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The Selection and Validation of a Marker Set for the Differentiation of Onion Cultivars from the Czech Republic

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Abstract

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The onion (*Allium cepa* L.) constitutes an important part of the human diet and is of economic importance on the vegetable market. Methods that allow unambiguous differentiation of cultivars and breeding lines are required for market control and the protection of plant breeders' rights. DNA analyses, such as single sequence repeat polymorphism (SSRs), represent such a tool. Based on the evaluation of polymorphism in 21 SSR loci, we established a panel of 15 easy to score SSR markers that differentiated 16 commercial onion cultivars in the Czech Republic. The polymorphism ranged from 2–3 alleles per locus, indicating a low level of diversity among the cultivars. The panel can also be used to evaluate genetic resources.

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

Allium cepa L., commonly referred to as the onion, is a species from the genus *Allium*. The onion is an important vegetable crop worldwide that is extensively used in the food supply. It is rich in secondary health-promoting metabolites, namely isoallin (Keusgen *et al*. 2002; Auger *et al*. 2004; Lanzotti 2006). There are different types of onions available with respect to shape, size and colour. In addition, the onion is a long day vegetable, and there is a differentiation between spring and winter types.

Characterization of onions based on phenotype is affected by the environment. Researchers have adopted several DNA profiling techniques that allow for precise diversity estimates, quantitative trait loci (QTL) mapping, and characterization of different quality traits in onions (Chinnappareddy *et al*. 2013). Generally, DNA profiling is used for genotype identification in GenBank® and cultivar identification on the market.

Several markers are currently available. RAPD (Random Amplified Polymorphic DNA) is still used for diversity estimates (Paredes *et al*. 2008; Mukherjee *et al*. 2013; Tedeschi *et al.* 2014), as well as AFLP (Amplified Fragment Length Polymorphism), which could be converted into other marker types (Lampasona *et al*. 2003; Filjushin *et al*. 2011; Santos *et al.* 2011; Simo *et al.* 2014). Plant genomes contain polymorphic repetitive sequences that are currently used as DNA markers. SSR (single sequence repeats) markers are the most popular, owing to their reliability, robustness, and cost effectiveness. They have been used for diversity estimates (Mallor *et al.* 2014), cultivar identification (Anandhan *et al.* 2014), and diversity studies (McCallum *et al.* 2008).

The goal of this study was to select a set of fluorescently labelled SSR primers that allowed for easy identification of onion cultivars for seed purity testing, which is required by breeders. It would be beneficial for breeders to have such a tool for unique identification of their cultivars because certification is often requested by growers.

MATERIAL AND METHODS

Leaves of sixteen onion cultivars were provided by local commercial onion producers. The cultivars represented basic onion types cultivated in large scale in the Czech Republic (Table 1). Total genomic

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DNA was extracted from frozen pooled leaves of ten plants per cultivar in parallel using the CTAB method, as described by Ovesná *et al*. (2011). DNA quality was verified by electrophoresis and quantified spectrophotometrically.

A set of 15 microsatellite markers selected out of a group of 21 pairs were used to establish the DNA profiles of the onion cultivars that were analysed. The microsatellite markers were taken from Jakše *et al.* (2005), Lee *et al.* (2011), and Cunha *et al.* (2012). The primers are listed in Table 2 and include the annealing temperature and the number of detected alleles per microsatellite locus across the analysed set. PCR with fluorescently labelled primers (6-fam, vic, ned and pet; Life Technologies, Foster City, USA) was performed in a reaction as described by Ovesná *et al*. (2014). The amplification products were separated by capillary electrophoresis in the sequencer ABI PRISM 3130 (Applied Biosystems, Foster City, USA). A multiplexed configuration of three or four reactions was used per analysis. LIZ500 (Applied Biosystems, Foster City, USA) was used as an internal size standard. Electrophoretograms were processed by GeneMapper software (Applied Biosystems, Foster City, USA). 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. An agglomerative hierarchical cluster analysis for the set of cultivars was performed using the UPGMA algorithm for Euclidean distances. The pvclust package (Suzuki & Shimoraira 2011) of the R software (R Core Team 2013) was used to assess uncertainty in hierarchical cluster dendrograms. AU (approximately unbiased) *P*-values were calculated by multi-scale bootstrap resampling. The number of bootstrap replicates was 10 000.

Table 1. List of onion cultivars

Variety		Skin colour	Maintainer
Elenka	H	yellow	Cora Seeds Srl (Cesena, IT)
Dormo	H	yellow	Nickerson Zwaan BV(MADE, NL)
Albienka	NH	white	SEMO a.s (Smržice, CZ)
Grenada	NH	violet	Moravoseed CZ a.s. (Mikulov, CZ)
Tosca	NH	yellow	Moravoseed CZ a.s. (Mikulov, CZ)
Unico	H	yellow	Moravoseed CZ a.s. (Mikulov, CZ)
Triumf	H	yellow	Moravoseed CZ a.s. (Mikulov, CZ)
Spirit	H	yellow	Bejo Zaden BV(Warmenhuizen, NL)
Lusy	NH	yellow	SEMO a.s (Smržice, CZ)
Elbrus	NH	yellow	SVS Holland BV (Enkhuizen, NL)
Sherpa	H	yellow	Advanta Seeds BV (Kapelle, NL)
Lamyca	H	yellow	Cora Seeds Srl (Cesena, IT)
Sturon	NH	yellow	Kees Broersen Zaden (Tuitjenhorn, NL)
Karmen	NH	violet	Meo Voto Zaden BV (Andijk, NL)
Django	H	yellow	Advanta Seeds BV (Kapelle, NL)
Bingo	NH	yellow	Bejo Zaden BV (Warmenhuizen, NL)

H – hybrid, NH – non-hybrid; NL – Nederland; CZ – Czech Republic; SK – Slovakia; PL – Poland; IT – Italy

The probability of non-identity (*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 is a frequency of *i*-allele. The polymorphic information content (PIC) index for each marker was calculated as previously described (Botstein *et al*. 1980).

RESULTS AND DISCUSSION

We tested a set of markers spanning 21 SSR loci to evaluate their ability to differentiate between onion cultivars that are representative of basic species types (white, yellow or violet; early or late) that are commercialized in the Czech Republic.

Several SSR loci (GBAS102, GBAS001, GBAS027, GBAS089 ASA06 and ASA04) were monomorphic across the studied set of 16 cultivars and were excluded from further evaluation. The number of alleles per SSR locus amplified by 15 SSR primer pairs ranged from 2 to 3, with a mean of 2.2 alleles. The eight loci (ACM017, ACM018, ACM066, ACM068, ACM093, ACM094, ACM105, and ACM170) amplified only two different alleles, and these values were lower than those described previously (Kim *et al*. 2003; Baldwin *et al.* 2012). On the other hand, we found three alleles generated at the SSR loci ACM091, ACM112, and ACM146 instead of the two reported by Jakše *et al*. (2005). It is apparent that diversity within the set of investigated cultivars is rather low as assessed by number of generated alleles compared to other studies that included both long and short

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day onions. However, the newly described allele sizes indicate a different genetic background for these onions compared to Spanish or Asian cultivars.

Variability of allele sizes was found to range between 165–342 bp (Table 2). Allele sizes for the SSR loci ACM018, ACM105, ACM146, GBAS001, and GBAS102 were identical to alleles sizes listed in Jakše *et al.* (2005) and Cunha *et al*. (2012). Some SSR alleles found on ACM004, ACM066, ACM091, ACM093, ACM094 and ACM112 differ by 1 or 2 bp compared to Jakše *et al*. (2005). Such a shift may result from the reading accuracy of different devices (e.g., slab vs. capillary electrophoresis) and the estimate of the operator, so those alleles can be considered to be identical. Allelic standards, however, should be used across laboratories to confirm such conclusion (This *et al*. 2004). We identified new allele sizes that are typical for our set of cultivars (see Table 2). Additionally, other authors reported different allele sizes compared to those described in the original publications. This

Table 2. Number of alleles per marker, probability of non-identity (*H*), polymorphism information content (PIC), and allele size

SSR[1]	Locus label	No. of alleles	*H*	PIC	Size (bp)	
					observed	expected
Panel M						
ACM013*	6FAM	3	0.394	0.311	165, 168, 174	183, 186, 192
ACM017*	VIC	2	0.208	0.258	250, 253	265, 268
ACM018*	NED	2	0.346	0.278	275, 278	275, 278
Panel N						
ACM004*	6FAM	3	0.478	0.363	201, 205, 212	203, 206, 213
ACM066*	VIC	2	0.111	0.105	185, 187	184, 186
ACM068*	NED	2	0.111	0.105	260, 263	277, 281
Panel O						
ACM091*	6FAM	3	0.353	0.289	172, 178, 184	177, 183
ACM093*	VIC	2	0.346	0.277	133, 135	134, 137
ACM094*	NED	2	0.291	0.248	109, 112	110, 114
Panel P						
ACM105*	6FAM	2	0.104	0.093	151, 154	151, 154
ACM112*	VIC	3	0.353	0.28	178, 181, 184	177, 180
ACM115*	NED	2	0.388	0.312	220, 223	239, 242
Panel R						
ACM146*	6FAM	3	0.25	0.217	210, 213, 216	213, 216
ACM151*	VIC	3	0.491	0.371	243, 245, 247	264, 266
ACM170*	NED	2	0.263	0.217	208, 218	227, 236
Panel F						
GBAS001**	6FAM	1	0	0	184	184
GBAS027**	VIC	1	0	0	340	254
GBAS089**	NED	1	0	0	342	389
GBAS102**	PET	1	0	0	211	211
Panel G						
ASA04***	6FAM	1	0	0	268	264
ASA06***	VIC	1	0	0	216	192

Annealing temperature (TA, °C) 60°C; *Jakše *et al*. (2005); **Lee *et al*. (2005); ***Cunha *et al*. (2012); [1]panel-set of primers for multiplex reaction

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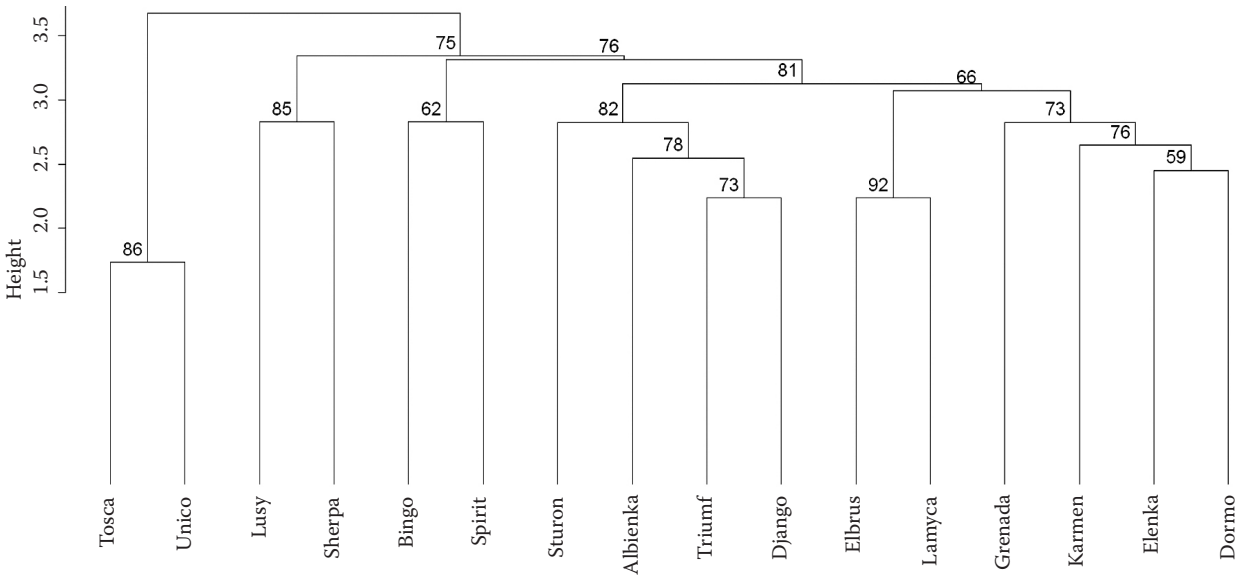

Figure 1. Dendrogram showing associations between the 16 onion cultivars computed with Euclidean distances and an average linkage (UPGMA) clustering method; the numbers above each node represent approximately unbiased (AU) *P*-values, calculated by the multi-scale bootstrap re-sampling (nboot = 10 000); height indicates the dissimilarity between each cluster

reflects the diversity and genetic variation of varieties originating from different regions of the world (Jakše *et al*. 2005; McCallum *et al*. 2008).

Amplification profiles of the selected SSR loci formed discrete peaks. These can be easily read and thus contribute to the reproducibility of the assay. *H* values ranged from 0.104 for ACM105 to 0.491 for ACM004.

The average *H* value of the marker set was 0.30. Such an *H* value has been shown to be appropriate for the differentiation of various species, either propagated vegetatively or through 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 cultivars.

High reproducibility of the testing method is a basic prerequisite for its application in practice (Bustin *et al*. 2009; Poczai *et al*. 2013). The panel of SSR markers clearly fulfils this parameter because it was verified by replication of the assays in parallel. As profiles of tree SSR markers can be analysed with fluorescent labelling at once, this assay is an efficient tool for onion genotyping and for control purposes to detect possible admixtures or cross pollination (Buso *et al*. 2008). Alleles of analysed cultivars will be used as a size standard for future testing.

Associations between analysed cultivars (as graphically shown by the dendrogram in Figure 1) have not revealed any specific grouping of locally grown cultivars. Only the Czech cultivars Tosca and Unico (Moravoseed), which descend from the same breeding company, associated together. Two other cultivars, Grenada and Karmen (Moravoseed), were located in another cluster. Foreign cultivars that are currently widely used for commercial onion production were dispersed in different clusters. Cultivars did not associate according the bulb shape, probably due to the low number of analysed accessions. Early or later types did not form discrete clusters, as the trait values are overlapping. Two violet cultivars associated together. We conclude that the genetic basis of commercial cultivars is rather limited, but we presented a panel of SSR markers suitable for their differentiation. Future experiments will focus on applying the analysis of currently available genetic resources used in breeding programs and identifying lines used for hybrid seed production.

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