Microsatellite Analysis Indicates the Specific Genetic Basis of Czech Bolting Garlic

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Abstract

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Garlic, *Allium sativum* L., is a vegetable long used for culinary and medical purposes. A certain level of garlic quality is required by the local consumers, which is usually preserved by the varieties grown in that region. The aim was to establish an assay offering fast and inexpensive differentiation of garlic varieties. Length polymorphism of microsatellite loci (SSR, ILP markers) is often used in such a case. No assays have been described earlier. A set of SSR and newly used ILP markers has been assembled and verified. SSR loci ASM53, ASM072, ASA08 and ASA17 were the most polymorphic. Up to 18 alleles were scored per these loci. Monomorphic loci were identified, and excluded from the assay. The assay allows for the authenticity and confirmation of Czech garlic varieties. Moreover, a cluster analysis separated the Czech bolting varieties, indicating their specific genetic basis. The breeding potential of contemporary garlic varieties and lines is discussed.

Keywords: *Allium sativum* L.; diversity; genotyping; SSR markers; variety testing

Allium sativum L., commonly known as garlic, is a species from the genus *Allium.* Garlic is native to central Asia (Kamenetsky *et al.* 2005), with a history of more than 7000 years. Garlic properties have been widely studied, and these studies highlight its antibacterial, antiviral, and antiplatelet activities (Choi *et al.* 2007; Aviello *et al.* 2009; Iciek *et al.* 2009; Chan *et al.* 2013).

Garlic, being a vegetatively propagated species (Cheng *et al.* 2012; Shemesh *et al.* 2013), exhibits a wide range of diversity in morphological, reproductive and bulb traits (Senula & Keller 2000) because of its apomictic nature, which has led to numerous somatic mutations (Ata 2005). It is known that garlic is able to adapt itself to various climatic conditions and numerous ecotypes differing in the content of organosulphur compounds (ASCOs) have been described (Horníčková *et al.* 2010, 2011; Soto Vargas *et al.* 2010; Khar *et al.* 2011; Ovesná *et al.* 2011).

Because garlic field production is laborious, the cultivation of local garlic varieties in EC and accordingly in the Czech Republic has decreased. As a result, imported garlic has been supplied to the market that does not fully meet consumer requirements even though it corresponds to the characteristics laid down in Commission Regulation 2288/97. Depending on the region, consumers may require bolting garlics or hardneck garlics, i.e. garlics producing scapes, i.e. long flowering stems growing through the centre of the bulb producing bulbils, whereas non-bolting garlics or softneck garlics that do not form scapes are not so popular. Semi-bolters could not be differentiated from non-bolting garlics on the market and taxonomically are identical with them (Block 2010). Consumers call for garlic with a certain pungency. This specific trait is usually provided by local varieties. Tools to differentiate local and foreign garlic varieties have not been available up to now.

In the Czech Republic, seed garlic can be produced and certified only from varieties that are registered in the Czech Republic's catalogue (National Listing of Plant Varieties) or in the European catalogue. Therefore, confirmation of the varietal identity in seed garlic certified production is mandatory. We aimed to develop a system of unambiguous identifiers that can differentiate the local and foreign garlic varieties that are grown in or imported into the Czech Republic.

We focused on DNA profiling because morphological identifiers could not be applied to stored garlic bulbs. Several approaches could be considered (Zhao *et al.* 2011; Garcia-Lampasona *et al.* 2012; Morales *et al.* 2013). SSR markers have been reported to be easily applicable for the identification of plant varieties (Gupta *et al.* 1996). Several comparative studies (Nagaoka & Ogihara 1997; Varshney *et al.* 2005) indicated that microsatellite analysis represents a highly appropriate method and although new genotyping and sequencing techniques have emerged, microsatellite analysis is still used because it is cost effective (Guichoux *et al.* 2011; Kalia *et al.* 2011).

The development of DNA profiles specific to garlic varieties which have been approved and grown in the Czech Republic, breeding lines used by local breeders and the identification of their specific features in comparison with varieties and commodities imported from abroad was the main aim of this study.

MATERIAL AND METHODS

Plant material. Forty-three garlic varieties were obtained from breeders and farmers in the Czech Republic directly from the field. French and Spanish breeders provided seven of the varieties, and three (Chinese and Spanish) were obtained from retailers (Table 1). The leaves of five plants per accession were pooled and frozen at –80°C. To assess the possibility of running the DNA analysis directly from cloves, DNA was extracted in parallel from the fresh clove tissue. A protocol using a CTAB detergent was performed according to Saghai-Maroof *et al.* (1984) with modifications as described in Ovesná *et al.* (2011). The quality and concentration of DNA were verified using agarose gel electrophoresis. The λ HindIII (Fermentas, Vilnius, Lithuania) ladder was used as a size and concentration standard.

Microsatellite analysis. A set of 14 microsatellite markers selected out of 23 originally chosen pairs and 2 Intron Length Polymorphism (ILP) markers from introns 1 and 3 of garlic alliinase were used to establish the DNA profiles of the set of garlic accessions. The fourteen microsatellite markers were taken from several publications (Ma *et al.* 2009; Cunha *et al.* 2012), and the two ILP pairs (Intron 1 and Intron 3) were developed in our laboratory. The primers are listed in Table 2, including the repeat motif, the annealing temperature and the number of detected alleles per microsatellite locus. PCR, using fluorescently labelled primers (6-fam, vic, ned and pet produced by Life Technologies, Foster City, USA), was performed in a reaction volume of 15 µl containing 1× Mg-free buffer (Biotools, Madrid, Spain), 2mM $MgCl_2$, 0.33mM of each dNTP (Invitrogen, Foster City, USA), 0.33µM of each primer, 1U Tth polymerase (Biotools, Madrid, Spain) and 100 ng DNA template. The PCR was performed in a Labcycler (Sensoquest, Goettingen, Germany) under the following conditions: an initial denaturing step of 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at annealing temperature (Table 1), 40 s at 72°C and 72°C for 5 min. The amplification products were separated by capillary electrophoresis in the ABI PRISM 3130 sequencer (Applied Biosystems, Foster City, USA). A multiplexed configuration of four reactions was used in one analysis. LIZ500 (Applied Biosystems) was used as an internal size standard. Electrophoretograms were processed by GeneMapper software (Applied Biosystems).

Data analysis. For each locus, the presence or absence of bands in each size category through all genotypes was scored. The data were set in a binary matrix. The genetic similarities were calculated using the Jaccard coefficients and the unweighted neighbour-joining method (UNJ) was used for the dendrogram. The analyses were performed in Darwin software (Perrier *et al*. 2003). The probability of nonidentity, H, is a measure of the genetic variation of a population (gene diversity, Nei 1973). This index equals the probability that two genotypes taken at random from the set of genotypes will not possess the same allele type and may therefore be used as a convenient estimate of marker utility (Powell *et al.* 1996).

H values were calculated as follows:

$$H = 1 - \Sigma p_i^2$$

where:
p_i – frequency of i-allele

RESULTS AND DISCUSSION

All 21 microsatellite loci and 2 newly described ILPs loci (data not shown here) were analysed across 20 samples representing the Czech garlic varieties, both the bolting and non-bolting types, and the two French varieties cultivated in the Czech Republic to cover the expected variability of the set. We found the highest length variability at SSR loci ASM53 (9 alleles), ASM072 (11 alleles), ASA08 (18 alleles) and ASA17 (11 alleles), which was supported by

Table 1. List of garlic varieties, breeding lines and commodities used in the study

Variety	Country of origin	Maintainer or provider	Form	Type	Variety	Country of origin	Maintainer or provider	Form	Type
Anin*	Czech Republic	Kozák Jan	winter	hardneck	Therador	France	Agri Obtentions	winter	softneck
Anton	Czech Republic	Kozák Jan	winter	softneck	Thermidrome	France	Agri Obtentions	winter	softneck
Arno	France	Top Semence	spring	softneck	Tristan	Czech Republic	TAGRO	winter	hardneck
Benátčan	Czech Republic	Kozák Jan	winter	softneck	Unikat	Czech Republic	MORAVOSEED	winter	hardneck
Bjetin	Czech Republic	Kozák Jan	winter	hardneck	Vekan	Czech Republic	Kozák Jan	winter	hardneck
Blanin*	Czech Republic	Kozák Jan	winter	hardneck	Violet Spring Garlic	Spain	Garmez group	spring	softneck
Brick	Czech Republic	MORAVOSEED	winter	hardneck	Záhorský II	Czech Republic	Kozák Jan	winter	softneck
Dukát	Czech Republic	MORAVOSEED	winter	hardneck	Záhorský*	Slovakia	Zelseed	winter	softneck
Džambul*	Czech Republic	Kozák Jan	winter	hardneck	Breeding lines				
Edenrose	France	Agri Obtentions	spring	hardneck	Al II	Czech Republic	Kozák Jan	winter	hardneck
Germidour	France	Agri Obtentions	winter	softneck	BL II	Czech Republic	Kozák Jan	winter	hardneck
Goulurose	France	Agri Obtentions	spring	hardneck	BL127	Czech Republic	Kozák Jan	winter	hardneck
Harnaš	Poland	KHNO POLAN PLC	winter	hardneck	LAN	Czech Republic	Kozák Jan	winter	hardneck
Havran	Czech Republic	Kozák Jan	winter	hardneck	Red American Garlic[1]	Spain	Jose Martínez	spring	softneck
Japo*	Czech Republic	Kozák Jan	spring	softneck	Rusák[2]	Czech Republic	Hrdlička	winter	hardneck
Japo II	Czech Republic	Kozák Jan	spring	softneck	Rusák 4[3]	Czech Republic	BRANCO	winter	hardneck
Jolimont	France	Sicacefel	spring	softneck	Rusák_Hradecký[4]	Czech Republic	Hradecký	winter	hardneck
Jovan	Czech Republic	Kozák Jan	winter	hardneck	Rusák_Riegel[5]	Czech Republic	Riegel	winter	hardneck
Karel IV.	Czech Republic	SEMO a.s.	winter	hardneck	Spanish Roja[1]	Spain	Jose Martínez	spring	hardneck
Lukan	Czech Republic	Kozák Jan	winter	softneck	Vinar	Czech Republic	Kozák Jan	winter	hardneck
Lumír	Czech Republic	MORAVOSEED	spring	softneck	White American Garlic[1]	Spain	Jose Martínez	spring	softneck
Matin	Czech Republic	Kozák Jan	spring	softneck	White Spring Garlic[1]	Spain	Jose Martínez	spring	softneck
Mirka	Czech Republic	Kozák Jan	winter	hardneck	Záhorský	Czech Republic	Kozák Jan	winter	softneck
Mojmír	Slovakia	Zelseed	winter	softneck	Commodities				
Morado de Cuenca	Spain	Coopaman	spring	softneck	Čínský česnek	China	Tesco Stores ČR, Ltd.	winter	softneck

high H values. Several SSR loci, including AMS025, GBAS001, GBAS027, GBAS089 and ASA04, were monomorphic across the studied group of the Czech and French varieties (Table 1) with an H value of 0. Therefore, these loci were excluded from the set of appropriate markers. Likewise, the SSR locus ASA04, which had a significantly low H value (0.035), was also excluded. Finally, 14 selected microsatellites and 2 ILPs loci were used to generate specific DNA profiles of the fifty-three varieties that are currently available in the Czech market. The average H value of the marker set was calculated to be 0.68. The value is comparable to that (0.62) obtained by Smith *et al.* (1997) for wheat SSRs. Moreover, such an H value was shown to be appropriate for the differentiation of various species, either vegetatively propagated or self-pollinating, as indicated by other authors (Favoretto *et al*. 2011; Gong & Deng 2012; Wang *et al.* 2013). Thus, we concluded that our set of markers generated a sufficient number of data points to allow for an unambiguous distinction of the analysed varieties.

Leaf tissue was used as a matrix to generate the data and represented the appropriate material for DNA extraction and further DNA profiling. Consumers often demand garlic seed certification and variety identification in the market, and therefore, we tested

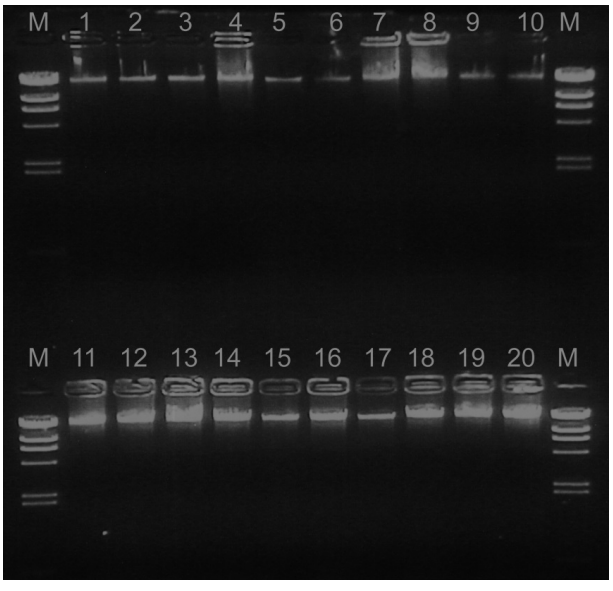

Figure 1. Separation of DNAs isolated from garlic leaves (lanes 1–10) and garlic cloves (lanes 11–20) from 10 different varieties under UV light after electophoresis in 0.8% agarose gel and ethidium bromide staining; a λ HindIII (Fermentas, Vilnius, Lithuania) ladder was used as a size standard (lanes M)

Table 1 to be continued

Variety	Country of origin	Maintainer or provider	Form	Type	Variety	Country of origin	Maintainer or provider	Form	Type
Slavín	Czech Republic	Kozák Jan	winter	hardneck	Sologarlic	China	Tesco Stores ČR, Ltd.	winter	hardneck
Staník	Czech Republic	Kozák Jan	winter	hardneck	Tjakka	China	Tesco Stores ČR, Ltd.	winter	softneck
Tantal	Czech Republic	TAGRO	winter	hardneck					

*Plant material providers; providers: [1]breeding line by courtesy of Mr. Jose Martínez, Cordóba, Spain; [2]breeding line by courtesy of Mr. Hrdlička, Dolánky n.O., Czech Republic; [3]breeding line by courtesy of BRANCO, Ltd., Hamr n. J., Czech Republic; [4]breeding line by courtesy of Mrs. Míhulková and Mr.Hradecký, Czech Republic; [5]breeding line by courtesy of Mrs. Riegelova, Šlapanice, Czech Republic; commodities: fromTesco Stores ČR, Ltd., Czech Republic
Varieties and/or breeding lines by courtesy of breader or maitainer of the garlic variety: Agri Obtentions, Guyancourt, France; MoravoSeed, Ltd. Mušlov 1701 T/4, 692 01 Mikulov, Czech Republic; TOP Semence, BP 2, 26160 La Bâtie-Rolland, France; Sicacefel, Domaine de Capou, Montauban, France; Zelseed s.r.o. Horná Potôň 16, Slovakia; Kozák Jan, Ing., Poběžovice 31, 534 01 Holice; KHNO POLAN PLC (Krakowska Hodowla i Nasiennictwo Ogrodnicze POLAND Sp. z o.o.); Garmez group, s.r.o. Na spravedlnosti 1386//25 , 59401 Velké Meziříčí, Czech republic; Coopaman, S. Coop. de Castilla-La Mancha, Las Pedroñeras, Cuenca

Table 2. List of primers used in the study to amplify SSR and ILS loci

SSR primer-locus-label	Repetition unit	T_A (°C)	No. of alleles	H	Reference
ASM035-6-FAM	(GCC)3, (TCC)3	60	3	0.619	Ma *et al.* (2009)
ASM040-VIC	(AC)6, (AC)14-(AT)5	60	5	0.748	Ma *et al.* (2009)
ASM53-NED	(CA)15, (AC)8	60	9	0.854	Ma *et al.* (2009)
ASM59-PET	(TG)11, (TG)5	60	5	0.765	Ma *et al.* (2009)
ASM072-6-FAM	(TA)7-(TG)5 GC (GT)9 T (TG)8	60	11	0.803	Ma *et al.* (2009)
ASM078-VIC	(GT)12	60	4	0.576	Ma *et al.* (2009)
ASM080-NED	(CCG)5	60	2	0.344	Ma *et al.* (2009)
ASM109-PET	(ACC)4	60	3	0.561	Ma *et al.* (2009)
Intron 1-6-FAM		60	4	0.606	CRI
Intron 3-NED		60	3	0.650	CRI
ASA07-NED	(TG)7	60	5	0.605	Cunha *et al.* (2012)
ASA08-PET	(GT)8	60	18	0.909	Cunha *et al.* (2012)
ASA10-6FAM	(AC)7	50	5	0.731	Cunha *et al.* (2012)
ASA14-VIC	(GT)7	50	8	0.821	Cunha *et al.* (2012)
ASA16-NED	(TG)5 C (GT)6	60	4	0.430	Cunha *et al.* (2012)
ASA17-PET	(CA)12 (CT)28	60	11	0.858	Cunha *et al.* (2012)
AMS025-PET	(AC)21 (AT)3	50	1	0.000	Fischer and Bachmann (2000)
GBAS001-6-FAM	(TA)4	60	1	0.000	Lee *et al.* (2011)
GBAS027-VIC	(GGA)4	60	1	0.000	Lee *et al.* (2011)
GBAS089-NED	(AG)4, (TAG)3	60	1	0.000	Lee *et al.* (2011)
GBAS102-PET	(AAAT)3	60	1	0.000	Lee *et al.* (2011)
ASA04-6FAM	(TCC)5 (TCC)4 (TCC)5	60	1	0.035	Cunha *et al.* (2012)
ASA06-VIC	(TG)5	60	1	0.000	Cunha *et al.* (2012)

T_A – annealing temperature; H – probability of nonidentity; CRI – Crop Research Institute, Prague-Ruzyně, Czech Republic

DNA extraction from mature cloves. Using the same CTAB-based protocol, we were able to extract DNA of adequate quality as verified by gel electrophoresis (Figure 1) and namely by downstream processing, i.e. the same results of microsatellite analysis. The DNA profiles generated using these DNAs were identical to those available from leaf tissue, which concurrently confirms the accuracy of the assay. High reproducibility of the testing method is, among others, a basic prerequisite for its application in practice (Bustin *et al.* 2009; Poczai *et al.* 2013), and the presented method clearly fulfils this parameter. Thus, the method can be applied for garlic clone genotyping and for control purposes to detect possible mechanical varietal admixtures after *in vitro* multiplication or other types of propagation (Buso *et al*. 2008). Checks can be performed at different stages of seed production or in the market products.

To ensure the comparability and reproducibility of the independent analyses conducted in different years or different laboratories, standard alleles should be included in the analysis (This *et al*. 2004). In our study, nine varieties (Benátčan, Bjetin, Havran, Jovan, LAN, Slavín, Staník, Vekan, Záhorský II) that represent widely grown genotypes were selected as a source for the standard alleles. Ideally, such a set of reference varieties, representing a ladder of all known alleles, should be included in each test, but such analyses would be too expensive. Based on our experience three standard alleles were sufficient for achieving the reliable sample allele identification.

For the uniformity assessment of vegetatively propagated garlic varieties, a population standard of 1% (the percentage of off-type plants that do not comply with varietal characteristics) with an acceptance probability of at least 95% should be applied.

The maximum number of off-types allowed for the uniformity standards for 6–35 plants is 1 off-type (CPVO EU 2004). Variability was assessed within the varieties selected as standards using at least six individual plants. No indication of intravarietal diversity was found. There is no evidence to indicate that the varieties lack uniformity.

The DNA profiles generated also allowed us to identify the combination of microsatellite alleles that distinguished Czech garlic varieties from foreign varieties grown by Czech farmers and varieties appearing in the market.

The DNA profiles showed close relationships between some of the Czech varieties. Two pairs of varieties differed only in one allele out of the 108 generated. Vars. Tantal and Staník, coming from different breeding stations, or Slovak var. Mojmír and Czech var. Lukan thus document the preference of local breeders to a certain garlic type. However, all of the varieties were clearly distinguished. We confirmed that SSR length variability could be successfully applied to check for variety designation and for breeding material characterization as shown for other crop species (Ijaz 2011).

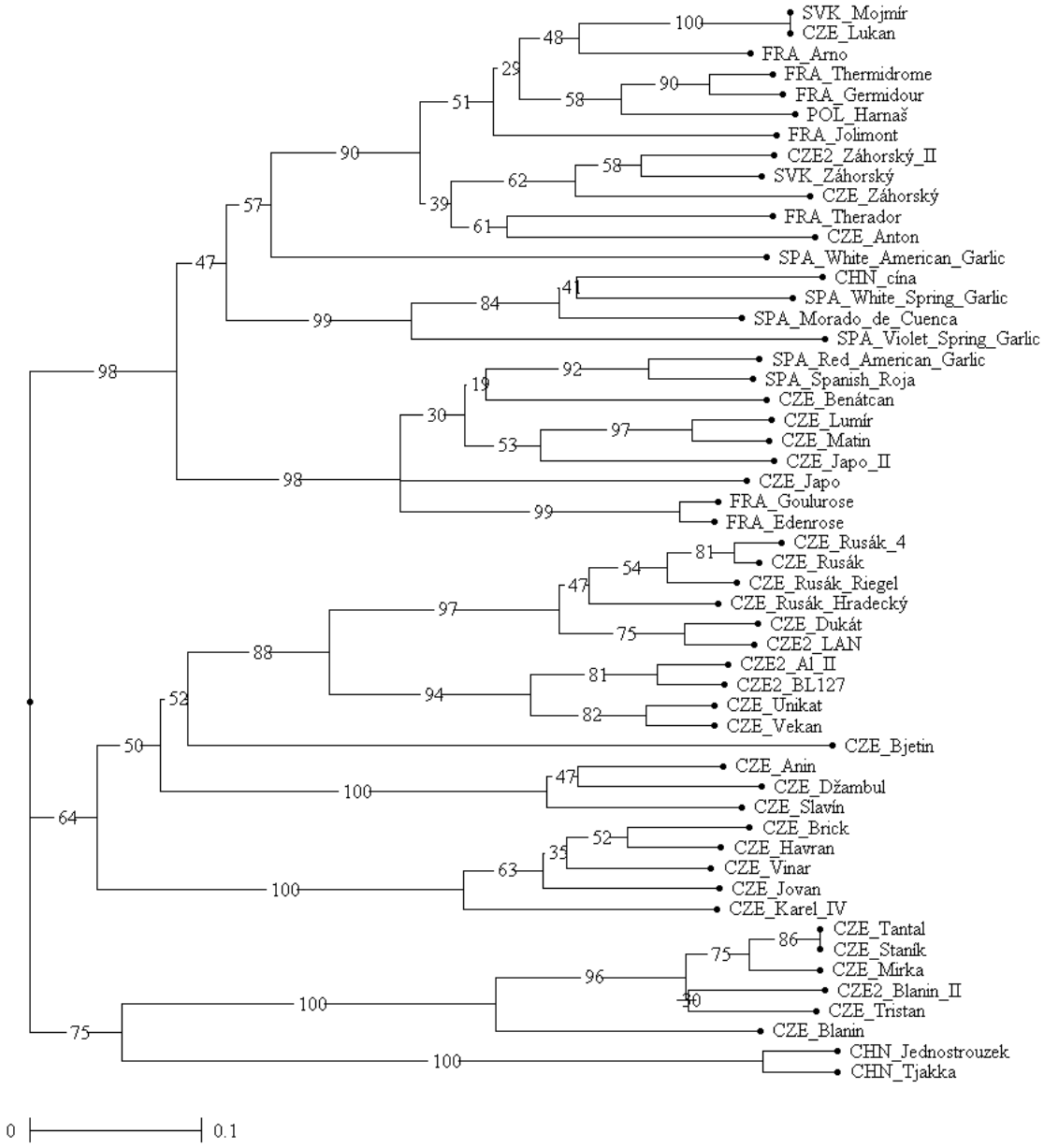

Figure 2. Dendrogram indicating association among analyzed garlic cultivars based on variability at SSR loci (the cultivar name is preceded by the country abbreviation: CZE – Czech Republic, CZE2 – Czech Republic, new variety; CHN – China; FRA – France; SPA – Spain; SVK – Slovakia; POL – Poland)

As shown in the dendrogram (Figure 2), the cluster analysis divided the analysed materials into several groups. Chinese garlic appeared in the Czech market and solo or pearl garlic formed an individual branch (multiple accessions were analysed and identical profiles were recorded, data not shown here). Three clusters were formed solely by the Czech bolting garlic, whereas Czech non-bolting garlic and French, Spanish and Chinese varieties, both bolting and non-bolting, fit into two other clusters. High bootstrap values at most of the nodes supported the credibility of the clustering. It is clear from the dendrogram that varieties grouped according to the territory of their origin and, taking into account sub-clustering, also grouped according to the scape type. Foreign varieties, either bolting or non-bolting ones, do not associate with Czech bolting varieties, which indicate the specific features of the Czech bolting garlic. This analysis suggests that Czech bolting garlic should be preferentially used for the breeding of new varieties of the Czech garlic type.

Garlic breeding is based on the selection of differing clones from a working collection. The lack of sexual processes prohibits conventional breeding in garlic (Neta *et al.* 2011). Thus, possible clonal variability and adaptability of garlic make the development of new lines possible. We compared the DNA profiles of the Slovak variety Záhorský with a local line cultivated under the same name for breeding purposes and a newly registered variety, Záhorský II. We detected changes of allele sizes in three loci only. On the other hand, varieties Blanin and the newly registered Blanin II differ dramatically. Changes were detected in 10 loci out of the 16 analysed. These data document the variability retained in some Czech varieties.

To assess the breeding potential of non-registered landrace varieties or breeding lines, we analysed lines known under the common name "Rusák," which designates a bolting garlic that originated in Russia. These lines came from different places in the Czech Republic, and their genetic basis groups them together in the dendrogram. These lines are similar to Czech bolting garlic, and breeders intend to use them in their breeding programs. Another local line named Vinar, according to its place of origin, was also fully associated with Czech bolting garlic and a descending variety, Karel IV, which was registered in 2013.

The genetic basis of Czech bolting garlic is apparently different from the other varieties available in the Czech market, either those produced locally or imported, and consumers are demanding the right to check the authenticity of the varieties.

Analyses of microsatellite loci and ILPs length polymorphism have been proved to be suitable for the identification of Czech garlic varieties and to distinguish them from foreign genotypes. This system can be used for germplasm analysis in the gene banks and in the market.

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