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Genetic features of Czech blue poppy (*Papaver somniferum* L.) revealed by DNA polymorphism

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Abstract: Seeds of Czech poppies used for culinary purposes are frequently mixed with seeds of imported low-quality poppies with higher alkaloid content and poor taste properties. That not only decreases the quality of the product on the market, but it can also represent a potential health hazard for consumers. To prevent such adulteration, the method is needed that could clearly detect the presence of the low-quality poppies in the products on the market and which can also confirm the authenticity of the genetic material for growers. For that reason, length polymorphism of Simple Sequence Repeats was evaluated in the blue and non-blue poppy varieties of Czech and foreign origin. Used markers clearly distinguished 'Czech blue poppy' cultivars from blue poppies of another origin as well as from poppies of non-blue seed colour. We conclude that used markers can be applied to a commodity verification on the market to detect/exclude the presence of other genotypes.

Keywords: *Papaver somniferum*; Czech blue poppy; length polymorphism; Simple Sequence Repeats

Papaver somniferum L. (opium poppy) is an annual herb used in the food and pharmaceutical industry. In the Mediterranean and the Middle East poppies have been grown for millennia, spreading from their homeland in East and Central Asia (Schiff 2002). Poppy is also traditionally grown in the Czech Republic and nowadays represents a lucrative export article (Vašák 2008). Similar to other countries, the poppy cultivation is regulated in the Czech Republic, specifically by the Act on Addictive Substances No. 167/1998 Coll., effective as of 1. January 1999, reporting requirements for poppy and cannabis growers.

Being the source of various alkaloids, high-content poppies can be abused for illicit drug production (Liscombe & Facchini 2008; Labanca et al. 2018). Since these alkaloids are derived from poppy capsules, seeds are insignificant to the pharmaceutical industry. However, after some treatment, they can be used in the food industry. Such seeds are poor in taste and often contain above-the-limit amounts of alkaloids. When mixed with seeds of poppies aimed for the culinary purpose, they not only decrease the quality of the product on the market, but they can also represent a potential health hazard for consumers (Lopez et al. 2018; Eisenreich et al. 2019).

Among poppy cultivars used for culinary purposes, the most popular are those with blue-coloured seeds, which exhibits better culinary and taste properties. The seeds of these poppies, i.e. poppies with low alkaloid content, are used in the food industry and as a source of oil for culinary, cosmetic and technical applications. To be considered a blue poppy, the composition of the commodity and products on the Czech market must follow the Czech Guild Standard 'Czech Blue Poppy' (http://eagri.cz/public/web/file/625876/_32018_02.pdf).

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It has been clearly demonstrated that procedures based on nucleic acid polymorphism analysis are suitable for verification of any organism identity (Bohme et al. 2019). One of the methods operating on this principle is the analysis of length polymorphism of Simple Sequence Repeats (SSR) or microsatellites. A microsatellite represents a stretch of DNA in which some DNA motif in the length of 2 to 6 base pairs is repeated up to 50 times. The genomes of eukaryotes are rich in the content of such repetitive DNA sequences (Morgante & Olivieri 1993; Kejnovsky et al. 2009; Garrido-Cardenas et al. 2018;) and they are reflecting genetic diversity (Brinkmann et al. 1998). Microsatellite analysis is also the popular method recommended *inter alia* for cultivar identification (McGregor et al. 2000; Iqbal et al. 2011; Iquebal et al. 2013; Ovesna et al. 2014; Corrado 2016; Zhao et al. 2019).

This paper aims to describe the usage of SSR markers that can distinguish poppies mentioned in the Czech Guild Standard 'Czech Blue Poppy' from other poppies.

MATERIAL AND METHODS

In total, 29 poppy accessions of Czech and foreign origin were collected. Seeds of individual accessions were grown in separated flowerpots under controlled conditions. Following the phenological phase 13–15 according to BBCH (Biologische Bundesantalt, Bundessortenamt and Chemische Industrie) scale, few leaves of plants representing each accession were sampled for DNA isolation by DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's recommendation. The quality and quantity of isolated DNA was verified by UV spectrometry (S-111107AW nanophotometer; Implen, Germany) and electrophoretic separation in 1% (w/v) agarose gel. Thereafter, DNA of individual accessions was subjected to PCR reaction with primers and conditions described in Vašek et al. (2020).

The amplification products from PCR reactions were then subjected to fragment analysis using genetic analyser 3500 (Applied Biosystems, USA) with GeneScan LIZ500 (Applied Biosystems, USA) as an internal size standard. Electrophoretograms were processed by GeneMapper software 6.0 (Applied Biosystems, USA). For each locus, the presence or absence of bands in each size category through all genotypes was scored and 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 & Shimodaira 2006) of the R software (R Foundation for Statistical Computing, Austria, 2009; https://www.R-project.org) was used to assess uncertainty in hierarchical cluster dendrograms. AU (approximately unbiased) *P*-values were calculated by multiscale bootstrap resampling. The number of bootstrap replicates was set to 10 000. The polymorphic information content (PIC) for each marker was calculated as described in Botstein et al. (1980).

RESULTS AND DISCUSSION

Poppy seeds produced by cultivars bred for culinary purposes are frequently mixed with low-quality seeds of cultivars with higher alkaloid content. That decrease the quality of the product on the market and represents the potential health hazard for consumers (Lopez et al. 2018; Eisenreich et al. 2019). As low-quality seeds are cheaper, the situation result in economic losses of food poppy producers. Thus, the method is needed that could verify the commodity and food products authenticity.

SSR analysis was employed in order to distinguish poppies mentioned in the Czech Guild Standard 'Czech blue poppy' from poppies, which do not fill the criteria demanded by this standard. In the present work, we analysed a set of 29 poppy accessions using 17 SSR markers described in Vašek et al. (2020). Of originally tested 17 SSR markers all proved to be polymorphic across the studied set. The number of alleles ranged from 2 to 9 per locus and PIC values ranged from 0.092 to 0.284 (Table 1). As expected, the number of polymorphic alleles was lower in comparison with the study of Vašek et al. (2020), which is given by a lower number of studied varieties. However, the SSR profiles were identical for genotypes common to both studies.

Among the 17 SSR loci tested, we identified five (OPEST- 048c, 086d, 099, 102b and 169) that were both monomorphic across all Czech blue poppies and polymorphic in other poppies. However, two of these loci (OPEST- 086d and 169) amplified only two different alleles that resulted in lower PIC values. Therefore, they were excluded from further evaluation. The three remaining markers (OPEST- 048c, 099 and 102b) were used for the initial cluster analysis along with OPEST026, which was also monomorphic across Czech blue poppies except for 1 out of 7 alleles. The preliminary cluster analysis based on these four SSR markers distinguished the Czech blue poppies from the most of other varieties. In order to increase the robustness of the analysis and to differentiate the Czech blue poppies from the remaining ones, the set of four markers was extended by another

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Table 1. Used markers, detected alleles, number of alleles per locus and PIC values of markers

Marker	Detected alleles	Number of alleles	PIC
OPEST026	118, 121, 127, 139, 142, 145, 148	7	0.195
OPEST048c	186, 189, 192, 195, 198, 204	6	0.161
OPEST051c	147, 153, 156, 159, 162	5	0.200
OPEST053c	188, 191, 194, 197, 200, 203	6	0.181
OPEST061	221, 224, 227, 230, 233, 236	6	0.209
OPEST081c	164, 167, 170, 173, 176	5	0.197
OPEST086d	223, 244	2	0.092
OPEST099	276, 284, 285, 288	4	0.210
OPEST102b	194, 197, 200, 203	4	0.182
OPEST106	182, 185, 188, 191	4	0.284
OPEST120b	150, 153, 156	3	0.283
OPEST126b	222, 225, 228	3	0.235
OPEST131	232, 244, 247, 250	4	0.203
OPEST156	264, 267, 270	3	0.246
OPEST169	172, 205	2	0.120
OPEST177b	96, 100, 103, 106, 112	5	0.246
OPGSSR001	217, 220, 223, 226, 229, 232, 235, 241, 244	9	0.162

PIC – the polymorphic information content

three (OPEST- 051c, 061 and 106). These three markers were selected with regard to their ability distinguish Czech blue poppies from varieties such as 'Kék Duna' or 'Rosemarie', which was not clearly separated from Czech blue poppies using the original four markers.

When tested, the final set of aforementioned seven markers (OPEST- 026, 048c, 051c, 061, 099, 102 and 106) proved to have enough discrimination capacity to distinguish Czech blue poppies from all other poppies. According to the results of hierarchical clustering (dendrogram, Figure 1), poppies mentioned in the Czech Guild Standard 'Czech Blue Poppy' are clustered together and the cluster is highly supported by the data (AU *P*-value ≥ 0.99). The result of hierarchical cluster-

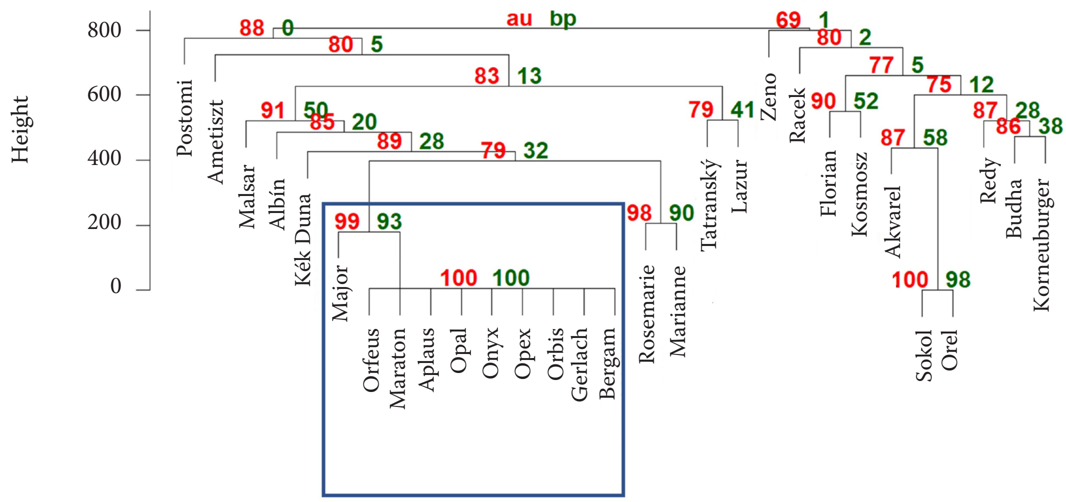

Figure 1. Dendrogram showing the association between the 29 poppy accessions based on euclidean distances and average linkage (UPGMA) clustering method; the numbers above each node represent bootstrap probability (bp) and approximately unbiased (au) *P*-value calculated by the multi-scale bootstrap re-sampling (nboot = 10 000); height indicates the dissimilarity between each cluster. The blue rectangle within the plot highlights the blue poppies mentioned in the Czech Guild Standard 'Czech blue poppy'

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Table 2. Country of origin of individual poppy accessions and their seed colour

Accession name	Origin	Seed colour	Accession name	Origin	Seed colour
Gerlach*	CZE	blue	Albín	CS	white/beige
Bergam*	SVK	blue	Kék Duna	HUN	blue
Orbis*	CZE	blue	Kosmosz	AUT	gray
Opex*	CZE	blue	Ametiszt	HUN	blue
Onyx*	CZE	blue	Marianne	NL	gray
Opal*	SVK	blue	Redy	CZE	brown to ochre
Aplaus*	CZE	blue	Buddha	HUN	blue
Maraton*	SVK	blue	Tatranský	SVK	white/beige
Orfeus*	CZE	blue	Korneuburger	AUT	white/beige
Major*	SVK	blue	Akvarel	CZE	white/beige
Florian	AUT	gray	Racek	CZE	white/beige
Rosemarie	NL	blue	Orel	CZE	white/beige
Sokol	CZE	white/beige	Lazur	PL	blue
Postomi	HUN	blue	Zeno	AUT	blue
Malsar	SVK	gray	–	–	–

CZE – Czech Republic; SVK – Slovakia; CS – Czechoslovakia; AUT – Austria; PL – Poland; H – Hungary; NL – Netherlands; *poppy accessions mentioned in the Czech Guild Standard 'Czech Blue Poppies'

ing also confirmed that discrimination capacity of used markers was high enough to differentiate Czech blue poppies from blue poppies of foreign origin such as 'Kék Duna' and 'Buddha' (Hungary), 'Rosemarie' and 'Marianne' (Netherlands), 'Lazur' (Poland) or 'Zeno' (Austria). In addition, the Czech white poppies such as 'Sokol', 'Orel' or 'Akvarel' were also distinguished from Czech blue poppies.

In several cases the discrimination capacity of used markers was high enough to distinguish foreign poppy accessions according to their origin, as in the case of 'Rosemarie' and 'Marianne', which are both of Netherland origin or 'Florian' and 'Kosmosz', being both the product of Austria breeding. In addition, these varieties were clearly distinguished from another Austria accession 'Zeno', which is however of blue seed colour contrary to grey seed colour of 'Florian' and 'Kosmosz' having both the blue-coloured seeds. It was stated that some SSR markers are strongly associated with various agronomical traits such as seed size, plant height, capsule index and others (Celik et al. 2016; Gol et al. 2017).

On the contrary, the association of individual poppy accessions did not always reflect the country of their origin or seed colour, as displayed in Figure 1 and Table 2. For example, blue poppies 'Buddha' and 'Postomi' both with the same origin (Hungary) are not together in one cluster. Nevertheless, used markers capacity to distinguish Czech blue poppies from other poppies, which is the main object of the current study, is sufficient.

It should be noted that used markers were tested on DNA extracted from solitaire plants of each accession. Considering the possible intra-population variability, the discrimination capacity of used markers regarding Czech blue poppy authentication should be further verified on DNA pool extracted from more individuals of the same accession.

CONCLUSION

In our study, we analysed a set of the 29 poppy accessions in order to distinguish poppies mentioned in Czech Guild Standard 'Czech blue poppy' from other poppies. The set of 7 SSR markers was used to explore DNA polymorphism of individual accessions, and data outputs were analysed using hierarchical clustering. The results revealed that 'Czech blue poppies' form one cluster, which is highly supported by the data. Therefore, used markers can be effectively used for distinguishing blue poppies mentioned in Czech Guild Standard.

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