppl. 1)[1]. These genotypes were subjected to two full diallel crosses (5 × 5, 6 × 6). Parental and hybrid plants were grown for phenotypic evaluation in an unheated greenhouse in randomized blocks with 35 × 50 cm area for each plant in five replications. Phenotypic data were randomly collected from the middle 15 genotypes of each accession. The main yield components recorded were fruit weight per plant (FWP), fruit number per plant (FNP), average weight of one fruit (AWF), and fruit length (FL).

Microsatellite DNA assay. The 11 parental lines were fingerprinted following standard protocols with twelve simple sequence repeat (SSR) markers: Hpms1-1, Hpms1-5, Hpms1-111, Hpms1-143, Hpms1-168, Hpms1-172, Hpms2-13, Hpms2-21, CAMS-864, CAMS-236, CAMS-647, CAMS-811 (Lee et al., 2004; Minamiyama et al., 2006; Mimura et al., 2012) (Suppl. 2). The resulting amplification products were resolved on an Applied Biosystems Genetic Analyzer 3500 automated sequencer. Fragment sizes were recorded by Gene-Mapper Software Version 4.1 and manually checked.

Methyl-sensitive arbitrary polymorphism assay. Methyl-sensitive amplified polymorphism (MSAP) analysis was performed to identify methylation-susceptible anonymous 5′-CCGG sequences and assess their methylation status in sweet pepper lines and their F₁ hybrids in both seedling and flowering stages. The upper thirds of young leaves were used.

MSAP is a modification of the standard AFLP technique. It employs *Eco*RI as a rare cutter and methylation-sensitive fre-

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[1] Supplemantary Materials 1–7 are available in the online version of the paper: http://www.bionet.nsc.ru/vogis/download/pict-2018-22/appx13.pdf

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quently cutting restriction enzymes *Hpa*II and *Msp*I, which are a pair of isoschizomers recognizing the same tetranucleotide 5′-CCGG but differently sensitive to methylation at the inner or outer cytosine (Reyna-López et al., 1997). We tried several selective primers and chose a set that gave many consistently scorable bands. The primer sets used were the *EcoR*I reverse primer with one of *Hpa*II/*Msp*I forward primers (Suppl. 3).

The analysis of MSAP results was based on comparisons of binary presence–absence matrices for individuals obtained with the *EcoR*I-*Hpa*II and *EcoR*I-*Msp*I primer combinations. The presence of both *EcoR*I-*Hpa*II and *EcoR*I-*Msp*I products was denoted as the nonmethylated state. The presence of either *EcoR*I-*Hpa*II or *EcoR*I-*Msp*I corresponded to the methylated state. The analyses showing neither *EcoR*I-*Hpa*II nor *EcoR*I-*Msp*I were interpreted as uninformative, since such cases might be caused by either fragment absence or hypermethylation (Ashikawa, 2001).

The resulting MSAP products were resolved using the automated sequencer Applied Biosystems Genetic Analyzer 3500. The data on fragment size were recorded by GeneMapper Software Version 4.1 and manually checked.

Data analysis. Mid-parent (MPH) and high-parent (HPH) heterosis indices were calculated from equations

$$MPH = 100 \times \frac{F_1 - \overline{P}}{\overline{P}}, \text{ where } \overline{P} = \frac{P_1 + P_2}{2},$$

$$\text{and } HPH = 100 \times \frac{F_1 - BP}{BP},$$

where BP is the best of the parents. If a hybrid was inferior to the worse of its parents, the negative heterosis was calculated against the worse parent.

The software Treecon was used to calculate genetic distances (GD) and to construct a neighbor-joining phylogenetic tree with 100 bootstraps (Nei et al., 1983).

Genetic distances were calculated from SSR data based on the Nei and Li algorithm (Nei, Li, 1979). Differential methylation (DM) was evaluated by counting the number and ratio of methylated and nonmethylated loci for each cross-combination of lines.

The relationships between genetic distances, differential SSR allelic polymorphism (DP), differential methylation, and heterosis were assessed by correlation analysis.

Results

Quantitative analysis and hybrid performance of two diallel sets

ANOVA revealed significant ($p < 0.05$) to highly significant ($p < 0.01$) differences among pepper lines for all traits under investigation. For mating design, the lines were divided into 2 sets (I, red and II, yellow) and crossed in the 5×5 and 6×6 full diallel manner. The subsequent trial of 50 hybrids F$_1$ and its parents with analysis of variance components showed that the general (GCA) and specific (SCA) combining abilities differed from zero significantly for all traits. The ratio of the GCA:SCA variance component exceeded zero except for AWF in the 2nd group (6×6), where SCA > GCA. Therefore, GCA (or the additive effect) is expected to be responsible for the greatest part of variation in hybrid performance in this factorial.

There was a significant difference among pairwise combinations in heterotic effect for yield component traits. The mean heterosis values for two sets of hybrids were positive for all traits. Frequencies of heterosis manifestation were higher for FWP and FNP. The levels of heterosis varied broadly from one cross to another within each diallel set, and differed between two sets. The distribution of the levels of mid-parent and high-parent heterosis is presented in Table 1. The widest range of variation in MPH for FWP was observed among the crosses in the 1st group, but for FNP and FL, in the 2nd group. Crosses with high hybrid superiority (>30 %, FWP) over the mid-parent level were found in both diallel sets, but negative implementations of heterosis were more frequent in set I.

High-parent heterosis was observed in both sets of hybrids for all traits under study. Its level for FWP varied from 0.3 to 68.8 % in set I and from –14 to +68 % in set II. Among 50 diallel hybrids, only 23 expressed heterosis for this trait: ⅓ in set I and ½ in set II. The HPH levels for other traits were significantly lower.

Genetic diversity by SSR analysis

A high level of genetic diversity at the 12 SSR loci was observed. Of the 60 detected alleles, 54 were polymorphic, including 9 unique alleles. The mean number of alleles per SSR locus was 5.0, ranging from 2 to 7 (see Suppl. 2). There

Table 1. Means, range of F$_1$ performance, mid-parent heterosis (MPH), high-parent (HPH) heterosis for fruit weight per plant (FWP, kg), fruit number per plant (FNP), average weight of one fruit (AWF, g) and fruit length (FL, cm) in two sets of diallel hybrids

Set	Trait	F$_1$ performance		MPH (%)		HPH (%)	
		Mean	Range	Mean	Range	Mean	Range
I	FWP	0.81	0.5~1.3	20.3	–35.7~101.6	24.0	0.3~68.8
	FNP	9.01	6.7~12.0	14.8	–30.0~75.0	17.6	–4.1~42.8
	AWF	89.6	67.5~112.0	4.39	–15.4~30.5	1.57	–10.9~20.2
	FL	13.58	11.5~16.0	11.8	1.9~25.7	8.0	0.8~21.2
II	FWP	1.18	0.7~1.7	19.9	–24.0~77.0	15.1	–14.0~68.0
	FNP	7.72	6.0~13.0	16.15	–33.3~71.1	17.2	–25.1~58.5
	AWF	156.6	116.8~213.7	5.29	–19.8~35.5	5.82	–17.9~22.0
	FL	10.4	8.7~12.7	6.49	–13.0~33.7	7.96	–5.8~24.2

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were seven loci in which more than 5 alleles were resolved. Among two sets of lines, there were six specific alleles in three loci, from which two were represented only in set I and four in set II (Suppl. 4).

Nei's coefficient of genetic dissimilarity for the SSRs data ranged from 0.136 to 0.434, the mean being 0.287. The two sets showed some specific features: inbred lines in set II demonstrated higher genetic diversity than set I.

UPGMA clustering based on Nei's distances was in accordance with the line diverging into two sets, i. e. it coincided with phenotypic features of the fruit (Fig. 1). Probably, some of the analyzed loci are associated with specific germplasm and particular traits.

Fig. 1. Dendrogram based on the SSR-based genetic distances (GD) among 11 pepper accessions.

Correlation among genetic distances, heterosis F₁, and hybrid performance

Genetic distances based on SSRs accounted for diallel set I pointed to strong positive correlations with MPH and HPH for AWF and FL (Table 2). Its coefficient of determination calculated from the regression of heterosis FL on GD was higher
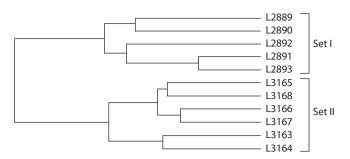
for half-diallel hybrids (r^2 = 49 %; 50.4 %), but of heterosis AFW, for reciprocal hybrids (r^2 = 38.4 %; 49 %). Significant negative correlations (r = 0.52) were observed for FNP with a higher impact of reciprocal hybrids (r = –0.67).

In set II, despite of a significant reciprocal effect, the relationships of GDs and the manifestation of heterosis in

Table 2. Correlation between differential SSR polymorphism, F₁ hybrid performance (x_i), mid-parent heterosis (MPH), and high-parent heterosis (HPH) for fruit weight per plant (FWP, kg), fruit number per plant (FNP), average fruit weight (AFW, g), and fruit length (FL, cm) in two sets of hybrids

Hybrids		Index	x_i				MPH				HPH			
			FWP	FNP	AFW	FL	FWP	FNP	AFW	FL	FWP	FNP	AFW	FL
Set I	Full-diallel hybrids	GD	0.05	–0.06	0.18	0.33	–0.31	–0.56**	0.42**	0.62**	–0.32	–0.48**	0.47**	0.52**
		NPL	0.12	0.10	0.09	0.36	–0.16	–0.42	0.35	0.68**	–0.15	–0.27	0.32	0.51*
		NML	–0.30	–0.11	–0.41	–0.45*	0.31	0.54*	–0.26	–0.68**	0.32	0.46*	–0.09	–0.62**
		R	0.16	0.08	0.19	0.46*	–0.23	–0.47*	0.30	0.70**	–0.21	–0.31	0.20	0.61**
	Half-diallel hybrids	GD	0.18	0.23	0.03	0.54	–0.16	–0.48	0.25	0.70**	–0.28	–0.32	0.12	0.71*
		NPL	0.23	0.35	–0.04	0.56	0.00	–0.23	0.20	0.75*	–0.02	–0.02	0.18	0.67*
		NML	–0.47	–0.46	–0.29	–0.55	0.14	0.31	–0.12	–0.61*	0.23	0.31	0.13	–0.69*
		R	0.25	0.32	0.06	0.65*	–0.11	–0.32	0.15	0.71*	–0.10	–0.13	0.06	0.75*
	Reciprocal hybrids	GD	–0.11	–0.36	0.35	0.23	–0.43	–0.67*	0.62*	0.55	–0.36	–0.61	0.70*	0.43
		NPL	0.00	–0.17	0.23	0.27	–0.35	–0.56	0.58	0.62*	–0.37	–0.47	0.66*	0.44
		NML	–0.12	0.25	–0.53	–0.42	0.55	0.71*	–0.48	–0.74*	0.51	0.59	–0.52	–0.62
		R	0.04	–0.18	0.33	0.38	–0.41	–0.58	0.54	0.69*	–0.41	–0.46	0.52	0.55
Set II	Full-diallel hybrids	GD	–0.04	0.20	–0.34	0.38*	0.04	0.08	–0.13	0.56**	0.12	0.10	0.01	0.46**
		NPL	0.00	–0.01	0.06	–0.08	0.00	–0.09	0.07	0.07	–0.07	–0.12	0.06	–0.01
		NML	0.03	0.10	–0.05	–0.15	0.10	0.02	0.11	–0.06	0.18	0.14	0.13	0.04
		R	–0.01	–0.03	0.05	–0.03	–0.01	–0.07	0.03	0.07	–0.08	–0.13	0.02	–0.03
	Half-diallel hybrids	GD	–0.26	–0.19	–0.17	0.14	–0.24	–0.25	0.04	0.42	–0.20	–0.23	0.04	0.22
		NPL	–0.04	–0.16	0.20	–0.12	–0.11	–0.23	0.15	0.09	–0.15	–0.21	0.22	–0.21
		NML	–0.26	–0.21	–0.12	–0.23	0.04	–0.16	0.28	0.04	0.27	–0.07	0.20	0.30
		R	0.04	–0.06	0.18	0.04	–0.08	–0.11	0.01	0.10	–0.15	–0.12	0.11	–0.26
	Reciprocal hybrids	GD	0.18	0.42	–0.48	0.60	0.26	0.38	–0.27	0.73**	0.33	0.40	0.01	0.55*
		NPL	0.03	0.07	–0.03	–0.05	0.09	0.06	0.01	0.06	–0.04	–0.04	–0.11	0.10
		NML	0.32	0.28	–0.01	–0.11	0.15	0.19	–0.04	–0.14	0.16	0.34	0.06	–0.10
		R	–0.06	–0.01	–0.03	–0.05	0.05	–0.03	0.06	0.07	–0.06	–0.14	–0.07	0.10

N o t e: GD, genetic distances; NPL, number of polymorphic loci; NML, number of monomorphic loci; R, NPL/NML ratio; *p < 0.05; **p < 0.01.

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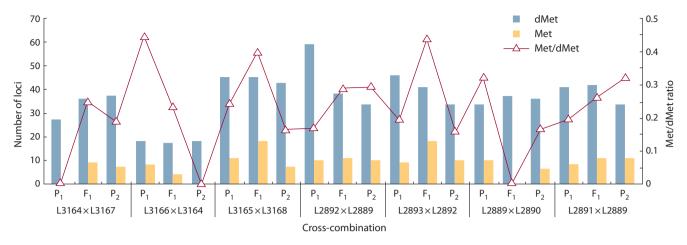T.V. Pechkovskaya, L.A. Mishin, L.V. Khotyleva

Fig. 2. Methylation status of sweet pepper hybrids and their parental lines at the flowering stage.

all 30 F_1 for FL assumed the values 0.56 and 0.46 for MPH and HPH, respectively, whereas measurement correlations in reciprocal hybrids exceeded ($r = 0.73$; 0.55) both the overall and half-diallel levels.

There were no significant correlations between GDs and hybrid performance for all measured quantitative traits; only a poor correlation between GDs and FL was observed in set II.

Correlation between differential SSR polymorphism, heterosis F_1, and hybrid performance

Simple correlation coefficients – the differential polymorphism (DP) of the parents, F_1 performance, mid- and high-parent heterosis – were strong for FL, as GDs in set I (see Table 2). The impact of polymorphic locus number into MPH manifestation reached 46 % (r^2); into HPH, 26 %. The ratio of poly- and monomorphic loci ($R_{NPL/NML}$) was responsible for 49 % and 37 % of the F_1 heterotic response for MPH and HPH, respectively. The tightest links between NPL and $R_{NPL/NML}$, corresponding to (r) 0.71, were identified in half-diallel F_1 hybrids.

The number of monomorphic loci was directly associated with FNP and inversely, with heterosis for FL. The correlations increased in the reciprocal F_1 ($r = 0.7$) and decreased in the half-diallel F_1. A similar tendency was found for AFW, where a significant positive link ($r = 0.66$) was detected for the number of polymorphic loci and HPH of this trait.

The analysis of relationships among indexes under study in set II revealed no significant associations.

Methyl-sensitive amplified polymorphism

Hybrids of 7 cross-combinations (L3164 × L3167, L3166 × × L3164, L3165 × L3168, L2892 × L2889, L2893 × L2892, L2889 × L2890, L2891 × L2889) with different manifestation of heterosis, from negative to positive effects, were analyzed by MSAP with four AFLP markers. A total of 203 loci were detected in plant seedlings P_1, P_2, F_1, of which 24 showed variability in DNA methylation (Suppls. 5–7). We found differences between parental and maternal lines in both the polymorphism of amplified loci and its epiallelic variability. The following cross combinations of allelic variants were detected (P_1/P_2): Met/dMet; dMet/dMet; Met/Met; Met/0; dMet/0. It is worth noting that in all analyzed hybrids at the

seedling stage status *dMet* (demethylated) was predominant regardless of the statuses of parental lines, except for five alleles, where both parents were methylated (ver. Met/Met) and the hybrid inherited the methylation status. It may be presumed that the early superiority of F_1 seedlings could be caused by demethylation and the resultant rise in gene expression, which, in turn, contributed to heterosis.

At the flowering stage, we evaluated 95 AFLP loci, which were differentially methylated in two parents and their hybrids. The most informative primers were *Hpa*II/*Msp*I-*tctt* and *Hpa*II/*Msp*I-*tctc*, which allowed us to consider 46 and 32 loci, respectively, including 30 and 28 differentially methylated among parental lines (Fig. 2). With *Hpa*II/*Msp*I-*tcaa*, we analyzed 17 loci, of which 14 were differentially methylated, including 13 *de novo* in hybrids. The highest number of *de novo* F_1 methylation patterns were detected with *Hpa*II/*Msp*I-*tctt*. Three (L3164 × L3167, L2893 × L2892, L3165 × × L3168) of the seven analyzed hybrids had elevated methylation levels as compared to parents. Hybrid L2889 × L2890 had a reduced level of methylation, whereas L3166 × L3164 and L2892 × L2889 were methylated additively, i. e. within the parental range (Fig. 2, Table 3). The total number of nonmethylated loci in F_1 hybrids was comparable to parents.

The Met/dMet ratio varied among cross-combinations from zero to 0.44. Its highest rate was found in L2893 × L2892 and L3165 × L3168, and the lowest, in L2889 × L2890. In the last case, there were found no Met loci and this hybrid (L2889 × L2890) displayed the highest HPH levels for most traits analyzed.

Impact of methylation to heterosis

Analysis of F_1 methylation status in relation of heterosis found out that the total number of non-methylated loci in F_1 positively link ($r = 0.647$) with mid-parent heterosis (MPH) for FWP, whereas both the number of methylated loci and the Met/dMet ratio have lower impacts (Table 4).

For high-parent heterosis (HPH), we found a positive effect of the prevalence of dMet loci and negative effects of both the number Met loci and the Met/dMet ratio on FWP and FNP. In contrast, the heterosis for AWF was negatively predetermined by the numbers of both Met and dMet loci.

Аллельная и эпигенетическая вариация ДНК
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М.Н. Шаптуренко, С.В. Вакула, Л.А. Тарутина, Т.В. Никитинская
Т.В. Печковская, Л.А. Мишин, Л.В. Хотылева

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Table 3. Methylation status, high-parent (HPH), and mid-parent (MPH) heterosis for some traits

Hybrids		Number of dMet loci	Number of Met loci	Ratio Met/dMet	Fruit weight per plant		Fruit number per plant		Mean fruit weight		Fruit length	
					HPH	MPH	HPH	MPH	HPH	MPH	HPH	MPH
Set I	L2889 × L2890	37	0	0.000	68.8**	29.1*	42.8**	14.0*	20.2*	15.2*	–	15.9*
	L2891 × L2889	42	11	0.261	27.5*	84.3**	41.1**	63.7**	–	17.0*	0.8	16.8*
	L2892 × L2889	38	11	0.289	–	63.0**	–4.1	75.0**	–	–0.4	–	9.0
	L2893 × L2892	41	18	0.439	–	76.0**	–	51.0**	–	17.5*	21.2	25.7*
Set II	L3166 × L3164	17	4	0.235	–6.0	–7.8	–25.1*	–33.3*	18.0	35.5**	5.8	12.2*
	L3164 × L3167	36	9	0.250	–	–7.5	–	–6.94	–	–0.5	–	–3.1
	L3165 × L3168	45	18	0.400	24.0	33.8	21.0*	29.3*	–	2.2	–4.4	–13.0*
Mean		36.6	10.1	0.267		38.6**		31.7**		12.2*		7.5

N o t e : "–", intermediate inheritance; dMet, demethylated loci; Met, methylated loci. *$p < 0.05$; **$p < 0.01$.

Among hybrids of set I, the HPH values for FWP and FNP decreased with increasing Met/dMet ratio and the number of methylated loci. However, there were not significant relationships for MPH. Interestingly, hybrid L2889 × L2890 was characterized by the full absence of methylated loci, and it demonstrated the highest heterotic effect on FWP, FNP, and AWF.

As opposed to set I, hybrids of set II displayed a rise in HPH and MPH for FWP and FNP with increasing Met/dMet ratio. The highest level of heterosis was noted in hybrid L3165 × × L3168 with the greatest Met/dMet ratio and the numbers of both methylated and nonmethylated loci.

Discussion

The accessions under study represented the major components of the gene pool of sweet pepper breeding program in Belarus targeted at raising long-fruited and high yielding hybrids F$_1$. Set I comprises lines with red-colored triangular fruit, and set II, with orange-colored rectangular fruit. It is apparent from the data that a considerable proportion of the crosses expressed high degrees of heterosis, indicating that heterosis is generally high in sweet pepper.

The SSR analysis of the two sets of accessions revealed some interesting features of allelic variability in sweet pepper. The diversity measure based on SSR clearly divided accessions into two groups concordant with phenotypic trait expression among set I and II. On the one hand, this was due to possible associations of some SSR loci with specific germplasm and particular traits, on the other hand, due to the differential selection for certain phenotypes (such as fruit shape and color).

One of the most important issues about heterosis is its predetermination by the extent of heterozygosity, assessed from DNA polymorphism in parental lines. Several attempts have been done to assess the adequacy of this approach (Melchinger, 1999). To characterize heterozygosity and its impact on heterosis manifestation, we used two measures. The first was the Nei–Lee genetic diversity (GD), and the second, differential polymorphism (DP) evaluated by counting the numbers of polymorphic and monomorphic loci in each pairwise combination. Our data indicated that the strength of

Table 4. Correlations among differential DNA methylation, high-parent (HPH) and mid-parent (MPH) heterosis for fruit weight per plant (FWP), fruit number per plant (FNP), mean fruit weight (AFW), and fruit length (FL) in heterotic and nonheterotic hybrids

Index		HPH	MPH
FWP	dMet	0.341	0.647
	Met	–0.422	0.500
	Met/dMet	–0.675	0.343
FNP	dMet	0.652	0.737
	Met	–0.083	0.525
	Met/dMet	–0.385	0.340
AWF	dMet	–0.668	–0.701
	Met	–0.844*	–0.393
	Met/dMet	–0.756*	–0.188
FL	dMet	0.051	–0.169
	Met	0.540	–0.211
	Met/dMet	0.614	–0.165

*$p < 0.05$.

relationships between GD and heterosis varied from one data set to another depending on the trait. The highest relationships were observed between GDs and heterosis manifestation for fruit length in both sets, with some differences in groups of half-diallel and reciprocal hybrids. We also found significant associations between GDs and SCA. These observations appear to be promising for selection of heterotic cross-combinations. As in the case of GDs, the differential polymorphism of SSR loci was the most significant for fruit length in set I. The number of polymorphic loci was large and directly associated with F$_1$ performance and heterosis for this trait. The number of monomorphic loci was inversely linked with fruit length, but directly with heterosis for fruit number per plant. The correlation values varied among half-diallel and reciprocal hybrids, which might be caused by maternal (cytoplasmic) effects. No significant associations among analyzed

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M.N. Shapturenko, S.V. Vakula, L.A. Tarutina, T.V. Nikitinskaya
T.V. Pechkovskaya, L.A. Mishin, L.V. Khotyleva

parameters were identified in set II. Possible explanations are: (i) different selection forces acted between initial germplasms in set I and set II; (ii) some SSR loci are likely to be linked with QTL fruit length. These suggestions are supported by correlations of SSR GD, DP with F_1 hybrid performance and heterosis for this trait. The contribution of SSR GD and DP had a greater effect on set I, whose selection was aimed at increasing fruit length. The presence of inverse relationships of GDs with FWP and FNP looks logical when we assumed links between SSR markers and FL, which could not affect the plant yield, not being associated with FWP or FNP. Our result argues for the suggestion that measures of heterozygosity are useful for predicting the heterotic response only when a significant portion of the selected markers are linked with heterotic QTLs or HTL of the trait at issue.

The predicting value of molecular markers for trait heterosis is expected to be low. SSR diversity characterizes the genome-wide diversity, while the heterozygous loci of target traits are expected to be localized (Xu et al., 2002). Quantitative traits of interest are complex. Consequently, they involve many genes with small effects, and it is difficult to find markers associated with such genes. One more issue is the expression of heterozygous or potentially heterotic loci in F_1. The molecular basis of heterosis may be attributed to the altered regulation of gene expression in the hybrid (Shen et al., 2012). Two different parental alleles brought together in F_1 may create a combined pattern and cause deviations from a simple additive model, acting in favor of heterosis manifestation (Swanson-Wagner et al., 2006; Li et al., 2015). One of the mechanisms of transcriptional regulation is DNA methylation. Correlation studies suggest that epigenetic effects, including cytosine methylation of DNA, carry important phenotyping consequences and that they may be involved in pathways contributing to heterosis (Tsaftaris et al., 1997; Chodavarapu et al., 2012; Ryder et al., 2014; Ong-Abdullah et al., 2015). It was observed in various plant taxa that a great majority of the cytosine methylation sites manifested stable inheritance from inbred parents to hybrids, but some sites showed deviation from expected parental additivity (Zhang et al., 2010; Becker et al., 2011; Lauria et al., 2014). Vergeer et al. (2012) suggested that inbreeding depression was linked with increased DNA methylation, reduced in outbreeds. However, other works (Sanghera et al., 2011; Shen et al., 2012) argue in favor of the importance of methylation for hybrid vigor. According to Sanghera et al. (2011), inbreeding depression is caused by lower genes expression due to homozygosity of methylated DNA in regulating factors, whereas heterosis, on the contrary, stems from higher levels of gene expression due to heterozygosity for methylated and nonmethylated alleles. To date, the relative impacts of hypermethylation and hypomethylation on heterosis are not clear (Kawanabe et al., 2016).

In this study, we analyzed differential methylation among P_1, P_2, and their F_1 hybrids and found some contrasting patterns in both the seedling and flowering stages. Our data suggest that the early superiority in some F_1 seedlings can be caused by demethylation and the resulting rise in gene expression, which should contribute to heterosis. Further heterosis manifestation, though, should be associated with the methylation/nonmethylation status. Correlations between heterosis and the numbers of methylated and nonmethylated loci at the flowering stage suggest that cross-hybridization promotes the rise of epigenetic modifications in the hybrid genome. These modifications are likely to be associated with methylation, as their effects are eliminated at different developmental stages. Probably, these modifications influence the functional status of various genes, causing a cascade response in gene networks, which in turn modulates metabolism and contributes to the heterotic response.

Our results support the relative importance of epigenetic changes in F_1, in addition to the structural DNA-polymorphism, for heterotic expression. Epigenetic modifications bring some nuances into the explanation of heterosis, and their genetic effects need to be tested. Their actions explain (i) the lack of linear relationships between genetic diversity and heterosis and (ii) the high heterotic effects in F_1 from closely related lines.

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Conflict of interest
The authors declare no conflict of interest.

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М.Н. Шаптуренко, С.В. Вакула, Л.А. Тарутина, Т.В. Никитинская
Т.В. Печковская, Л.А. Мишин, Л.В. Хотылева

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ORCID ID

M.N. Shapturenko orcid.org/0000-0003-4694-3197

S.V. Vakula orcid.org/0000-0002-2242-7107

T.V. Nikitinskaya orcid.org/0000-0002-3263-4732

L.V. Khotyleva orcid.org/0000-0003-0295-5022