Genetic diversity and structure analysis of Croatian garlic collection assessed by SSR markers

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ABSTRACT

This study examines genetic diversity and structure of a Croatian garlic germplasm collection using 13 simple sequence repeat (SSR) markers. A total of 71 alleles were observed across 64 accessions representing 3 Croatian regions (Istria, Dalmatia and continental Croatia) and 16 foreign landraces, with an average of 5.46 alleles per locus. Among the 80 accessions analysed, 61 distinct multilocus genotypes (MLG) were identified, of which 51 represented unique genotypes and the remaining accessions were divided into 10 MLG groups, comprising potential duplicates or redundant genotypes. Model-based Bayesian and hierarchical UPGMA clustering approaches revealed five major groups within the collection which partially correlated with geographical origin. The analysis of molecular variance (AMOVA) showed that the majority (87.71%) of the total molecular diversity is within the Croatian groups of accessions, even though a significant share (12.29%) of diversity derived from genetic diversity among groups. These results support regional structuring, as well as the existence of significant diversity within local populations. This study is the first comprehensive report on an extensive evaluation of genetic resources of garlic maintained by Croatia with the aim of setting the course for future preservation strategies with particular emphasis on the value of diversity in the context of climate change both on macro and micro levels.

Keywords: collections management, garlic, genetic diversity, genetic structure, plant genetic resources, SSR

Abbreviations: AFLP, amplified fragment length polymorphism; AMOVA, analysis of molecular variance; EST, expressed sequence tags; MLGs, multilocus genotypes; RAPD, random amplified polymorphic DNA; SSR, simple sequence repeat.

INTRODUCTION

Garlic (*Allium sativum* L.) is a valuable crop and one of the oldest-known horticultural species. It has been used as a condiment, food and medicinal plant since ancient

times (Ipek et al., 2005). The production of garlic in 2018 reached 28,494,130 metric tons, with China being the largest producer and accounting for almost 80% of

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the world production (FAOSTAT, 2018). Its centre of origin is considered to be in Central Asia (Kyrgyzstan, Tajikistan, Turkmenistan and Uzbekistan) (Hong and Etoh, 1996) with *Allium longicuspis* proposed to be its wild ancestor (Egea et al., 2017). It belongs to the genus *Allium* whose recent classification is mostly based on nuclear ribosomal DNA sequences involving the internal transcribed spacer region and includes >800 species in 15 subgenera and >70 sections (Li et al., 2010; Friesen et al., 2020). Two regions with broad species diversity are from the Mediterranean basin to Central Asia and North-West of America (Fritsch and Friesen, 2002; Li et al., 2010).

Garlic is diploid ($2n = 16$) and asexually (vegetative) propagated species, primarily from cloves due to obligatory apomixis (Bradley et al., 1996), although a few fertile types had been reported (Etoh et al., 1988). Therefore, clonal selection is the method of choice for garlic breeding. Sexual reproduction and fertility are impaired among commercial cultivars (García-Lampasona et al., 2003), relying solely on mutations (spontaneous or induced), somaclonal variation and genetic transformation. Vegetative reproduction usually assures crop uniformity, but in garlic, there is a great degree of diversity in phenotypic traits, as well as in some agronomic traits such as drought tolerance and other stress responses (Bradley et al., 1996; Stavěliková et al., 2008). Large genetic diversity has been observed in garlic clones that are cultivated around the world and also in collected non-cultivated garlic accessions found in nature (Maass and Klaas, 1995; Bradley et al., 1996; Volk et al., 2004). Classification of cultivars based on morphological traits is difficult due to a wide variation in clove number, colour and size within the same cultivar, varying further phenotypically under different agro-climatic conditions, despite the lack of sexual reproduction.

Conservation of garlic genetic resources is important as such collections present a source of traits with potential economic value. A large number of garlic genetic resources are maintained in gene banks, which are a source of variability and present an opportunity for genetic research and breeding programs. Two main strategies for preserving garlic collections are field maintenance and cryopreservation, each of them having their benefits and drawbacks, but for long-term preservation cryopreservation generally becomes less costly than field culture after 8–13 years (Keller et al., 2013). Maintenance of field collections is expensive and laborious and demands equipment and consumables. Hence, characterisation and evaluation of accessions require a combination of agro-morphological traits and molecular markers to help decision-making in minimising the number of accessions and maximising the genetic diversity. Molecular studies of garlic are mainly focused on the analysis of genetic diversity of cultivated garlic clones, mostly studied through the use of several molecular marker techniques, such as random

amplified polymorphic DNA (RAPD) (Maass and Klaas, 1995; Paredes et al., 2008), amplified fragment length polymorphism (AFLP) (García-Lampasona et al., 2003; Volk et al., 2004; Ipek et al., 2005, 2006) and isozymes (Ipek et al., 2003). These techniques are preferential in discriminating garlic taxonomy because they are not affected by environmental conditions, which is an issue with morphological traits.

Simple sequence repeats (SSRs) or microsatellites have been extensively used in the analysis of genetic diversity and population genetic studies on many plant species, including garlic (Ma et al., 2009; Wang et al., 2010; Zhao et al., 2011; Jo et al., 2012; Cunha et al., 2014; Ipek et al., 2015; Barboza et al., 2018; Kumar et al., 2019). Besides next-generation sequencing techniques and high-throughput genotyping-by-sequencing (GBS) approaches, SSR markers are still preferred and intensively used due to genome abundance, high polymorphism, co-dominance, high reproducibility, low cost and transferability in laboratories (Wang et al., 2010). Barboza et al. (2018) developed 110 SSR markers from expressed sequence tags (EST) and summarised in total 312 previously reported genomic and EST SSR markers in garlic species (Ma et al., 2009; Lee et al., 2011; Cunha et al., 2012; Chand et al., 2015; Ipek et al., 2015; Liu et al., 2015). However, their usage has been limited by the large (15.9 Gbp/1C) and duplicated genome that result, in some cases, in more than two alleles per SSR locus (Ipek et al., 2008; Ma et al., 2009; Egea et al., 2017; Peška et al., 2019). In the Adriatic region, garlic has been grown since ancient times (Dumičić et al., 2013). Six Croatian garlic landraces (traditional cultivars or varieties) – *Brgudski ozimi* *sinonim Benkovački*, *Cerički ozimi*, *Istarski crveni*, *Podravski ozimi*, *Slavonski ozimi* and *Šokac* – are registered in the EU Plant Variety Database (v.3.2.1).

The objective of this study was to assess the genetic diversity and structure of the Croatian garlic collection comprising 64 accessions from 3 Croatian regions (Istria, Dalmatia and continental Croatia) and 16 foreign landraces using SSR markers. To our knowledge, this is the first attempt to extensively evaluate garlic accessions from Croatia. Obtained results would give better insight on garlic genetic resources, and thus support the effective and sustainable management of Croatian collections.

MATERIAL AND METHODS

Plant material

A total of 80 accessions maintained as a working collection of the Institute of Agriculture and Tourism, Poreč, Croatia, were evaluated, of which 64 accessions were originated from 3 Croatian regions and 16 from abroad. Some of the Croatian accessions evaluated here are already a part of the working Collection of Vegetables of the National Program for the Conservation and Sustainable Use of Plant Genetic Resources for Food and Agriculture. The information on the

accessions included in the national collection (CPGRD ID in Table S1 in Supplementary Materials) is available in the Croatian Plant Genetic Resources Database: <https://cpgrd.hapih.hr/>.

Garlic accessions used in the analysis are given in Table S1 in Supplementary Materials. Eleven of sixteen foreign landraces were sourced from the United States Department of Agriculture (USDA, Pullman, WA) through the Germplasm Resources Information Network (GRIN) in 2017. Three accessions were sourced from Plant Genetic Resources Network held by Embrapa Genetic Resources and Biotechnology, Brazil. The remaining two foreign accessions were obtained from the nursery (Semenarna Ljubljana) or directly from the breeder (see Table S1 in Supplementary Materials; IPTPO ID: AGRE11 and IPT189).

SSR genotyping

Total genomic DNA was extracted from fresh young leaves of a single individual plant (100 mg) per accession, using the commercial kit PureLink® Plant Total DNA Purification Kit (Invitrogen), in accordance with the manufacturer's instructions.

Thirteen SSR markers [GB-ASM-040, GB-ASM-053, GB-ASM-059, GB-ASM-072, GB-ASM-080 (Ma et al., 2009), Asa14 (Cunha et al., 2012) and As739, As623, As2655, As96, As449, As6389 and As5944 (Ipek et al., 2015)] were used for genotyping (Table 1) by following the optimised procedures described in Ma et al. (2009) and Ipek et al. (2015). Forward SSR primers were end-labelled with 6FAM fluorophore. The amplified fragments were run on an ABI 3730XL DNA analyser (Applied Biosystems, Foster City, CA, USA) and the results were analysed using GeneMarker®

Software V2.7.0 (SoftGenetics, USA) and 400HD ROX™ dye-labelled internal size standard marker. SSR peak estimates were determined using inbuilt software on pre-set settings. Each peak was individually evaluated. Where more than two alleles (peaks) were apparent, their pattern was cross-examined with other available samples to determine their unique descriptive allelic values. To eliminate the possibility of error, all samples in which the appearance of multiple peaks was detected were analysed several times. Multiple peaks can be addressed by picking two 'main' peaks to allow further analysis of co-dominant markers (Ma et al., 2009) or by treating SSRs as dominant markers and recording the presence or absence of all specific alleles (Pan, 2006; Arroyo et al., 2010; Trapnell et al., 2011; Wang et al., 2014; Meena et al., 2020) to avoid bias in the elimination of specific and reproducible peaks. We used the latter approach in genetic structure analysis.

Data analysis

Alleles from each microsatellite marker were scored as present (1) or absent (0) to create a binary matrix. After the identification of accessions sharing the identical multilocus genotype, replicates originating from the same region were removed from the dataset to calculate molecular diversity parameters. Molecular diversity was inferred by distinct clones (N_g), genotypic (clonal) richness (N_{gr}), number of private genotypes [genotypes found exclusively in a particular group of accessions] (N_{pr}), private genotypic (clonal) richness (N_{par}), percentage of polymorphic markers and Shannon's information index (Shannon and Weaver, 1949; Lewontin, 1972). N_{gr} and N_{par} were defined as the number of distinct genotypes and the numbers of private genotypes independent of the sample size, respectively, and they were calculated using the rarefaction method of Hurlbert (1971) as described by Lazović et al. (2018).

Pairwise distances between 80 accessions were calculated using Dice's coefficient (Dice, 1945), and a UPGMA tree based on Dice's distance matrix was constructed. Statistical support of the branches was tested with a bootstrap analysis using 1,000 replicates (Felsenstein, 1985). The calculations were made using PAST v4.01 (Hammer et al., 2001).

The assessment of the genetic structure was performed using STRUCTURE v2.3.4 (Pritchard et al., 2000) and BAPS v6.0 (Corander et al., 2003), including 61 distinct multilocus genotypes (MLGs). Thirty runs of STRUCTURE were done by setting the number of clusters from 1 to 11 ($K = 1-11$) on the Isabella computer cluster at the University of Zagreb, University Computing Centre (SRCE). Each run consisted of a burn-in period of 200,000 steps followed by 10^6 MCMC (Monte Carlo Markov Chain) replicates assuming admixture model and correlated allele frequencies. No prior information was used to define the clusters. The choice of the most likely number of clusters (K) was carried out by comparing the average

Table 1. Allelic diversity of 13 microsatellite loci scored in 80 garlic accessions.

No.	Locus	Repeat motif	Size range	N_a
1	Asa14	(GT) ₇	221–243	5
2	GB-ASM-040	(AC) ₆ , (AC) ₁₄ -(AT) ₅	253–283	6
3	GB-ASM-053	(CA) ₁₅ , (AC) ₈	156–214	8
4	GB-ASM-059	(TG) ₁₁ , (TG) ₅	264–295	5
5	GB-ASM-072	(TA) ₇ , (TG) ₅ GC(GT) ₉ T(TG) ₈	161–273	6
6	GB-ASM-080	(CCG) ₅	142–155	4
7	AS739	(AGC) ₁₀	196–212	6
8	AS623	(GCT) ₆	289–292	2
9	AS2655	(AGAAA) ₅	239–262	6
10	AS96	(AACGGC) ₄	123–141	5
11	AS449	(AGATGG) ₅	195–221	6
12	AS6389	(AGCCTG) ₅	100–235	5
13	AS5944	(AC) ₂₈	159–231	7
Average				5.46

N_a is the number of alleles.

estimates (and standard deviations) of the likelihood of the data, $\ln[\Pr(X|K)]$, for each value of K as well as by calculating an *ad hoc* statistic ΔK (Evanno et al., 2005) as implemented in STRUCTURE HARVESTER v0.6.94 (Earl and Vonholdt, 2012). Runs were clustered and averaged using CLUMPAK (Kopelman et al., 2015). BAPS was run with the maximal number of clusters (K) set to 10 and each run was replicated 30 times. The best value of K was estimated using the log marginal likelihood values of the best partitions and the distribution of posterior probabilities for different K values. Results of the mixture analysis were used as input for population admixture analysis (Corander and Marttinen, 2006) with default settings.

The analysis of molecular variance (AMOVA; Excoffier et al., 1992) was used to partition the total molecular variance among and within groups of accessions originating from the three Croatian regions and abroad. The variance components were tested using 10,000 permutations in Arlequin v3.5.2.2 (Excoffier and Lischer, 2010). Pairwise comparisons between groups that were examined with AMOVA resulted in ϕ_{ST} values, which were equivalent to the proportion of the total variance that was partitioned between the two groups.

RESULTS

Thirteen SSR markers were selected for this study (Table 1). We found 1–5 alleles per locus for each accession. Overall, 71 alleles were detected with a mean of 5.46 alleles per locus. The number of alleles per locus ranged from two at AS623 to eight at GB-ASM-053 (Table 1).

Among the 80 accessions evaluated, we identified 61 distinct MLGs, of which 51 represented unique genotypes. The remaining accessions were divided in 10 MLG groups, comprising potential duplicates or redundant genotypes. Group size ranged from two to seven accessions per group.

Our molecular diversity study of garlic accessions revealed a total of 48 distinct genotypes in the group of 64 Croatian accessions, while all of the 16 foreign accessions were distinct genotypes (Table 2). Croatian

and foreign sets shared three genotypes (Figure 1; accessions P1S02/P4S75, P3S40/P4S70 and P4S78/P1S01/P3S47/P3S48/P3S52/P3S57/P3S59). Within the group of Croatian accessions, the highest number of distinct genotypes (19) was observed in the group originating from Dalmatia. The lowest number of distinct genotypes (13) was found in accessions from continental Croatia. However, the values of genotypic richness, i.e. the number of genotypes independent of sample size was relatively similar across regions, ranging from 12.66 to 13.61. The Croatian region of Istria had the highest share of private genotypes, as well as the highest private genotypic richness (Table 2), while the region of continental Croatia showed the highest Shannon's information index.

A total of 48 distinct genotypes were identified in the Croatian set of accessions. The genotypes within groups (from one or more Croatian regions, but not among foreign accessions) were counted as one distinct genotype; e.g. accessions P1S02 (MLG G06) and P3S40 (MLG G09) are not considered as distinct genotypes (Table S1 in Supplementary Materials). The number of private genotypes attributed to the Croatian regions refers to all genotypes, except for foreign accessions (Table S1 in Supplementary Materials). The higher percentage of polymorphic loci and Shannon's index of the foreign set of accessions reflected the geographically distant origins.

UPGMA tree based on Dice's distance matrix was constructed to assess the relationships among accessions (Figure 1). Five main clusters (A–E) and two separate lineages (P2S30 and P4S77-SYR) were identified. Clusters B, C and E were supported with bootstrap values >50%.

The assessment of the genetic structure was performed on 61 distinct MLGs using STRUCTURE and BAPS softwares. The most probable number of clusters (K) was inferred by the *ad hoc* statistic ΔK based on the rate of change in the log probability of data between successive K values (Evanno et al., 2005). The highest ΔK value was detected at $K = 3$ ($\Delta K = 391.14$) followed by $K = 4$ ($\Delta K = 21.18$) and $K = 5$ ($\Delta K = 9.37$), whereas the highest average estimate of the likelihood of the data, $\ln[\Pr(X|K)]$, was obtained at $K = 5$ (-944.85) (Figure S1 in Supplementary Materials).

As expected, the results obtained by STRUCTURE (Figure 1B) were congruent with the UPGMA tree (Figure 1A). At $K = 3$ clusters A and B corresponded to the UPGMA clustering, whereas cluster C included the UPGMA clusters C–E. At $K = 4$, cluster C splitted into UPGMA clusters C and D, and finally, at $K = 5$, cluster D splitted into UPGMA clusters D and E. Therefore, STRUCTURE and UPGMA clustering were in accordance. Two separate lineages (P2S30 and P4S77-SYR) observed in the UPGMA tree represented accessions of mixed ancestry (Figure 1B).

The presence of distinct clusters among accessions was further evaluated using a Bayesian approach as implemented in BAPS. The partition with the highest log

Table 2. Molecular diversity of 80 garlic accessions.

No	Origin	n	N_g	N_{gr}	N_{pr}	N_{par}	% P	I
1	Croatia	64	48	–	45	–	84.51	0.508
1.1	Continental Croatia	16	13	13.00	11	11.00	64.79	0.493
1.2	Istria	22	18	13.61	17	12.88	67.61	0.412
1.3	Dalmatia	26	19	12.66	16	10.43	70.42	0.420
2	Foreign	16	16	16.00	13	13.00	94.37	0.626
Total		80	61					

n is the number of accessions; N_g is the number of distinct genotypes; N_{gr} is the genotypic richness; N_{pr} is the number of private genotypes; N_{par} is the private genotypic richness; % P is the percentage of polymorphic loci; I is the Shannon's information index.

marginal likelihood (−1,305.93) consisted of five clusters that were highly congruent with both the UPGMA tree clustering and the results of STRUCTURE at $K = 5$ (Figure 1C).

Most accessions (38/80) were grouped into cluster A, followed by cluster B (24/80), and clusters C–E (with 8, 8 and 2 accessions, respectively) (Figure 1). Accessions from the Istria region prevailed in cluster A (18 of 38),

followed by those from Dalmatia (9) and continental Croatia (7). Only four foreign accessions were grouped in this cluster. Accessions from Dalmatia grouped mostly into cluster B (15/24), together with four accessions from continental Croatia and five foreign accessions. Eight accessions grouped into cluster C, of which five were from continental Croatia, one from Dalmatia and two foreign accessions. Cluster D comprised three foreign and four Istrian accessions, as well as a Dalmatian one, whereas two foreign accessions grouped into cluster E. An accession from Istria and a foreign accession (Syria) assigned to cluster D showed highly admixed ancestry and formed separate lineages on the UPGMA tree.

Accessions from Slovenia and Serbia grouped in cluster A, including one accession from the USA and one from Brazil. Cluster B comprised accessions from Balkan countries (Bulgaria, Montenegro and Serbia), Spain and the USA. Accessions from the Czech Republic and Uzbekistan grouped in cluster D, whereas two Brazilian accessions formed the cluster E.

The AMOVA was used to partition the total molecular diversity among and within groups of accessions originating from the three Croatian regions and abroad (Table 3).

AMOVA showed that approximately 97.25% of the total genetic diversity was attributable to differences among accessions within the two origin groups resulting in a non-significant ϕ_{ST} value (Table 3(A)). However, the partition of the total genetic diversity among and within three Croatian regions revealed that a significant ($p < 0.0001$) proportion of total variation (12.29%) was due to genetic differences of accessions among the three Croatian regions (Table 3(B)). The same was true in AMOVA that included the accessions from three Croatian regions and a group of foreign accessions (Table 3(C)) although the proportion of total variation (8.89%) attributable to the among-group component was somehow lower, but still highly significant ($p < 0.0001$).

The pairwise comparisons between the four groups of accessions revealed that the partition between the group of foreign accessions (P4) and those from continental Croatia (P1) was non-significant as it was between the Dalmatian group (P3) and foreign

Figure 1. UPGMA tree (A) based on SSR marker data of 80 garlic accessions and its proportion of membership in each cluster as defined by STRUCTURE (B) and BAPS (C). Region of origin of each accession is indicated as follows: P1 – continental Croatia, P2 – Istria, P3 – Dalmatian and P4 – foreign (followed by three-letter country codes). Bootstrap support values >50% of 1,000 replicates are given above branches. Each accession is represented by a single horizontal rectangle divided into colours. Each colour represents one cluster, and the length of the coloured segments shows the estimated proportion of membership in that cluster. SSR, simple sequence repeat.

Table 3. AMOVA for the partition of the total molecular diversity between and within Croatian and foreign garlic accessions (A), among and within three Croatian regions (B) and among and within three Croatian regions including foreign accessions as a fourth group.

Analysis	Source of variation	df	Variance components	% total variation	ϕ -statistics	$P(\phi)$
(A)	Among groups	1	0.244	2.75	0.027	0.099
	Within groups	62	8.639	97.25		
(B)	Among groups	2	1.023	12.29	0.123	<0.0001
	Within groups	47	7.304	87.71		
(C)	Among groups	3	0.790	8.89	0.089	<0.0001
	Within groups	62	8.090	91.11		

AMOVA, analysis of molecular variance.

accessions (P4) (Table 4). However, all the partitions including the Istrian group of accessions (P2) were significant, and the highest ϕ_{ST} was obtained when analysing the partition between the Istrian (P2) and Dalmatian (P3) groups.

DISCUSSION

The cultivation and use of garlic are a part of the Croatian tradition, and each region or village has its local landraces preserved for generations (Dumičić et al., 2014). In the last decades, significant efforts have been made to collect and characterise national genetic resources to ensure their long-term conservation in ex situ collections, and thus, their sustainable management. The Institute of Agriculture and Tourism (IPTPO) collection was established in 2014 and is continually expanded ever since. A molecular characterisation of the collection has not been carried out so far, and the available published data are sparse (Poljuha et al., 2018).

In this study, we aimed to molecularly characterise the current Croatian garlic collection and to assess its genetic diversity and structure. We genotyped 64 accessions originating from three Croatian regions, along with 16 introduced cultivars, using 13 polymorphic SSR markers. The study revealed a total of 48 distinct genotypes in the group of Croatian accessions, while all analysed foreign accessions were distinct genotypes. The diversity of the latter set (also confirmed by a higher percentage of polymorphic loci and Shannon's index) was expected because the foreign garlic materials analysed in the present study were intentionally selected to broaden genetic variation in the sample set. They were obtained from the collection of the USDA (Pullman, WA, USA) and chosen to represent 7 out of 10 different phylogenetic groups identified in previous AFLP-based diversity analysis of garlic (Ipek et al., 2003). In addition, within this set of samples, we analysed three accessions from the Brazilian collection

at Embrapa. The diversity of Croatian garlic accessions observed through different parameters was relatively similar across all three regions (Table 1). The most diverse group of Croatian accessions originated from the Dalmatia region, with 19 distinct genotypes, out of the 26 analysed. Although the observed diversity is probably related to the sample size, Dalmatia is a well-known location of garlic cultivation (Ožanić, 1955; Dumičić et al., 2013), with a few widely distributed and highly valued ecotypes, such as *Ljubitovački šarac* and *Brgudski ozimi*. However, the highest genotypic richness and the number of private genotypes were found in the set of accessions from the Istria region, indicating the potential of this area as a valuable source of genetic material for breeding. Genotypic richness could correlate with specific and diverse microclimate conditions in a relatively small area.

In this research, we chose 13 polymorphic SSR markers, developed in previous studies (Ma et al., 2009; Cunha et al., 2012; Ipek et al., 2015) and applied in the analyses of garlic genetic resource collections and germplasms (Zhao et al., 2011; Jo et al., 2012; Cunha et al., 2014; Barboza et al., 2020). The SSR markers revealed considerable genetic diversity among 61 MLGs identified, with Dice's distance ranging from 0.019 to 0.773, with an average value of 0.346. Usually, SSR-based methods rely on identifying a maximum of two peaks in an electropherogram, measuring the fluorescence of correctly matched tags for either homozygous or heterozygous individuals. This becomes challenging in species where genome duplication is common. In this study, we observed from one to five peaks on a single SSR marker per accession (Table S2 in Supplementary Materials). Moreover, the observed variations in garlic SSR loci could also occur in part as a result of replication slippage (Hosseinzadeh-Colagar et al., 2016). Since we were able to observe clear peaks and other authors also reported more than two alleles per SSR locus in diploid *Allium* species (Fischer and Bachmann, 2000; Ipek et al., 2008, 2015; Ma et al., 2009; Jo et al., 2012), we disregarded slippage in favour of suggested genome duplication. Even if the number of peaks could be matched, there is no objective method to determine a cut-off value that would accurately describe the actual phylogenetic relationship of the accessions, as confirmed by other molecular methods, breeding programme records or even phenotypic observation. This does not devalue the use of SSR for phylogenetic conclusions if a stringent methodology for recognising peaks is observed. A few authors reported a significant diversity within garlic collections, using both morphological and molecular data (Zhao et al., 2011; García-Lampasona et al., 2012; Chen et al., 2014; Cunha et al., 2014; Barboza et al., 2020). In this study, AMOVA analysis revealed 87.71% of the total molecular diversity within and 12.29% among Croatian groups. The same pattern was observed when evaluating the three Croatian regions and a group of foreign accessions. These

Table 4. AMOVA's pairwise ϕ_{ST} values among groups of garlic accessions.

Group of accessions	Region	P1	P2	P3	P4
P1	Continental Croatia		*	ns	ns
P2	Istria	0.103		***	**
P3	Dalmatia	0.049	0.189		ns
P4	Foreign accessions	-0.006	0.115	0.048	

Below diagonal: pairwise ϕ_{ST} values.

Above diagonal: $P(\phi_{ST})$.

AMOVA, analysis of molecular variance; ns, non-significant.

*Significant at $p < 0.05$.

**Significant at $p < 0.01$.

***Significant at $p < 0.001$.

results support regional structuring and the existence of significant diversity within local populations as a consequence of probable ancestral sexual reproduction followed by independent domestication history (Cunha et al., 2014). These values are in accordance with the results of Zhao et al. (2011) and Kumar et al. (2019), who observed similar percentages of variance (84.4% and 84.0%, respectively) attributable to within-population differences.

Clustering analysis revealed five main clusters among the 64 Croatian and 16 foreign accessions (clusters A–E, Figure 1). The distinction of clusters based on geographical origin was not clear, mainly due to unknown historical origin of accessions, frequent exchange of propagule material among farmers from different Croatian regions and the vegetative propagation of the species. Istrian garlic accessions were mostly grouped in cluster A and Dalmatian prevailed in cluster B, whereas the accessions from continental Croatia were evenly distributed in clusters A–C (Figure 1A). Since our primary intention was to catalogue the collection and detect duplicate or redundant genotypes, we were especially interested in the closely related samples. With 13 markers used, we detected only 10 MLG groups in 80 analysed samples. All identical and closely related accessions were observed in clusters A–C, and their grouping was based on geographical origin (e.g. P2S19, P2S20 and P2S21; P2S32, P2S33 and P2S34; P3S42, P3S45 and P3S49; P1S09, P1S10 and P1S11; P1S15 and P1S16; etc.) or traditional ways of plant material exchange in these areas (e.g. P3S40 and P4S70; P3S47, P3S48, P3S52, P3S57, P3S59 and P1S01; etc.).

We also noticed a great diversity among accessions from some narrow geographical areas, such as the Central Istrian Pazin area, where we identified diverse groupings of collected samples. Closely related accessions P2S23, P2S24 and P2S25 grouped in cluster A, together with the Istrian genotype P2S27 with a peculiar morphological and aromatic characteristics. In contrast, two accessions from the same area, P2S18 and P2S37, grouped in cluster D, together with geographically closed P2S26 accession. Such areas with pronounced diversity are a valuable source of plant material for breeding purposes. In this study, we also identified the relationship between some widely distributed and highly valued cultivars, such as ‘Ljubitovečki šarac’ (accession P3S53), which displayed admixture of two populations with membership shared between clusters A and B (0.63:0.37, respectively).

A few authors have reported a moderate level of correlation between genetic clustering based on SSR markers with geographical origin (Ma et al., 2009; Zhao et al., 2011; Jo et al., 2012; Kumar et al., 2019), whereas other studies reported none (Ipek et al., 2003, 2008; Volk et al., 2004; Paredes et al., 2008; Barboza et al., 2018). Clustering by phenotypic characteristics observed by different authors is also variable (Bradley et al., 1996; García-Lampasona et al. 2010; Ovesná et al., 2014; Ovesná et al., 2015; Barboza et al.,

2020). In our study, accessions were not clustered according to their flowering behaviour (Table S1 in Supplementary Materials). The lack of correlation between morphological variation and marker pattern may originate from the non-association of the markers used for genotyping with phenotypic characteristics but also can be rooted in epigenetic variation in garlic lines, propagated in some regions for centuries. Barboza et al. (2020) observed and explained the discrepancy between the level of genetic and phenotypic variation found in the Argentine garlic collection by studies of Gimenez and García-Lampasona (2018) and Gimenez et al. (2016). They indicated frequent and dynamic epigenetic changes in non-coding and coding regions of the garlic genome, even under normal field-cultivation conditions, in comparison to less frequent genetic changes, as possible causes for the observed discrepancies between molecular and phenotypic variation.

In our analysis, foreign accessions were present in all clusters (Figure 1). Brazilian cultivars ‘Chinês Real’ (P4S65) and ‘Ugarte’ (P4S67) formed the genetically most distant cluster E. However, the third Brazilian accession P4S66 (cv. ‘Peruano’) grouped in cluster A, in a subcluster with accession P2S28 from the Istrian region. All the above-mentioned Brazilian cultivars were previously analysed by SSRs in the study of Cunha et al. (2014) using a set of 130 accessions. In their study, cultivar ‘Peruano’ formed a separate lineage as the only accession with admixed ancestry. Two Polish accessions (P4S71 and P4S72) grouped in cluster C, whereas accession P4S69 from North Moravia in Czech Republic and P4S80 originated from Uzbekistan grouped in cluster D. Accession P4S80 (UO85) was classified as *A. longicuspis* L. in the GRIN (USDA) database but in our analysis did not separate from other accessions of *A. sativum* L. This is in accordance with the study of Ipek et al. (2015) in which accessions of both species were clustered together based on their flowering ability but not on their geographic origin. Volk et al. (2004), who examined the diversity of 211 commercial and federally held accessions maintained by the US National Plant Germplasm System (NPGS), including the clone UO85, also report the indistinguishability of *A. longicuspis* and *A. sativum*.

Our analysis revealed two accessions that formed separate lineage having admixture ancestry – Syrian P4S77 and P2S30 (Figure 1). Accession P4S77 is classified as cv. ‘Kisswani’ in the GRIN database (USDA) and the cultivar originates from Syria. Accession P2S30 is recorded as cv. ‘Music’ in the IPTPO collection. It belongs to *A. sativum* ssp. *ophioscorodon*, and the hardneck garlic type that produces scapes or flower stalks originates from Italy. This cultivar is characterised by high overwintering ability, increased vigour and high marketable yields (Walters, 2008).

This study confirms the usefulness of SSR genotyping for examination of diversity within garlic collections and provides important information for

further management of Croatian garlic collection, as well as its relationship to known international garlic cultivars.

CONCLUSIONS

Garlic is a staple crop present in the Mediterranean culture for hundreds of years. As highly valued vegetative species, it was enjoyed and traded wherever people moved, and it is evident that farmers went through great efforts to select unique varieties. Assessment of genetic diversity and structure of the Croatian garlic germplasm supported regional structuring and the existence of significant diversity within local populations. Genetic differentiation of the accessions based on origin provides insights into the movement of garlic genotypes, as well as ethnological and historical data. This study is the first attempt of a molecular characterisation of collected genetic resources of garlic in Croatia with the aim of setting the course for future preservation strategies with particular emphasis on the value of diversity in the context of climate change both on macro and micro levels.

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AUTHOR CONTRIBUTIONS

D. P., Z. Š. and S. G. B. worked on conceptualisation, methodology and validation. D. P., M. F., I. K., T. W. and Z. Š. performed formal analysis. D. P., D. B., N. T., G. D., S. K. and C. C. participated in providing the necessary resources. D. P., M. F., I. K., T. W. and S. G. B. curated the data. Z. Š., S. G. B., D. B., D. P. and S. K. contributed to the funding acquisition. D. P., M. F., T. W. and Z. Š. prepared original manuscript draft. All co-authors reviewed and edited the manuscript.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

REFERENCES

- ARROYO, J. M., RIGUEIRO, C., RODRÍGUEZ, R., HAMPE, A., VALIDO, A., RODRÍGUEZ-SÁNCHEZ, F., AND JORDANO, P. (2010). Isolation and characterization of 20 microsatellite loci for laurel species (*Laurus*, Lauraceae). *American Journal of Botany*, 97(5), e26–e30.
- BARBOZA, K., BERETTA, V., KOZUB, P. C., SALINAS, M. C., MORGENFELD, M. M., GALMARINI, C. R., AND CAVAGNARO, P. F. (2018). Microsatellite analysis and marker development in garlic: Distribution in EST sequence, genetic diversity analysis, and marker transferability across Alliaceae. *Molecular Genetics and Genomics*, 293, 1091–1106.
- BARBOZA, K., SALINAS, M. C., ACUÑA, C. V., BANNOUN, F., BERETTA, V., GARCÍA-LAMPASONA, S., BURBA, J. L., GALMARINI, C. R., AND CAVAGNARO, P. F. (2020). Assessment of genetic diversity and population structure in a garlic (*Allium sativum* L.) germplasm collection varying in bulb content of pyruvate, phenolics, and solids. *Scientia Horticulturae*, 261, 108900, doi: 10.1016/j.scienta.2019.108900.
- BRADLEY, K. F., RIEGER, M. A., AND COLLINS, G. G. (1996). Classification of Australian garlic cultivars by DNA fingerprinting. *Australian Journal of Experimental Agriculture*, 36, 613–618.
- CHAND, S. K., NANDA, S., ROUT, E., AND JOSHI, R. K. (2015). Mining, characterisation and validation of EST derived microsatellites from the transcriptome database of *Allium sativum* L. *Bioinformation*, 11, 145–150.
- CHEN, S., CHEN, W., SHEN, X., YANG, Y., QI, F., LIU, Y., AND MENG, H. (2014). Analysis of the genetic diversity of garlic (*Allium sativum* L.) by simple sequence repeat and inter simple sequence repeat analysis and agro-morphological traits. *Biochemical Systematics and Ecology*, 55, 260–267.
- CORANDER, J., AND MARTTINEN, P. (2006). Bayesian identification of admixture events using multilocus molecular markers. *Molecular Ecology*, 15(10), 2833–2843.
- CORANDER, J., WALDMANN, P., AND SILLANPÄÄ, M. J. (2003). Bayesian analysis of genetic differentiation between populations. *Genetics*, 163(1), 367–374.
- CUNHA, C. P., HOOGHEIDE, E. S., ZUCCHI, M. I., MONTEIRO, M., AND PINHEIRO, J. B. (2012). New microsatellite markers for garlic, *Allium sativum* (Alliaceae). *American Journal of Botany*, 99, e17–e19.
- CUNHA, C. P., RESENDE, F. V., ZUCCHI, M. I., AND PINHEIRO, J. B. (2014). SSR-based genetic diversity and structure of garlic accessions from Brazil. *Genetica*, 142(5), 419–431.
- DICE, L. R. (1945). Measures of the amount of ecologic association between species. *Ecology*, 26, 297–302.
- DUMIČIĆ, G., ČAGALJ, M., URLIĆ, B., RUNJIĆ, M., AND GORETA BAN, S. (2013). Yield components of

- introduced cultivars and local ecotypes of garlic (*Allium sativum* L.). Paper presented at the 48th Croatian and 8th International Symposium on Agriculture, Dubrovnik, Croatia, 349–352.
- DUMIČIĆ, G., PAVIĆ, Š., LJUBENKOV, B., URLIĆ, B., ŽANIĆ, K., AND RAKO, A. (2014). Bulb yield components of garlic ecotypes from Adriatic region. Paper presented at the 49th Croatian and 9th International Symposium on Agriculture, Dubrovnik, Croatia, 310–314.
- EARL, D. A., AND VONHOLDT, B. M. (2012). STRUCTURE HARVESTER: A website and program for visualising STRUCTURE output and implementing the Evanno method. *Conservation Genetic Resources*, 4(2), 359–361.
- EGEA, L. A., MÉRIDA-GARCÍA, R., KILIAN, A., HERNANFEZ, P., AND DORADO, G. (2017). Assessment of genetic diversity and structure of large garlic (*Allium sativum*) Germplasm Bank, by Diversity Arrays Technology “Genotyping-by-Sequencing” Platform (DArTseq). *Frontiers in Genetics*, 8, 98, doi: 10.3389/fgene.2017.00098.
- ETOH, T., NOMA, Y., NISHITARUMIZU, Y., AND MAKAMOTO, T. (1988). Seed productivity and germinability of various garlic clones collected in Soviet Central Asia. *Memoirs of the Faculty of Agriculture, Kagoshima University*, 24(1), 29–39.
- EVANNO, G., REGNAUT, S., AND GOUDET, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molecular Ecology*, 14(8), 2611–2620.
- EXCOFFIER, L., AND LISCHER, H. E. L. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, 10, 564–567.
- EXCOFFIER, L., SMOUSE, P. E., AND QUATTRO, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction sites. *Genetics*, 131, 479–491.
- FAOSTAT DATABASE. Food And Agriculture Organization Of The United Nations. (2018). Rome, Italy: FAO. Retrieved from <http://www.fao.org/faostat/en/#data/QC>. (March 26, 2020).
- FELSENSTEIN, J. (1985). Confidence limits on phylogenesis: An approach using the bootstrap. *Evolution*, 39, 783–791.
- FISCHER, D., AND BACHMANN, K. (2000). Onion microsatellites for germplasm analysis and their use in assessing intra- and interspecific relatedness within the subgenus *Rhizirideum*. *Theoretical and Applied Genetics*, 101, 153–164.
- FRIESEN, N., SMIRNOV, S. V., SHMAKOV, A. I., HARDEN, T., OYUNTSETSEG, B., AND HURKA, H. (2020). *Allium* species of section *Rhizomatosa*, early members of the Central Asian steppe vegetation. *Flora*, 263, 151536.
- FRI TSCH, R. M., AND FRIESEN, N. (2002). Evolution, domestication and taxonomy. In D. Rabinowitch and L. Currah (Eds), *Allium crop science: Recent advances* (pp. 5–30). Wallingford, UK: CAB International.
- GARCÍA-LAMPASONA, S. C., ASPRELLI, P., AND BURBA, J. L. (2012). Genetic analysis of a garlic (*Allium sativum* L.) germplasm collection from Argentina. *Scientia Horticulturae*, 138, 183–189.
- GARCÍA-LAMPASONA, S. C., BURBA, J. L., AND SIMON, P. W. (2010). Molecular markers: Are they really useful to detect genetic variability in local garlic collections? *The Americas Journal of Plant Science and Biotechnology*, 4, 104–112.
- GARCÍA-LAMPASONA, S. C., MARTÍNEZ, L., AND BURBA, J. L. (2003). Genetic diversity among selected Argentinean garlic clones (*Allium sativum* L.) using AFLP (amplified fragment length polymorphism). *Euphytica*, 132, 115–119.
- GIMENEZ, M. D., AND GARCÍA-LAMPASONA, S. C. (2018). Before-after analysis of genetic and epigenetic markers in garlic: A 13-year experiment. *Scientia Horticulturae*, 240, 23–28.
- GIMENEZ, M. D., YAÑEZ-SANTOS, A. M., PAZ, R. C., QUIROGA, M. P., MARFIL, C. F., CONCI, V. C., AND GARCÍA-LAMPASONA, S. C. (2016). Assessment of genetic and epigenetic changes in virus-free garlic (*Allium sativum* L.) plants obtained by meristem culture followed by in vitro propagation. *Plant Cell Reports*, 35, 129–141.
- HAMMER, Ø., HARPER, D. A. T., AND RYAN, P. D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, 4(1), 9.
- HONG, C. J., AND ETOH, T. (1996). Fertile clones of garlic (*Allium sativum* L.) abundant around the Tien Shan Mountains. *Breeding Science*, 46, 349–353.
- HOSSEINZADEH-COLAGAR, A., HAGHIGHATNIA, M. J., AMIRI, Z., MOHADJERANI, M., AND TAFRIHI, M. (2016). Microsatellite (SSR) amplification by PCR usually led to polymorphic bands: Evidence which shows replication slippage occurs in extend or nascent DNA strands. *Molecular Biology Research Communications*, 5(3), 167–174.
- HURLBERT, S. H. (1971). The nonconcept of species diversity, a critique and alternative parameters. *Ecology*, 52, 577–586.
- IPEK, M., IPEK, A., ALMQUIST, S. G., AND SIMON, P. W. (2005). Demonstration of linkage and development of the first low-density genetic map of garlic, based on AFLP markers. *Theoretical and Applied Genetics*, 110, 228–236.
- IPEK, M., IPEK, A., AND SIMON, P. W. (2003). Comparison of AFLPs, RAPD markers, and isozymes for diversity assessment of garlic and detection of putative duplicates in germplasm collections. *Journal of the American Society for Horticultural Science*, 128, 246–252.
- IPEK, M., IPEK, A., AND SIMON, P. W. (2006). Sequence homology of polymorphic AFLP markers in garlic (*Allium sativum* L.). *Genome*, 49, 1246–1255.

- IPEK, M., IPEK, A., AND SIMON, P. W. (2008). Rapid characterisation of garlic clones with locus-specific DNA markers. *Turkish Journal of Agriculture and Forestry*, 32, 357–362.
- IPEK, M., SAHIN, N., IPEK, A., CANSEV, A., AND SIMON, P. W. (2015). Development and validation of new SSR markers from expressed regions in the garlic genome. *Scientia Agricola*, 72(1), 41–46.
- JO, M. H., HAM, I. K., MOE, K. T., KWON, S. W., LU, F. H., PARK, Y. J., KIM, W. S., WON, M. K., KIM, T. I., AND LEE, E. M. (2012). Classification of genetic variation in garlic (*Allium sativum* L.) using SSR markers. *Australian Journal of Crop Science*, 6(4), 625–631.
- KELLER, E. R. J., ZANKE, C. D., SENULA, A., BREUING, A., HARDEWEG, B., AND WINKELMANN, T. (2013). Comparing costs for different conservation strategies of garlic (*Allium sativum* L.) germplasm in genebanks. *Genetic Resources and Crop Evolution*, 60(3), 913–926.
- KOPELMAN, N. M., MAYZEL, J., JAKOBSSON, M., ROSENBERG, N. A., AND MAYROSE, I. (2015). Clumpak: A program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources*, 15, 1179–1191.
- KUMAR, M., RAKESH SHARMA, V., KUMAR, V., SIROHI, U., CHAUDHARY, V., SHARMA, S., SARIPALLI, G., NARESH, R. K., KUMAR YADAV, H., AND SHARMA, S. (2019). Genetic diversity and population structure analysis of Indian garlic (*Allium sativum* L.) collection using SSR markers. *Physiology and Molecular Biology of Plants*, 5, 377–386.
- LAZOVIĆ, B., KLEPO, T., ADAKALIĆ, M., ŠATOVIĆ, Z., ARBEITER, A. B., HLADNIK, M., STRIKIĆ, F., LIBER, Z., AND BANDELIJ, D. (2018). Intra-varietal variability and genetic relationships among the homonymic East Adriatic olive (*Olea europaea* L.) varieties. *Scientia Horticulturae*, 236, 175–185.
- LEE, G. A., KWON, S. J., PARK, Y. J., LEE, M. C., KIM, H. H., LEE, J. S., LEE, S. Y., GWAG, J. G., KIMA, C. K., AND MA, K. H. (2011). Cross-amplification of SSR markers developed from *Allium sativum* to other *Allium* species. *Scientia Horticulturae*, 128, 401–407.
- LEWONTIN, R. C. (1972). The apportionment on human diversity. *Evolution Biology*, 6, 381–398.
- LI, Q. Q., ZHOU, S. D., HE, X. J., YU, Y., ZHANG, Y. C., AND WEI, X. Q. (2010). Phylogeny and biogeography of *Allium* (Amaryllidaceae: Allieae) based on nuclear ribosomal internal transcribed spacer and chloroplast rps16 sequences, focusing on the inclusion of species endemic to China. *Annals of Botany*, 106(5), 709–733.
- LIU, T., ZENG, L., ZHU, S., CHEN, X., TANG, Q., MEI, S., AND TANG, S. (2015). Large-scale development of expressed sequence tag-derived simple sequence repeat markers by deep transcriptome sequencing in garlic (*Allium sativum* L.). *Molecular Breeding*, 35, 204, doi: 10.1007/s11032-015-0399-x
- MA, K. H., KWAG, J. G., ZHAO, W., DIXIT, A., LEE, G. A., KIM, H. H., CHUNG, I. M., KIM, N. S., LEE, J. S., Ji, J. J., KIM, T. S., AND PARK, Y. J. (2009). Isolation and characteristics of eight novel polymorphic microsatellite loci from the genome of garlic (*Allium sativum* L.). *Scientia Horticulturae*, 122(3), 355–361.
- MAASS, H. I., AND KLAAS, M. (1995). Intraspecific differentiation of garlic (*Allium sativum* L.) by isozyme and RAPD markers. *Theoretical and Applied Genetics*, 91, 89–97.
- MEENA, R. K., BHANDARI, M. S., AND GINWAL, H. S. (2020). Usage of microsatellite markers for characterization of polyploids: A case study in reference to hexaploid bamboo species. *Silvae Genetica*, 69(1), 94–97.
- OVESNÁ, J., LEIŠOVÁ-SVOBODOVÁ, L., AND KUČERA, L. (2014). Microsatellite analysis indicates the specific genetic basis of Czech bolting garlic. *Czech Journal of Genetics and Plant Breeding*, 50, 226–234.
- OVESNÁ, J., MITROVÁ, K., AND KUČERA, L. (2015). Garlic (*A. sativum* L.) alliinase gene family polymorphism reflects bolting types and cysteine sulphoxides content. *BMC Genetics* 16, 53, doi: 10.1186/s12863-015-0214-z.
- OŽANIĆ, S. (1955). *Agriculture of Dalmatia in the past: Contributions to the history of Dalmatia agriculture*. Croatia: Society of Agronomists NRH-Split Branch.
- PAN, Y. B. (2006). Highly polymorphic microsatellite DNA markers for sugarcane germplasm evaluation and variety identity testing. *Sugar Tech*, 8, 246–256.
- PEREDES, C. M., BECERRA, V. V., AND GONZALEZ, A. M. I. (2008). Low genetic diversity among garlic accessions detected using RAPD. *Chilean Journal of Agricultural Research*, 68, 3–12.
- PEŠKA, V., MANDÁKOVÁ, T., IHRADSKÁ, V., AND FAJKUS, J. (2019). Comparative dissection of three giant genomes: *Allium cepa*, *Allium sativum*, and *Allium ursinum*. *International Journal of Molecular Sciences*, 20(3), 733, doi: 10.3390/ijms20030733.
- POLJUHA, D., KRALJ, I., ŠATOVIĆ, Z., BAN, D., TOTH, N., DUMIČIĆ, G., KEREŠA, S., AND GORETA BAN, S. (2018). Assessment of genetic diversity of garlic accessions from Croatia by SSR markers. Paper presented at the 4th Congress of Croatian Geneticists with International Participation, Krk, Croatia, 58.
- PRITCHARD, J. K., STEPHENS, M., AND DONNELLY, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, 155(2), 945–959.
- SHANNON, C. E., AND WEAVER, W. (1949). *The mathematical theory of communication*. Urbana, USA: University of Illinois Press.
- STAVĚLÍKOVÁ, H. (2008). Morphological characteristics of garlic (*Allium sativum* L.) genetic resources collection – Information. *Horticultural Science*, 35, 130–135.
- TRAPNELL, D. W., HAMRICK, J. L., PARKER, K. C., BRAUNGART, K. W., AND GLENN, T. C. (2011). Evaluating

- the utility of microsatellites for investigations of autopolyploid taxa. *Journal of Heredity*, 102(4), 473–478.
- VOLK, G. M., HENK, A. D., AND RICHARDS, C. M. (2004). Genetic diversity among U. S. garlic clones as detected using AFLP methods. *Journal of the American Society for Horticultural Science*, 129, 559–569.
- WALTERS, A. (2008). Production method and cultivar effects on garlic over-wintering survival, bulb quality, and yield. *HortTechnology*, 18(2), 286–289.
- WANG, J. M., YANG, J. M., ZHU, J. H., JIA, Q. J., AND TAO, Y. Z. (2010). Assessment of genetic diversity by simple sequence repeat markers among forty elite varieties in the germplasm for malting barley breeding. *Journal of Zhejiang University Science B*, 11(10), 792–800.
- WANG, Z., YU, G., SHI, B., WANG, X., QIANG, H., AND GAO, H. (2014). Development and characterization of Simple Sequence Repeat (SSR) markers based on RNA-sequencing of *Medicago sativa* and *in silico* mapping onto the *M. truncatula* genome. *PLoS ONE*, 9(3), e92029, doi: 10.1371/journal.pone.0092029.
- ZHAO, W. G., CHUNG, J. W., LEE, G. A., MA, K. H., KIM, H. H., KIM, K. T., CHUNG, I. M., LEE, J. K., KIM, N. S., KIM, S. M., AND PARK, Y. J. (2011). Molecular genetic diversity and population structure of a selected core set in garlic and its relatives using novel SSR markers. *Plant Breeding*, 130, 46–54.

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SUPPLEMENTARY MATERIALS

Table S1. List of accessions.

Accession no.	IPTPO accession ID	Universal accession ID	Landrace	Origin of accession	Region	State	MLG	UPGMA	Structure K3	BAPS K5
P1S01	IJK18 ^{NB}	—	—	Vojnić	P1	Croatia	G01	B	B	B
P1S02	AFZ014 ^{NB}	—	Unski	Unčani	P1	Croatia	G06	A	A	A
P1S03	AFZ013 ^{NB}	—	Kerestinečki	Kerestinec	P1	Croatia	U01	C	C	C
P1S04	AFZ015 ^B	—	Bjelovarski	Bjelovar	P1	Croatia	U02	A	A	A
P1S05	AGRE8 ^{NB}	—	—	Sesvetski Kraljevec	P1	Croatia	U03	B	B	B
P1S06	AGRE9 ^{NB}	—	—	Cerje Lekeničko	P1	Croatia	U04	A	A	A
P1S07	AFZ011 ^{NB}	—	—	Bjelovar	P1	Croatia	U05	C	C	C
P1S08	IPT246 ^{NB}	—	VGUK1,2-710	Križevci	P1	Croatia	U06	A	A	A
P1S09	IPT249 ^{NB}	VEG00093	VGUK Šokac	Cerić	P1	Croatia	G02	A	A	A
P1S10	IPT250 ^{NB}	—	VGUK675	Bakovčice	P1	Croatia	G02	A	A	A
P1S11	IPT251 ^{NB}	—	VGUK Đakovački	Đakovo	P1	Croatia	G02	A	A	A
P1S12	IPT252 ^{NB}	—	VGUK Sremski	Srijem	P1	Croatia	U07	B	BM	B
P1S13	IPT272 ^B	—	Bilogorski	Donji Mosti	P1	Croatia	U08	C	C	C
P1S14	IPT273 ^{NB}	—	Mraclinski	Mraclin	P1	Croatia	U09	B	B	B
P1S15	IPT274 ^{NB}	—	Kerestinečki	Kerestinec	P1	Croatia	G07	C	C	C
P1S16	IPT275 ^{NB}	—	Unski	Unčani	P1	Croatia	G07	C	C	C
P2S17	IJK7 ^{NB}	—	Tinjanski	Tinjan	P2	Croatia	G08	A	A	A
P2S18	IPT010 ^B	VEG00095	Domaći ozimi crveni	Pazin	P2	Croatia	U10	D	C	D
P2S19	IPT011 ^B	VEG00096	Domaći ozimi	Kaštelir	P2	Croatia	G03	A	A	A
P2S20	IPT012 ^B	VEG00097	Domaći ozimi with stalk	Kaštelir	P2	Croatia	G03	A	A	A
P2S21	IPT013 ^B	VEG00098	Istarski crveni	Lindar	P2	Croatia	G03	A	A	A
P2S22	IPT014 ^{NB}	VEG00094	Domaći jari	Tinjan	P2	Croatia	U11	A	A	A
P2S23	IPT015 ^{NB}	VEG00099	Istarski bijeli	Pazin	P2	Croatia	U12	A	A	A
P2S24	IPT016 ^B	VEG00100	Istarski domaći	Ograde	P2	Croatia	U13	A	A	A
P2S25	IPT017 ^{NB}	VEG00101	Istarski bijeli	Ograde	P2	Croatia	U14	A	A	A
P2S26	IPT019 ^B	VEG00102	Brgujski	Brgud (Opatija)	P2	Croatia	U15	D	C	D

(Continued)

Table S1. (Continued)

Accession no.	IPTPO accession ID	Universal accession ID	Landrace	Origin of accession	Region	State	MLG	UPGMA	Structure K3	BAPS K5
P2S27	IPTO20 ^B	VEG00103	Brsečki	Brseč	P2	Croatia	U16	A	A	A
P2S28	IPT224 ^{NB}	VEG00104	–	Ližnjan	P2	Croatia	U17	A	AM	A
P2S29	IPT338 ^{NB}	–	Istarski bijeli	Lindar	P2	Croatia	U18	A	A	A
P2S30	IPT339 ^B	–	Music	Lindar	P2	Croatia	U19	X	CM	D
P2S31	IPT341 ^B	–	Istarski crveni	Piće	P2	Croatia	U20	A	A	A
P2S32	IPT342 ^{NB}	–	Mješavina	Tinjan	P2	Croatia	G04	A	A	A
P2S33	IPT343 ^B	–	Istarski crveni	Gračičće	P2	Croatia	G04	A	A	A
P2S34	IPT344 ^{NB}	–	Istarski bijeli	Gračičće	P2	Croatia	G04	A	A	A
P2S35	IPT345 ^{NB}	–	Istarski crveni	Beram	P2	Croatia	U21	A	A	A
P2S36	IPT347 ^B	–	Istarski crveni (with stalk)	Oprtalj	P2	Croatia	U22	A	A	A
P2S37	IPT348 ^{NB}	–	Istarski crveni (without stalk)	Oprtalj	P2	Croatia	U23	D	C	D
P2S38	IPT349 ^B	–	Istarski crveni (with stalk)	Oprtalj	P2	Croatia	U24	A	A	A
P3S39	IJK3 ^{NB}	VEG00223	–	Zmijavci	P3	Croatia	U25	A	A	A
P3S40	IJK4 ^{NB}	VEG00224	–	Rastok (Vrgorac)	P3	Croatia	G09	B	B	B
P3S41	IJK8 ^{NB}	VEG00225	–	Kurtovići	P3	Croatia	U26	A	A	A
P3S42	IJK9 ^{NB}	VEG00227	–	Obrovac Sinjski	P3	Croatia	G05	A	A	A
P3S43	IJK10 ^{NB}	VEG00226	–	Stija	P3	Croatia	U27	A	A	A
P3S44	IJK13 ^{NB}	VEG00229	–	Primorski dolac	P3	Croatia	G08	A	A	A
P3S45	IJK14 ^B	VEG00230	–	Trnbusi 1	P3	Croatia	G05	A	A	A
P3S46	IJK17 ^{NB}	VEG00231	–	Opuzen (Raba)	P3	Croatia	U28	B	B	B
P3S47	IJK19 ^{NB}	VEG00232	–	Metković	P3	Croatia	G01	B	B	B
P3S48	IJK20 ^{NB}	VEG00236	–	Trnbusi 2	P3	Croatia	G01	B	B	B
P3S49	IJK22 ^B	VEG00234	–	Rama (Vinjani g.)	P3	Croatia	G05	A	A	A
P3S50	IJK23 ^{NB}	VEG00235	–	Kijevo	P3	Croatia	U29	A	A	A
P3S51	IJK24 ^{NB}	VEG00236	–	Golubić 2	P3	Croatia	U30	C	C	C
P3S52	IJK25 ^{NB}	VEG00237	–	Golubić 1	P3	Croatia	G01	B	B	B

(Continued)

Table S1. (Continued)

Accession no.	IPTPO accession ID	Universal accession ID	Landrace	Origin of accession	Region	State	MLG	UPGMA	Structure K3	BAPS K5
P3S53	IJK26 ^{NB}	VEG00130	Ljubitovački šarac	Ljubitovica	P3	Croatia	U31	A	AM	AM
P3S54	IJK27 ^B	VEG00238	–	Unešić Plameni	P3	Croatia	U32	D	CM	D
P3S55	IJK28 ^B	–	–	Unešić Jaki	P3	Croatia	U33	B	B	B
P3S56	AFZ024 ^{NB}	–	Bruški 1	Bruška	P3	Croatia	U34	B	B	B
P3S57	AFZ025 ^{NB}	–	Lisički 1	Lisičić	P3	Croatia	G01	B	B	B
P3S58	AFZ026 ^{NB}	–	Rodaljički	Rodaljice	P3	Croatia	U35	B	B	B
P3S59	VEG00010 ^{NB}	VEG00010	Brgudski ozimi syn. Benkovački	Brgud	P3	Croatia	G01	B	B	B
P3S60	IPT268 ^{NB}	–	Cabrunski	Cabrun	P3	Croatia	U36	B	B	B
P3S61	IPT269 ^{NB}	–	Bruški	Brusje	P3	Croatia	U37	B	B	B
P3S62	IPT270 ^{NB}	–	Lisički	Lisje	P3	Croatia	G10	B	B	B
P3S63	IPT271 ^{NB}	–	Rodaljički	Rodaljice	P3	Croatia	G10	B	B	B
P3S64	IPT276 ^{NB}	–	Brgudski ozimi syn. Benkovački 1	Brgud	P3	Croatia	U38	B	BM	B
P4S65	B_2	0033*	Chinês real	China	P4	Brazil	U39	E	CM	E
P4S66	B_5	0077*	Peruano	Peru	P4	Brazil	U40	A	AM	AM
P4S67	B_9	0089*	Ugarte	Brazil	P4	Brazil	U41	E	CM	E
P4S68	IPT177 ^B	VEG00118/ W610735**	B92-22	Bulgaria	P4	Bulgaria	U42	B	B	B
P4S69	IPT182 ^B	VEG00124/ PI493116**	174	Czech Republic	U43	Czech Republic	U43	D	C	D
P4S70	IJK2 ^{NB}	–	–	Pinčić (Skadar)	P4	Montenegro	G09	B	B	B
P4S71	IPT181 ^B	VEG00123/ PI497945)**	–	Poland	P4	Poland	U44	C	C	C
P4S72	IPT185 ^B	VEG00126/ PI540351**	0525-85-76	Poland	P4	Poland	U45	C	C	C
P4S73	IPT179 ^{NB}	VEG00128/ PI383817**	Domasen	Serbia	P4	Serbia	U46	A	A	A
P4S74	IPT189 ^{NB}	VEG00129	–	Subotica	P4	Serbia	U47	B	B	B
P4S75	AGRE11 ^{NB}	–	–	Semenarna Ljubljana	P4	Slovenia	G06	A	A	A
P4S76	IPT186 ^{NB}	VEG00119/ PI615422**	Blanco de Huelma Zamora I	Spain	P4	Spain	U48	B	B	B
P4S77	IPT188 ^{NB}	VEG00122/ PI3497951**	Kisswani	Syria	P4	Syria	U49	X	CM	DM
P4S78	IPT180 ^{NB}	VEG00125/ PI3515971**	Chet's	USA	P4	USA	G01	B	B	B

(Continued)

Table S1. (Continued)

Accession no.	IPTPO accession ID	Universal accession ID	Landrace	Origin of accession	Region	State	MLG	UPGMA	Structure K3	BAPS K5
P4S79	IPT187 ^B	VEG00120/W612832**	870825	USA	P4	USA	U50	A	AM	A
P4S80	IPT178 ^B	VEG00127/PI576914**	UO86	Uzbekistan	P4	Uzbekistan	U51	D	C	D

MLG, multilocus genotype; NPGS, National Plant Germplasm System.

Accession No.: Accession number; *IPTPO Accession ID*: Institute of Agriculture and Tourism Collection ID (flowering behaviour (^B – bolting, ^{NB} – non-bolting); *Universal Accession ID*: CPGRD (Croatian Plant Genetic Resources Database; cpgrd.hapih.hr), CENARGEN, Embrapa, Brazil*, and GRIN, NPGS, USA** Collection ID; *Landrace*: traditional name or name listed in the collection; *Origin of Accession*: original collecting site; *Region*: Region of origin (P1 – continental Croatia, P2 – Istria, P3 –Dalmatia, P4 Foreign accessions); *State*: State of origin; *MLG*: Multilocus genotype (G group of identical accessions, U – unique genotype); *UPGMA*: Cluster on UPGMA tree (X – separate lineage); *Structure K3*: Cluster based on STRUCTURE analysis at $K = 3$ (M – accession of mixed origin: the proportion of membership lower than 0.75 in any cluster); *BAPS K5*: Cluster based on BAPS analysis at $K = 5$ (M – accession of mixed origin: the proportion of membership lower than 0.75 in any cluster).

Table S2. Number of SSR electropherogram peaks per marker per number of accessions observed in the set of 80 accessions.

Locus	Number of peaks				
	1	2	3	4	5
AS2655	33	38	7	0	2
AS449	27	51	1	1	0
AS5944	13	62	4	1	0
AS623	37	43	0	0	0
AS6389	12	68	0	0	0
AS739	1	78	0	1	0
AS96	0	78	1	0	0
Asa14	4	44	23	2	0
GB-ASM-040	12	64	3	1	0
GB-ASM-053	4	26	49	1	0
GB-ASM-059	8	68	3	1	0
GB-ASM-072	36	27	14	0	0
GB-ASM-080	52	28	0	0	0

SSR, simple sequence repeat.

Figure S1. Inference of the most likely number of clusters based on the results of STRUCTURE by analysing SSR data of 80 garlic accessions. SSR, simple sequence repeat.