

EVALUATION OF DIVERSITY IN BULGARIAN PEPPER CULTIVARS BY AGRONOMICAL TRAITS AND ISSR MARKERS

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Information about the genetic variation among cultivars of vegetable crops is of vital importance for improvement of plant breeding programmes worldwide. The objectives of this study were to group 19 pepper (*Capsicum annuum* L.) cultivars from the collection of Maritsa Vegetable Crops Research Institute, Plovdiv, Bulgaria into clusters according to their distances as estimated by agronomic traits and 9 di- and tri -nucleotide inter simple sequence repeat polymorphism (ISSR) markers and to assess the relationships between them. The phenotypic characterization during 3 consecutive years revealed significant differences among Bulgarian cultivars for the studied 13 phenotypic traits. The biplot analysis of quantitative traits showed that the most strongly correlating traits with the first axis (55.6% of variance) were fruit width, fruit weight and pericarp thickness (in the negative direction of the axis), and plant height (PH) (in the positive direction). The most discriminative traits, considering the second axis (22.6% of variance) were fruit length (FL) and to a lesser extent the stem height (StH). The correspondence analysis of the qualitative traits showed that the intensity of the colour of the fruit (before and at maturity), fruit colour before maturity and fruit shape in longitudinal section were the most discriminative characteristics for the first two dimensions. The agronomic traits data and 7 dinucleotide ISSR primers were used to estimate the pairwise genetic distances. Higher mean phenotypic distance (0.414) in

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comparison to the genotypic ones (0.214) among the cultivars was observed, indicating higher phenotypic diversity among them. A highly significant, positive correlation between the agronomic data and ISSR marker-based matrices ($r=0.41$, $p=0.001$) was detected. This indicates that ISSR distance tended to reflect that of the agronomics ones. However, additional molecular studies and large collection of highly diverse genotypes are needed to reveal associations between the marker loci and specific QTLs. The research initiated is a base for more precise estimation of genetic distances between pepper genotypes from the available large collection of landraces, local and modern cultivars including large number of highly polymorphic markers.

Keywords: genetic distances, genetic diversity, ISSR markers, pepper (*Capsicum annum* L.), phenotypic diversity

INTRODUCTION

Genetic diversity is the essential source of genetic progress in plant breeding. This provoked a considerable interest in characterisation of genetic resources for most cultivated plant species at both national and international level since the mid of the last century. Extensive characterisation of germplasm collections consisting of wild species, landraces, local forms, elite breeding lines and modern cultivars allow the identification of allelic variants associated to specific phenotypic variations (i.e. quality traits, tolerance or resistance to biotic and abiotic stresses) and the selection of the best genotypes for further improvement of species.

Among the vegetable crops, pepper (*Capsicum* spp.) is one of the most globally important crops because of its pungency and high nutritional value. *Capsicum* genus is comprised of as many as 36 species (ESHBAUGH, 2012) of which five (*C. annum* L., *C. frutescens* L., *C. chinense* Jacq., *C. baccatum* L., and *C. pubescens* Ruiz et Pav) are cultivated ones (PICKERSGILL *et al.*, 1979; PICKERSGILL, 1991). Among the cultivated peppers a special attention is given to the most economically important species *C. annum*, which includes forms with both pungent (chilli or hot pepper) and non-pungent (sweet pepper) fruits. Hence, the detailed characterisation of its phenotypic and genetic diversity is pivotal.

In Bulgaria, pepper (*Capsicum annum* L.) is the second most important vegetable crop and nowadays it occupies an area of 4035 ha, with an annual production of 59277 tons (NIKOLOV *et al.*, 2014). The country is characterised with large number of well-adapted local populations with specific characteristics for shape, colour, taste and biological value (MASHEVA, 2014). They have been used as a starting material for the conventional breeding programs directed toward development of genotypes with improved economic and agronomic traits (TODOROV and TODOROVA, 2002).

The systematic improvement of pepper in Bulgaria was initiated in the mid-forties of the last century by the development of several nonhomogeneous cultivars (populations) such as Byala shipka, Byal kalinkov, etc. through selection from local populations and further stabilisation. After that, using individual selection in local populations a number of cultivars, including Syvria 600, Kalinkov 800/7, Kurtovska kapia 1619, Gorogled 6, etc. were obtained in Maritsa VCRI, Plovdiv and they were widely spread in Bulgaria and abroad for a long time period (TODOROV and TODOROVA, 2002).

In the last 30 years new germplasm was obtained as a result of the use of various local and foreign genotypes and application of different breeding methods, including hybridisation,

repeated individual selection, male sterility and others. The newly developed breeding materials were evaluated for the presence of valuable agronomic traits, bioactive components, and resistance to biotic and abiotic stress factors. Some of them such as cultivars IZK Delikates, Kurtovska kapia 1 (TODOROVA, 2011), Deven (TODOROVA *et al.*, 2011), Yasen F1 (TODOROVA *et al.*, 2014), Milkana F1 were created from materials of local origin (breeding lines, cultivars and local forms extracted from local population), while the cvs. IZK Rubin (TODOROVA and PEVICHAROVA, 2006), IZK Kalin (TODOROVA and PEVICHAROVA, 2012) and Kaloyan originate from hybridization schemes including only foreign genotypes. Cultivars Hebar, Maritsa and Stryama were developed with participation of both, local and foreign pepper genetic resources (TODOROV and TODOROVA, 2002). Unfortunately, such long term breeding programs usually lead to a gradual loss of genetic diversity (RIVERA *et al.*, 2016), that could seriously hinder the development of new genotypes with superior qualities and resistance to stress factors. In that sense, the availability of information about the genetic diversity among the parental lines is an important prerequisite for the efficiency of breeding programmes, as well as for identification of redundant genotypes in both national and world gene bank collections.

The differences in plant and fruit morphology and other agronomic traits, among the genotypes of a breeding collection, are easily scorable indicators of genetic diversity. However, some of these are quantitative traits and their expression is strongly affected by the environmental conditions, which can lead to confounding results. In contrast, the DNA markers are highly reproducible and their variation is not modified by external factors. The genetic diversity among *Capsicum annuum* genotypes was previously studied using different PCR-based techniques including AFLP (PARAN *et al.*, 1998; AKTAS *et al.*, 2009; GELETA *et al.*, 2005; KOCHIEVA and RYZHOVA, 2003), RAPD (OYAMA *et al.*, 2006; MANA *et al.*, 2014), SSR (RIVERA *et al.*, 2016), ISSR (THUL *et al.*, 2012; MANA *et al.*, 2014), and DNA microarray (HILL *et al.*, 2013).

The ISSRs (ZIETKIEWICZ *et al.*, 1994) are dominant, multilocus, PCR-based DNA markers, amplifying genomic regions delimited by simple sequence repeats (SSR) at both sides. Their main advantages over the other multilocus markers (e.g. RAPD & AFLP) are their high reproducibility and low labour intensiveness, which combined with the applicability to any species makes them very convenient for low-cost genetic studies even in poorly studied taxons. Since the mid-nineties the ISSR markers were widely used in genetic diversity studies in numerous plant species, such as wheat (KHAN *et al.*, 2015), maize (DO AMARAL JUNIOR *et al.*, 2011), rice (AL-TURKI and BASAHI, 2015) and representatives of *Solanaceae* family (BORNET *et al.*, 2002; KOCHIEVA *et al.*, 2002; LAHIJI *et al.*, 2013).

The genetic diversity of pepper in Bulgaria has not been extensively analysed yet, even though such studies could provide information about sources of genetic differentiation following both natural and human selection. Only few studies based on simple sequence repeat (SSR) analysis have been reported (OH *et al.*, 2012; GOSPODINOVA *et al.*, 2008) on the limited number of landraces and elite breeding lines.

Keeping in view the above considerations and importance of germplasm characterisation in Bulgaria, the aim of the present study was to evaluate the genetic diversity among 19, widely used in the production system, Bulgarian pepper cultivars from the collection of Maritsa VCRI, Plovdiv, using agronomic traits and ISSR markers and to compare the results of both methods.

MATERIALS AND METHODS

Plant material

Nineteen Bulgarian pepper cultivars (old, traditional and newly developed) were used in this study (Table 1). The selected cultivars showed differences in agronomic traits such as plant height, stem height, fruit size, yield, shape, colour and position of the fruits suitable for different market and culinary purposes.

According to classifications (POPOV, 1940; CHRISTOV *et al.*, 1966) based on fruit shape and size the cultivars could be grouped as follows:

With large fruits (*ssp. macrocarpum*)

- Group with wide fruits (*ser. var. grossum* Sendt.): pumpkin or round fruits (*var. ratundum*) - Balgarski ratund; bell or blocky type (*var. dolma*) - Byal kalinkov, Kalinkov 800/7 and heart shaped fruits (*var. cordatum*) - Kaloyan;

- Group with long fruits (*ser. var. longum* Sendt.): with elongated fruits - IZK Kalin and IZK Rubin, with conical shape (*var. conicum*): Sivria 600, Gorogled 6, Zlaten medal 7, Hebar, Stryama, Milkana F1 and Yasen F1; Kapia type (*var. kapia*): Kurtovska kapia 1, Sofiyska kapia; horn shaped type (*var. horniforme*) – IZK Delikates;

With small fruits (*ssp. microcarpum*)

- Small fruited, *ser. var. shipka*, (*var. conoides* Irish.): Dzhulyunska shipka 1021, Byala shipka,

With fruits in bunch, cluster (*ssp. fasciculatum* Sturt.) – Buketen 50

Field experiments and phenotypic trait measurements

The cultivars were tested in the experimental plot at the Maritsa VCRI during the period 2011-2013. The sowing was conducted in unheated greenhouses at the end of March. The experiment was performed in four replications (20 plants per replication) using a block method. The planting was performed in the middle of May on alluvial meadow soil and furrow surface by 70/15 cm scheme. The plants were grown by adopted technology for mid-early field production. Twenty randomly selected plants/cultivar (five plants per replication) were evaluated for six quantitative plant and fruit traits: plant height - PH (cm), stem height - StH (cm), fruit length – FL (cm), fruit width at the base - FW (cm), pericarp thickness - PT (mm) and fruit weight - FWT (g), and seven qualitative fruit traits according to the Protocol for distinctness, uniformity and stability tests for *Capsicum annuum* L. (CPVO, http://cpvo.europa.eu/sites/default/files/documents/TP/vegetales/TP_076-CAPSICUM_ANNUUM.pdf) and the Descriptor for Capsicum (IPGR, AVRDC & CATIE, 1995) as follows: Fruit: predominant shape of longitudinal section (FPLS), Fruit: attitude at maturity (FAM), Fruit: colour before maturity (FCB), Fruit: intensity of colour before maturity (FIB), Fruit: colour at maturity (FCM), Fruit: intensity of colour at maturity (FIM), Fruit: capsaicin in placenta (FCP). The measurements of the plant traits were made after the end of the active vegetative growth while these related to fruit traits, after the second harvest.

DNA extraction and molecular analyses

Leaf samples from five randomly chosen young plants of each cultivar were collected and total genomic DNA was extracted using CTAB protocol described by DOYLE (1990). The concentration of the isolated gDNA was adjusted to 10 ng/μL and stored at -20 °C until use.

Nine 3' anchored ISSR primers were used in the study. All PCR reactions were carried out

with 30 ng of gDNA, 0.3 μ M ISSR primer, and 1x MyTaq™ HS Mix (BIOLINE) in a volume of 25 μ L on an Applied Biosystems Veriti 96 Thermal cycler (THERMO FISHER SCIENTIFIC). The temperature conditions consisted of an initial denaturation 94°C for 60 sec and 35 cycles of: 94°C - 30 sec; Ta°C (see Table 3 for details) – 60 sec; 72°C – 90 sec; and a final elongation at 72°C for 10 min. The products were separated in 2% agarose gels and the lengths of amplicons were scored using PyElph 1.4 software (PAVEL and VASILE, 2012).

Data analysis

For ISSR analysis only reproducible bands with high intensity were scored either as present (1) or absent (0) and the data were used for construction of presence / absence binary matrix. The gene diversity (NEI, 1973) was estimated with the POPGENE (YEH *et al.*, 1997), and the polymorphic information content (PIC) was calculated as described by KAYIS *et al.* (2010).

The genetic distances between pepper accessions based on the phenotypic (combining quantitative and qualitative traits) and molecular data were evaluated using Gower distances and Jaccard coefficients - respectively. The resulting distance matrices were used as input data for construction of dendrograms using UPGMA (Unweighted Pairwise Arithmetic Method). The quantitative and qualitative data of phenotypic traits were subjected on principal component analysis and multiple correspondence analysis, respectively to clarify the most discriminative traits among the genotypes. All statistical procedures were done using R (v. 3.2.3; <http://www.R-project.org>).

Duncan's multiple range test (DUNCAN, 1955) was used to compare the means at the detected significant differences ($P < 0.05$).

RESULTS

The phenotypic characterization of 19 Bulgarian pepper (*C. annuum* L.) cultivars performed during three consecutive years at the experimental field of Maritsa Vegetable Crops Research Institute, Plovdiv, Bulgaria revealed significant differences among Bulgarian cultivars for the studied 6 agronomic characteristics (Table 1).

To determine how Bulgarian cultivars were grouping according to the quantitative phenotypic traits and which of the traits were the most discriminative among the genotypes, a principal component analysis was performed. The first three components accounted for 90% of the data variance (Table 2). The first component explained 55.6%, while the second axis explained 22.6% of the observed variance. The most strongly correlating traits with the first axis were fruit width (FW), fruit weight (FWT) and pericarp thickness PT (in the negative direction of the axis), and plant height (PH) (in the positive direction). The most discriminative traits, considering the second axis, were fruit length (FL) and to a lesser extend stem height (StH) (Figure 1). Biplot results showed that 19 pepper cultivars (*C. annuum* L.) are scattered in all four quadrants. However, almost half of the genotypes (9 cultivars) were grouped in the second quadrant or near the y axis in the first quadrant. These genotypes are characterised by higher FWT and / or FL. In the third quadrant were clustered the cultivars with combined higher FW, PT and FWT, and lower FL - No14 (Byal kalinkov), No15 (Kalinkov 800/7) and No16 (Balgarski ratund). The rest of genotypes were situated in the fourth quadrant. The most common characteristic of these genotypes is the small fruit size.

Table 1. Mean values of studied agronomic traits in 19 Bulgarian pepper cultivars

No	Cultivar	Plant height	Stem height	Fruit length	Fruit width	Pericarp thickness	Fruit weight
		cm	cm	cm	cm	mm	g
1	Kaloyan	60.79 ^{c-f}	20.39 ^{bcde}	9.79 ^{bcde}	5.60 ^c	4.65 ^{ab}	84.08 ^{ab}
2	Sivria 600	62.07 ^{c-f}	20.03 ^{bcde}	15.00 ^a	3.61 ^e	2.70 ^{cd}	49.62 ^d
3	Stryama	61.00 ^{c-f}	25.95 ^{abc}	10.27 ^{bcde}	4.71 ^d	5.27 ^a	71.13 ^{abcd}
4	Kurtovska kapia 1	59.93 ^{def}	23.07 ^{abcd}	11.51 ^{bc}	4.49 ^{de}	3.73 ^{bc}	64.72 ^{bcd}
5	Gorogled 6	64.24 ^{c-f}	22.31 ^{abcd}	7.64 ^{ef}	2.51 ^f	0.73 ^f	11.82 ^e
6	Buketen 50	43.71 ^f	23.77 ^{abcd}	8.15 ^{def}	2.57 ^f	1.58 ^{def}	18.97 ^e
7	IZK Rubin	65.07 ^{c-f}	24.40 ^{abcd}	8.93 ^{cde}	1.70 ^f	1.35 ^{ef}	13.93 ^e
8	IZK Kalin	82.00 ^{abc}	26.98 ^{ab}	7.92 ^{def}	1.96 ^f	1.49 ^{def}	17.40 ^e
9	Hebar	69.87 ^{cd}	29.87 ^a	10.07 ^{bcde}	4.19 ^{de}	4.15 ^{ab}	56.45 ^{cd}
10	Yasen F ₁	61.67 ^{c-f}	16.77 ^{de}	12.49 ^{ab}	4.59 ^{de}	3.88 ^{bc}	76.05 ^{abc}
11	Milkana F ₁	70.63 ^{bcd}	22.63 ^{abcd}	12.82 ^{ab}	4.61 ^{de}	3.45 ^{bc}	72.54 ^{abcd}
12	Zlaten medal 7	61.10 ^{c-f}	19.19 ^{bcde}	12.38 ^{ab}	3.60 ^e	3.43 ^{bc}	59.19 ^{bcd}
13	Sofiyska kapia	65.67 ^{c-e}	24.58 ^{abcd}	10.85 ^{bcd}	4.29 ^{de}	3.68 ^{bc}	59.40 ^{bcd}
14	Byal kalinkov	47.07 ^{ef}	12.23 ^e	7.33 ^{efg}	6.08 ^{bc}	4.64 ^{ab}	92.04 ^a
15	Kalinkov 800/7	53.90 ^{def}	13.37 ^e	8.13 ^{def}	6.64 ^{ab}	3.81 ^{bc}	76.57 ^{abc}
16	Balgarski ratund	57.92 ^{def}	17.97 ^{cde}	3.42 ^h	7.07 ^a	5.37 ^a	74.26 ^{abcd}
17	Dzhulyunska shipka 1021	72.59 ^{bcd}	18.54 ^{bcde}	5.26 ^{fgh}	1.89 ^f	2.03 ^{de}	9.22 ^e
18	Byala shipka	93.69 ^a	18.36 ^{bcde}	4.52 ^{gh}	2.30 ^f	1.82 ^{def}	9.01 ^e
19	IZK Delikates	90.50 ^{ab}	26.28 ^{abc}	14.69 ^a	1.61 ^f	1.61 ^{def}	12.88 ^e

a, b, c, ... - Duncan's multiple range test ($p < 0.05$)

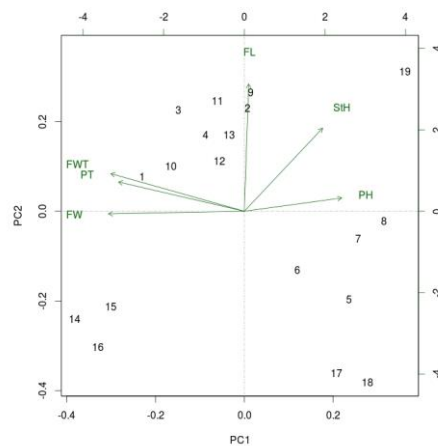


Figure 1. Biplot graphics of the first two principal components based on quantitative data of 19 Bulgarian pepper cultivars

Table 2. Principal component analysis – Eigen values, percentage of explained variance, correlation of traits with the first three components

	PC1	PC2	PC3
Eigen values	3.335	1.359	0.708
Explained variance (%)	55.6	22.7	11.8
PH	0.37	0.16	-0.77
StH	0.29	0.56	-0.16
FL	-0.04	0.76	0.39
FW	-0.53	-0.01	-0.16
PT	-0.48	0.14	-0.45
FWT	-0.52	0.26	-0.08

A multiple correspondence analysis was used to determine the grouping of studied genotypes according to their qualitative phenotypic traits. The resulting first three axis of MCA explained approximately 57.7 % of the observed variance - 24.87, 18.28 and 14.5 %, respectively. The fruit colour and the colour intensity of the fruits had the strongest contribution to the first two axes (Figure 2b & c). The genotypes grouped in the first quadrant (Figure 2a) have dark fruits before and at maturity (FIB_7 and FIM_7). These genotypes are opposed to the ones located in the second quadrant which are characterised with light colour fruits (FIB_3 and FIM_3). The three genotypes grouped in the lower area of the fourth quadrant have medium colour intensity (FIB_5 and FIM_5) – a category which had the greatest contribution to the second principal axis. The pungency of the fruit and the shape of the longitudinal section also have strong contribution to this axis.

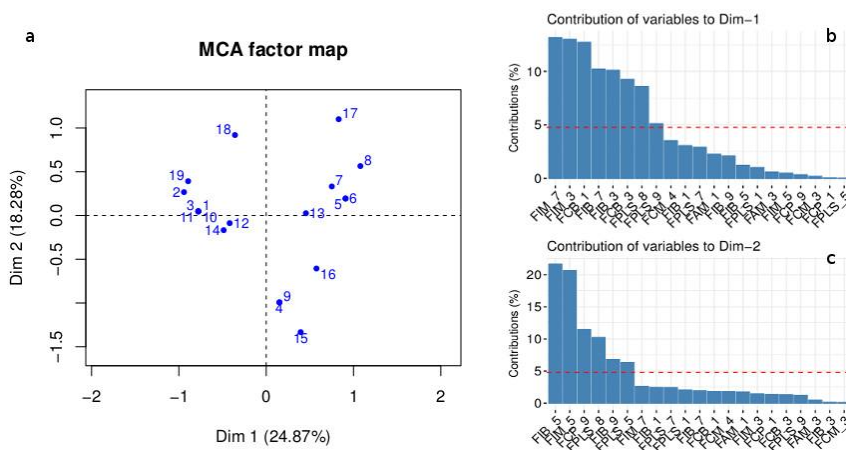


Figure 2. Scatter plot of the MCA analysis based on qualitative data of 19 Bulgarian pepper cultivars (a); variables contribution to first and second axis (b and c, respectively)

The quantitative and qualitative trait data were combined and used for estimation of the Gower's distances among the studied accessions. The phenotypic distances between the pepper genotypes ranged from 0.052 to 0.763 with a mean distance of 0.414. A dendrogram based upon the Gower's distances was constructed (Figure 3). All cultivars were grouped in two main clusters, each representing by 7 and 12 genotypes, respectively.

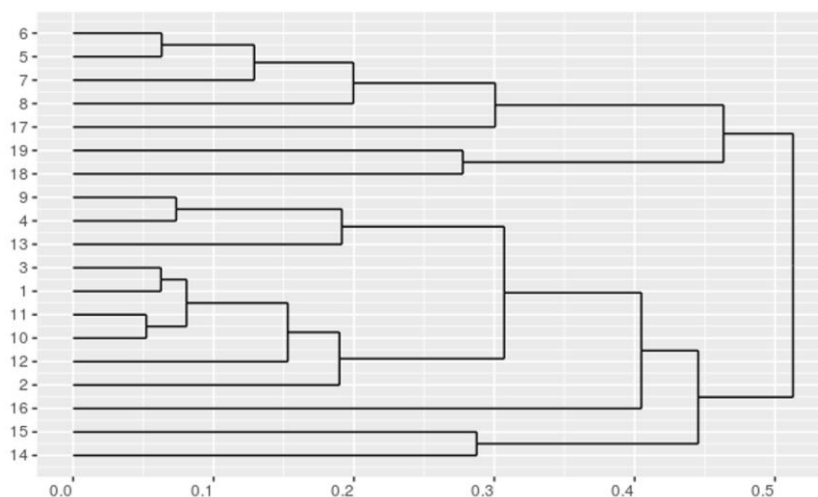


Figure 3. UPGMA-based dendrogram of 19 Bulgarian pepper cultivars, based on phenotypic data.

The first cluster included cultivars, forming lightweight, predominantly shorter and narrower fruits with thinner pericarp. The upper sub-cluster included the genotypes with dark coloured fruits, among which Buketen 50 (No6), Gorogled 6 (No5), IZK Rubin (No7) and IZK Kalin (No8) are for powder production. The cultivar Dzhulyunska shipka 1021 (No17) forms an outgroup in that subcluster, due to its pungent fruit taste. The other cultivar with pungent fruit taste Byala shipka (No18) is grouped together with IZK Delikates (No19) due to their light coloured fruits.

The second main cluster included cultivars with relatively large and heavy fruits with thicker pericarp (Table 1). The smaller subcluster combines the two cultivars, Byal kalinkov (No14) and Kalinkov 800/7 (No15), belonging to blocky type (*var. dolma*) peppers. In the second sub-cluster Balgarski ratund (No16) forms an outgroup, being the only genotype with an oblate shape in longitudinal section. The remaining nine genotypes are characterised by relatively triangular (FPLS_7) and horn shaped (FPLS_9) form of the longitudinal section. They form two distinct sub-clusters according to the colour before maturity and the fruit colour intensity. The first of them included cultivars with darker fruits colour such as Hebar (No9), Kurtovska kapia 1 (No4) and Sofiyska kapia (No13) while the second one is composed of cultivars with lighter fruit colour before and at maturity.

For assessment of genetic diversity between pepper genotypes nine ISSR primers were used. Seven of the primers consist of dinucleotide repeats, and two of them were with trinucleotide repeats. The two trinucleotide primers failed to amplify clear bands and were

rejected from the analysis. The rest of the primers produced in total 53 reproducible bands in the analysed 19 pepper genotypes. The number of bands per primer varied from 2 to 12 (Table 3).

Table 3. List of ISSR primers, their nucleotide sequence and temperature of annealing (T_a °C), total number of bands per primer, number of polymorphic bands, level of polymorphisms, described as percentage of the total number of bands; gene diversity (H_e ; Nei, 1973); Polymorphic Information Content (PIC)

Primer No	Primer sequence	T_a °C	Total No of bands	No of polymorphic loci	Polymorphic (%)	H_e	PIC
ISSR_1	(CT)8GC	56	8	1	12.50	0.04	0.10
ISSR_2	(CA)8G	56	10	2	20.00	0.09	0.23
ISSR_3	(CT)8AC	52	2	1	50.00	0.18	0.10
ISSR_4	(TC)8C	54	10	0	-	-	-
ISSR_5	(CT)9G	60	3	2	66.67	0.18	0.27
ISSR_6	(GT)6GG	59	8	3	37.50	0.08	0.23
ISSR_7	(GA)9C	60	12	4	33.33	0.08	0.14
ISSR_8	(CAC)7T		0	-	-		
ISSR_9	(CAC)7G		0	-	-		
Total			53	13	24.53		
Mean						0.071	0.177

Thirteen (24.53%) of the amplified bands were polymorphic and seven (53.85%) of them were unique and were observed in just one of the studied 19 cultivars. Absence of bands was observed in the profiles of 3 pepper cultivars, generated by: (CT)8GC in Buketen 50 (2010 bp); (CT)8AC in Kalinkov 800/7 (655 bp) and (GA)9C in Balgarski ratund (767 bp) while 4 new bands were identified in the profiles of three cvs.: (GA)9C in Gorogled 6 (636 bp and 736 bp); (CT)9G in Hebar (1220 bp) and (GT)6GG in Kalinkov 800/7 (792 bp). The gene diversity for the whole set of Bulgarian pepper cultivars ranged from 0.044 for primer (CT)8GC to 0.181 for primer (CT)9G with an average of 0.071. The highest PIC value was observed for (CT)9G (0.266), followed by (CA)8G (0.227).

The genotypic data were converted into genetic distance matrix using Jaccard coefficient. The genetic distance among the nineteen genotypes varied between 0 and 0.340 with a mean of 0.214. The genetic distance matrix was used for the construction of a dendrogram (Figure 4). The resulting tree consisted of two outgroups and two main clusters. The outgroups were formed by No15 and No5 (cvs. Kalinkov 800/7 and Gorogled 6), which were the genotypes with the highest number of unique alleles. The first cluster included cultivars No1 (Kaloyan), No3 (Stryama), and No14 (Byal kalinkov), while the second one consisted of the rest of genotypes. The second cluster is separated into 2 sub-clusters. The first one is composed of cultivars No6 (Buketen 50), No8 (IZK Kalin) and No19 (IZK Delikates). In second sub-cluster were located all genetically related cultivars that were unresolved with the applied 7 ISSR markers, excluding two small fruited,

pungent peppers No17 and No18 (Byala shipka and Dhzulyunska shipka 1021), one with pumpkin fruits No16 (Balgarski ratund) and two (No2 and 9) with elongated fruits and conical shape (Sivria 600 and Hebar, respectively).

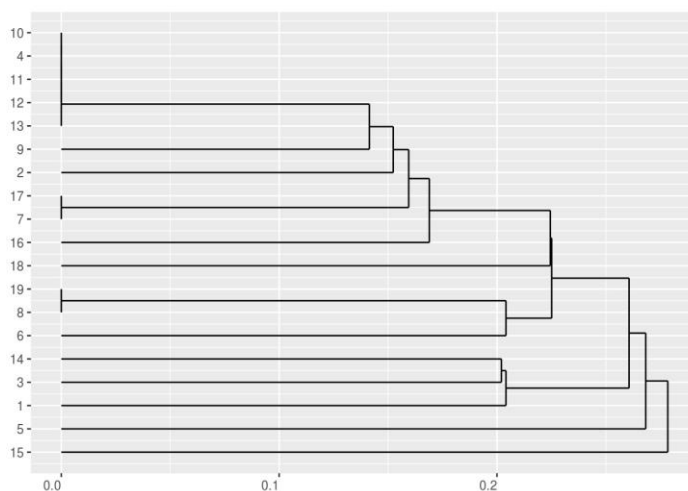


Figure 4. UPGMA-based dendrogram among 19 Bulgarian pepper cultivars, based on molecular data.

To analyse the correlation between the two distance matrices estimated using genotypic and agronomic data, a Mantel test was performed. The results showed a moderate, but highly significant positive correlation between the two matrices ($r=0.41$, $p=0.001$).

DISCUSSION

The primary aim of this study was to evaluate the existing levels and patterns of phenotypic and genetic diversity among nineteen Bulgarian pepper cultivars. The information about the existing diversity in economically important agronomic traits is an essential prerequisite for the success of any breeding program and allows the selection of genotypes with quantitative traits, such as high yield, fruit size and shape, acceptable to consumers.

In our study we analysed the phenotypic diversity based on 13 agronomic traits of nineteen Bulgarian pepper cultivars. Principal component analysis (PCA) was implemented for describing the phenotypic diversity of the pepper cultivars, using 6 quantitative traits, because of its usefulness in analyzing genetic variation among plant accessions and determining the most important variables contributing to variation (SHANKAR *et al.*, 2009). According to the PCA most of the variation among Bulgarian cultivars was manifested by the FW, PT, FWT, and PH which was negatively correlated with the previous three. These traits were also the most discriminative ones among the pepper accessions (*C. annuum* L.) used for the study of genetic and phenotypic landrace diversity in North-West Spain (RIVERA *et al.*, 2016). However, in the study of Bulgarian landraces by OH *et al.* (2012) the most discriminative trait of genotypes was the days from sawing to flowering. The average distance based on the studied phenotypic traits for the 19 pepper cultivars was 0.40. This value is close to the estimated by GELETA *et al.* (2005) (0.48) for a collection consisting of large number of *C. annuum* accessions from different geographic regions

around the world.

The multiple correspondence analysis showed that the studied cultivars vary most strongly with regard to the intensity of the fruit colour (before and at the maturity), the colour of the fruits before maturity, and the shape of the fruits in longitudinal section. RIVERA *et al.* (2016) also reported a strong contribution of fruit colour and shape to the variance of the studied by them landraces. OH *et al.* (2012) found that the fruit colour is not a discriminative trait among the Bulgarian landraces, but reported a strong correlation between fruit shape and the second principal component.

The assessment of genetic variability among genotypes is useful for the conservation of genetic resources, for broadening the genetic basis of cultivars, and for cultivar protection (YÜZBASIOGLU *et al.*, 2006). Among the different PCR-based markers, the ISSR markers were successfully used for molecular fingerprinting of *Capsicum annuum* (KUMAR *et al.*, 2001; THUL *et al.*, 2012) due to their greater efficiency in comparison to the other multilocus DNA markers as RAPD (THUL *et al.*, 2012). In our study we performed ISSR profiling of 19 Bulgarian pepper genotypes, using nine 3` anchored di- and tri-nucleotide repeat primers. In contrast to the results of KUMAR *et al.* (2001), who observed a greater total number of alleles using trinucleotide-repeat primers in pepper cultivars, in our experimental work these primers did not amplify clear bands. Similar to our results have been reported from OU and XUOXIAO (2012), in 5 cultivated pepper species, who selected only 13 dinucleotide ISSR primers showing reproducible profiles from a set of 50. The rest of the primers used in this study (in total 7 dinucleotide ISSRs) produced on average 7.6 alleles, which is less than the average number of alleles observed in peppers by the aforesaid teams or by another authors in other species belonging to *Solanaceae* family (BORNET *et al.*, 2002; KOCHIEVA *et al.*, 2002; LAHIJI *et al.*, 2013). ISSR primers (CT)8GC, (CT)8AC, (GA)9C, (CT)9G and (GT)6GG produced unique alleles in the profiles of five (Gorogled 6, Buketen 50, Hebar, Kalinkov 800/7 and Balgarski ratund) out of total 19 Bulgarian pepper cultivars used in the present study and they can be further sequenced and converted into SCARs for cultivar discrimination and other breeding purposes depending on their informativeness.

The lower number of observed polymorphisms, combined with the unique appearance of half of the alleles in just one of the studied cultivars was the reason for the estimated lower mean value of gene diversity (0.071), when compared to that reported by OYAMA *et al.* (2006) (0.081 for the cultivated accessions) and by THUL *et al.* (2012) for 14 *C. annuum* accessions collected from different regions in India but having common trait's characteristics with ISSR markers (47% genetic similarity). However, this value is higher from the reported by AKTAS *et al.* (2009) for 6 breeding lines of the state research institute Alata in Turkey (0.017) and for five non-Turkish genotypes (0.050). The observed two-fold higher level of genetic diversity in the collection consisting of 14 Turkish genotypes (0.171), reported by the above mentioned team, is due to the higher level of genetic variation in the selected 8 local genotypes.

The grouping of Bulgarian peppers according to the agronomic traits is generally consistent with the varietal type which means that genotypes with similar fruit characteristics are generally clustered together. In some extend similar grouping was also observed in the dendrogram, derived from ISSR data using distance-based UPGMA cluster analysis. Such grouping was observed for most of cultivars belonging to conical and kapia type-fruit cultivars.

The average estimated distance, using genotypic data, was almost twice as low when compared to the one based on agronomic ones. These data are in contradiction to the results of GELETA *et al.* (2005), who observed higher genetic distance based on AFLP markers in 39 *C.*

annum genotypes than the phenotypic one based on 20 morphological and agronomic traits records. According to LEFEBVRE *et al.* (2001) the genotypic distance may or may not reflect the phenotypic one, and the degree of correlation between them reflects the association between the marker loci and QTLs. In our study the correlation between the two matrices was highly significant and positive ($r=0.41$, $p=0.001$), but lower than that reported by LEFEBVRE *et al.* (2001) ($r=0.62$, $p<0.001$) and higher than the reported by GELETA *et al.* (2005) ($r=0.101$, $p<0.01$). The last authors also found that the association of morphological and AFLP genetic distance estimate values were significant and positive only between cherry and pungent elongated-fruit ($r=0.320$, $p<0.01$) and pungent elongated-fruit and paprika cultivars ($r=0.193$, $p<0.05$) but not between the remaining pepper types.

Our study showed a moderate but highly significant positive correlation between the agronomic data and ISSR marker-based matrices. This indicates that ISSR distance tended to reflect that of the phenotypic one. However, additional molecular studies using large set of highly diverse pepper genotypes are needed to reveal associations between marker loci and specific QTLs.

CONCLUSIONS

In the present study Bulgarian pepper cultivars showed lower genotypic variation in comparison to the phenotypic one which is most probably due to the limited number of ISSR markers used. Nevertheless, it was revealed that genetic distances, though not entirely, correctly reflect the existing phenotypic diversity in the studied cultivars and despite of the limited genome coverage of markers these results have potential implications in pepper breeding. This study also revealed specific for some of the studied genotypes alleles, which could be further used as potential markers for cultivar discrimination and other breeding purposes. However, for a better resolution and estimation of genetic relatedness between genotypes, higher number of polymorphic markers are needed.

The research initiated is a base for more precise estimation of genetic distances between pepper genotypes from a large Bulgarian collection of landraces, local and modern cultivars using different types of DNA markers (ISSR, SSR, SNP).

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AUTHORS' CONTRIBUTION:

Elena Todorovska and Stefan Tsonev equally contributed to the experimental design and molecular research, statistical data analysis, writing and general design of the article.

Velichka Todorova contributed to multiyear phenotypic characterization, maintenance and detailed description of cultivars, providing with seeds for molecular research, writing and design of article.

Stanislava Groseva and Teodora Popova contributed to the design of the experimental work and growing of pepper plants for DNA isolation

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EVALUACIJA DIVERZITETA SORATA BUGARSKE PAPIRIKE POMOĆU AGRONOMSKIH SVOJSTAVA I ISSR MARKERA

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Izvod

Informacija o genetičkoj varijabilnosti sorata povrća je od vitalnog značaja za poboljšanje oplemenjivačkih programa u svetu. Cilj ovog rada bio je grupisanje 19 sorata paprika (*Capsicum annuum* L.) iz kolekcije Maritsa institute za povrtarstvo i ratarstvo, Plovdiv, Bugarska u klustere na osnovu njihovih distance, utvrđene pomoću agronomskih svojstava i 9 di- i tri- nukleotidna ISSR markera, kao i da se utvrdi njihova međuzavisnost. Fenotipska karakterizacija za 13 osobina tokom tri uzastopne godine pokazala je značajne razlike ispitivanih sorata. Bi-plot analiza kvantitativnih osobina pokazala je da su najjače korelisane osobine sa prvom osom (55.6% varijanse) širina ploda, težina ploda i debljina perikarpa (negativno), kao i visina biljke (pozitivno). Diskriminativne osobine, u odnosu na drugu osu, bile su dužina ploda, i u nešto manjem stepenu visina stable. Korespondenciona analiza kvačlilitativnih osobina pokazala je da je intenzitet boje ploda (pre i u fazi sazrevanja), boja ploda pre faze zrelosti i oblik ploda u longitudinalnoj sekciji, bili najznačajnije diskriminatorne osobine. Agronomska svojstva i 7 dinukleotidnih ISSR prajmera su korišćeni za ocenu genetičke distance. Utvrđena je veća fenotipska distanca (0.414) u odnosu na genotipsku (0.214), ukazujući na veći fenotipski diverzitet. Visoko signifikantna pozitivna korelacija utvrđena je između agronomskih podataka i ISSR markera ($r=0.41$, $p=0.001$). Ovo istraživanje predstavlja osnovu za preciznije proučavanje genetičkih distance genotipova paprika unutar velikih kolekcija populacija, lokalnih i modernih kultivara, uz primenu većeg broja visoko polimorfnih markera.

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