Bulgarian Journal of Agricultural Science, 30 (Suppl. 1) 2024
Agricultural Academy

Genetic diversity assessment of selected rose genotypes using CEAP markers

Mariya Zhelyazkova[1*], Veselina Badzhelova[2], Svetlana Georgieva[1], Lyudmila Lozanova[3], Peter Hristov[3] and Stela Lazarova[3]

[1] *Trakia University, Faculty of Agriculture, 6000 Stara Zagora, Bulgaria*
[2] *Agricultural Academy, Institute for Roses and Aromatic Plants, 6100 Kazanlak, Bulgaria*
[3] *Bulgarian Academy of Sciences, Institute of Biodiversity and Ecosystem Research, 1113 Sofia, Bulgaria*
[*] *Corresponding author:* mariya.zhelyazkova@trakia-uni.bg

Abstract

Zhelyazkova, M., Badzhelova, V., Georgieva, S., Lozanova, L., Hristov, P. & Lazarova, S. (2024). Genetic diversity assessment of selected rose genotypes using CEAP markers. *Bulg. J. Agric. Sci., 30 (Supplement 1)*, 144–151

Bulgaria has a long-standing tradition of cultivating medicinal and aromatic plants, especially oil-bearing roses. Research on the genetic diversity of local rose populations using DNA markers is crucial for conservation and breeding applications. In this study, we used cis-element amplified polymorphism (CEAP) markers to assess the genetic diversity and relationships among eight *Rosa* genotypes, including two varieties of *Rosa damascena* Mill., *Rosa* sp. and five other species (*R. alba* L., *R. canina* L., *R. gallica* L., *R. centifolia* L., and *R. moschata* Herrm.), mostly sourced from the collection of Institute for Roses and Aromatic Plants. Sixteen CEAP primers targeting seven cis-elements produced 201 bands, of which 78% were polymorphic, indicating a significant genetic variation. Polymorphism Information Content (PIC) values ranged from 0.12 to 0.88, with an average of 0.56. Genetic diversity indicators, such as Shannon's Information Index (I = 0.6) and expected heterozygosity (He = 0.4), confirmed a high level of variability. CEAP markers also identified unique loci for specific genotypes. *Rosa damascena*, *R. gallica*, *R. 'raduga'* and *R. canina* showed greater genetic similarity, while *R. moschata*, *R. centifolia*, and *R. alba* were more distantly related.

Our study provides the first evidence of the utility and effectiveness of the CEAP markers in analysing genetic diversity across different *Rosa* genotypes, providing valuable data for future breeding programs. This research highlights the importance of genetic resource conservation and extend our knowledge on the genetic structure of oil-bearing *Rosa* species in Bulgaria.

Keywords: CEAP markers; genetic diversity; *Rosa* species; oil-bearing roses; *Rosa damascena*

Introduction

The importance of conserving and utilising crop diversity is acknowledged in both national and international laws and policies. PCR-based DNA markers have been widely used for variety identification, studying diversity and genetic relationships, thus facilitating marker-assisted selection in breeding and crop improvement programs (Godwin et al., 1997; Meszaros et al., 2007). They are also valuable tools for the conservation, protection, and reintroduction of endangered and economically important plant species. Bulgaria has a rich tradition of cultivating medicinal and aromatic plants, particularly oil-bearing roses. To support this heritage, fundamental studies on the genetic diversity of local plant populations using DNA markers are essential. These studies not only provide valuable insights, but also have significant practical applications for conservation, breeding, and sustainable rose cultivation.

The commercial essential oil production is based mainly on three rose species/hybrids (*Rosa gallica* L., *R. damascena* Mill. and *R. centifolia* L.), and their cultivars (Van de Pol, 2003). *Rosa gallica* has been cultivated for so long as both an ornamental and medicinal plant that botanists find it difficult to distinguish true wild forms from hybrids and products of ancient cultivation (Trees and Shrubs Online, 2024). A recent DNA-based study revealed that *R. damascena* have a triparental origin that includes two consecutive crosses (*R. moschata* Herrm. x *R. gallica* L.) x *R. fedtschenkoana* Regel and *R. moschata* (Iwata et al., 2000). *Rosa centifolia* is believed to have originated through hybridisation in the Mediterranean region, however its exact origin is not fully known, but it is thought to be the result of crossing several rose species (*R. gallica, R. phoenicia* Boiss*., R. moschata,* and *R. canina* L. (Widrlechner, 1981)).

The rose production in Bulgaria is primarily based on *R. damascena* Mill. f. *trigintipetala* Dieck, known also as the 'Kazanlik rose'. A smaller area with a local population of *Rosa alba* L. has been also cultivated. The Institute of Roses and Aromatic Plants (IRAP) in Kazanlak, which is the main supplier of essential oil rose planting material in the country, has a rich collection of oil-bearing and dog roses collected during the last century. A valuable genetic repository containing numerous accessions, phenotypes, and chemotypes has been established, providing a rich resource for research and breeding activities (Nedkov et al., 2005).

The aim of our study was to test the application and effectiveness of cis-element amplified polymorphism (CEAP) markers on eight *Rosa* genotypes. A recent study has shown that selected cis-elements (AAAG, ACGTG, CCGA, ACTCAT, GGTCA, TATCC, TGAC, and GATAA) are intricately linked to plant growth, development, signal transduction, and stress responses (Chen et al., 2022). Highly conserved within species and located in genes and promoters, cis-elements can be used for studies on genetic diversity analysis, relationship studies, and breeding across different species (Chen et al., 2022a; 2022b; Wang et al., 2024).

Material and Methods

Plant material

For this study, we collected seven rose genotypes from the experimental collection at the Institute for Roses and Aromatic Plants, and one genotype from an industrial plantation (Figure 1). *Rosa damascena* is represented by an improved local selection ('Population 5') of the 'Kazanlik rose'. *Rosa* '*raduga*' is a complex hybrid developed through the cross-breeding of 'Vesna' (*R. damascena* Mill. x *R. gallica* subs. *eryostila* Kell var. *austriaca* Br.) and 'Krimskaya krasnaya' (*R. gallica*) variety (Nazarenko, 1983). *Rosa* sp. is a newly discovered genotype, found in an old rose plantation near the town of Klisura. The genetic material for *R. gallica* var. *officinalis* was sourced from a specimen introduced from the Crimea region. *Rosa centifolia* and *R. moschata* were obtained from introduced plants cultivated in the IRAP collection. *Rosa canina* was represented by the Bulgarian variety 'Plovdiv 1', which was developed through selection from a

Fig. 1. A) *R. damascena* (Da), B) *R.* var. *raduga* (Ra), C) *Rosa* sp. (Kl), D) *R. centifolia* (Ce), E) *R. alba* (Al), F) *R. gallica* var. *officinalis* (Ga), G) *R. moschata* (Mo), H) *R. canina* (Ca)

local population of *R. canina*. Ten to twenty young leaves were collected from each rose plant, placed in plastic bags, and immediately stored at -20 °C until DNA extraction.

DNA extraction

Total DNA was isolated using the Plant DNA Preparation Kit (Jena Bioscience) and the Quick-DNA™ Plant/Seed Miniprep Kit following modified manufacturer's instructions. The quality and yield of the DNA samples were assessed using a NanoVue Plus spectrophotometer and 1% agarose gel electrophoresis, visualised with a Transilluminator (Bio-Imaging System).

CEAP analysis

A set of twenty-three CEAP primers targeting seven cis-elements was tested, and 16 of the most reproducible primers were selected for DNA amplification (Table 1). The PCR amplifications were performed in a 20 µL reaction volume containing 1 µL (50 ng) genomic DNA, 10 µL Red Taq DNA Polymerase 2×Master Mix, 1 µL (10 pmol) primer, and 8 µL nuclease-free ddH2O. The CEAP-PCR amplification was carried out using a Doppio Gradient 2 × 48 well thermal cycler (VWR®, Germany) following the protocol described by Chen et al. (2022). All amplified products were separated on 1.7% agarose gel electrophoresis in a 1×TBE buffer for 100 minutes at 100 V/cm and stained with GelRed® (Biotium, USA). The banding patterns were photographed under a UV transilluminator system (Bio-Imaging System, Israel).

Data analysis

Selected characteristics (e.g. the number of polymorphic (PB) and monomorphic bands (MB), the allelic diversity (A), specific number of alleles (SpL) for each CAEP primer were assessed. The CEAP banding profiles obtained were converted into a binary data matrix, where ‚1' indicates the presence of a band, ‚0' indicates its absence, and ‚-1' represents smeared or weak bands. The following parameters were employed to evaluate the primer efficiency, polymorphic information content (PIC, Roldan-Ruiz et al., 2000), effective multiplex ratio (EMR, Nagaraju et al., 2001), marker index (MI, Varshney et al., 2007), and resolving power (RP, Prevost & Wilkinson, 1999). To calculate the genetic diversity parameters, such as expected heterozygosity (He) and Shannon's Information Index (I), along with genetic distances we used GenALEx 6.5. Nei's (1973) gene diversity (H) for all primers was analysed in PopGen32. The genetic relationships were visualized through – Principal Coordinate Analysis (PCoA), carried out using GenALEx 6.5 (Peakall and Smouse, 2006) and Past 4.17 (Hammer and Harper, 2001). The shade plot, illustrating the polymorphism detected by each primer and cis-element for the studied genotypes, was generated using Primer v.7 software (Anderson et al., 2008).

Table 1. List of CEAP primers used to assess genetic variation among *Rosa* genotypes, their sequence and characteristics: number of polymorphic (PB) and monomorphic bands (MB), effective multiplex ratio (EMR), polymorphic information content (PIC), resolving power (Rp), marker index (MI), allelic diversity (A), specific number of alleles (SpL), Nei's genetic diversity (H)

Cis-element	Primer ID	Band size bp	PB	MB	PB %	EMR	PIC	Rp	MI	A	SpL	H
TATCC	TATCC5	350–3000	16	0	100	3.1	0.81	40.2	2.5	3.1	3	0.38
GATAA	GATAA4	250–2000	10	0	100	3.7	0.73	24	2.7	3.7	2	0.39
CCGA	CCGA6	350–3000	14	0	100	3.7	0.74	28.9	2.7	3.7	1	0.39
	CCGA10	300–550	2	2	50	2.4	0.48	11	1.1	4.8	1	0.04
TGAC	TGAC2	250–1000	4	5	44.4	2.5	0.38	20.6	0.9	5.6	2	0.15
	TGAC5	200–1500	10	3	76.9	3.9	0.49	25.4	1.9	5.1	1	0.23
	TGAC6	280–700	5	4	55.6	2.7	0.49	19.6	1.3	4.9	1	0.24
ACTCAT	ACTCAT1	250–3000	16	1	94.1	4.4	0.6	31.1	2.6	4.7	1	0.29
	ACTCAT2	350–2000	10	6	62.5	3.75	0.36	27.4	1.3	6.0	1	0.32
	ACTCAT5	250–2000	13	3	81.3	4.1	0.52	29.3	2.1	5.0	1	0.31
ACGTG	ACGTG1	200–3000	17	0	100	2.2	0.88	58.2	1.9	2.2	8	0.32
	ACGTG5	400–3000	10	1	90.9	3.0	0.73	31.1	2.2	3.3	3	0.25
	ACGTG26	200–500	1	4	16.7	1.48	0.12	7.6	0.2	7.4	0	0.14
AAAG	AAAG1	300–2000	9	2	81.8	4.0	0.55	18.7	2.2	4.9	0	0.30
	AAAG2	200–3000	15	2	88.2	4.4	0.57	27.7	2.7	4.9	0	0.36
	AAAG14	200–3000	13	3	81.2	3.9	0.57	27.5	2.2	4.8	0	0.30
		Total	165	36							25	
		Mean	10.3	2.3	77.8	3.3	0.56	24.6	1.9	4.6	1.6	0.28

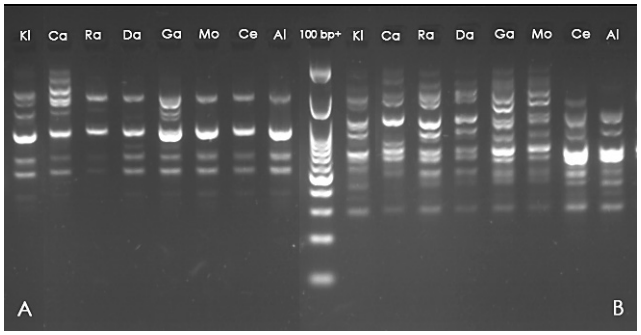

Fig. 2. PCR patterns generated by two CEAP primers: A) ACTCAT1 and B) ACTCAT2. The abbreviations for rose genotypes correspond to those in Figure 1

Results

CEAP polymorphism

The selected 16 CEAP primers generated a total of 201 bands, of which 165 were polymorphic (PB) and 36 were monomorphic (MB). The polymorphism ranged from 44% (primer TGAC2) to 100% (primers ACGTG1, CCGA6, GATAA4, and TATCC5). The number of amplified bands varied from four (CCGA10) to seventeen (ACTCAT1). The mean polymorphic information content (PIC) was 0.56, ranging widely from low (0.12 for ACGTG26) to very high (0.88 for ACGTG1). The total mean number of alleles across all primers was 56.3 (mean allelic diversity = 4.6). The average values for resolving power (Rp), marker index (MI), and effective multiplex ratio (EMR) were 24.6, 1.9, and 3.3, respectively, with the ranges for each primer presented in Table 1. The highest number of specific fragments for rose genotypes (SpL = 8) was recorded for primer ACGTG1, as follows: *Rosa* sp. (the genotype from Klisura – 200 bp), *R. moschata* (450, 1500, 2000, and 3000 bp), *R. alba* (850 bp), *R. centifolia* (900 bp), and *R. gallica* (500 bp). The average values for Nei's genetic diversity (H) were 0.28, with the highest value for GATAA4 and the lowest for CCGA10. An example photograph illustrating the CEAP-PCR amplification using two of the primers is shown in Figure 2.

Effectiveness of CEAP markers in assessing rose genotypes

The mean values for the Shannon's Information Index (I = 0.6) and expected heterozygosity (He = 0.4) indicated a relatively high genetic diversity and efficiency of the primers used across all loci and genotypes. The lowest values were recorded for *R. alba* (0.00) with primer CCGA6 and *R. centifolia* and *R. moschata* (0.14, 0.06) with primer TATCC5, respectively (Table 2). The shade plot in Figure 3 visualise the polymorphism detected by each primer across studied genotypes. Primers targeting the cis-element ACTCAT exhibited high polymorphism in all genotypes, and could be effectively used to assess genetic diversity and support marker-assisted

Table 2. Capability of CEAP primers in studied *Rosa genotypes* according Shannon's Information Index (I)

Cis-element	Primer	Al	Ce	Ga	Mo	Ca	Da	Ra	Kl	Mean
TATCC	TATCC5	0.51	0.14	0.56	0.14	0.56	0.60	0.69	0.61	0.48
GATAA	GATAA4	0.61	0.61	0.20	0.61	0.45	0.66	0.61	0.69	0.56
CCGA	CCGA6	0.00	0.66	0.41	0.53	0.62	0.69	0.66	0.48	0.51
	CCGA10	0.53	0.53	0.53	0.53	0.53	0.66	0.53	0.69	0.57
TGAC	TGAC2	0.64	0.68	0.69	0.69	0.69	0.69	0.64	0.68	0.68
	TGAC5	0.45	0.67	0.58	0.69	0.69	0.66	0.69	0.69	0.64
	TGAC6	0.57	0.57	0.64	0.68	0.68	0.64	0.64	0.68	0.64
ACTCAT	ACTCAT1	0.59	0.54	0.68	0.49	0.65	0.69	0.68	0.69	0.63
	ACTCAT2	0.64	0.67	0.56	0.60	0.65	0.69	0.68	0.56	0.63
	ACTCAT5	0.60	0.56	0.65	0.46	0.69	0.69	0.69	0.69	0.63
ACGTG	ACGTG1	0.23	0.49	0.49	0.54	0.31	0.38	0.38	0.49	0.41
	ACGTG5	0.37	0.45	0.37	0.58	0.52	0.63	0.45	0.45	0.48
	ACGTG26	0.68	0.68	0.68	0.68	0.68	0.68	0.68	0.68	0.68
AAAG	AAAG1	0.45	0.58	0.66	0.45	0.58	0.68	0.69	0.66	0.59
	AAAG2	0.61	0.61	0.69	0.51	0.68	0.69	0.69	0.69	0.65
	AAAG14	0.36	0.64	0.60	0.66	0.64	0.69	0.64	0.64	0.61

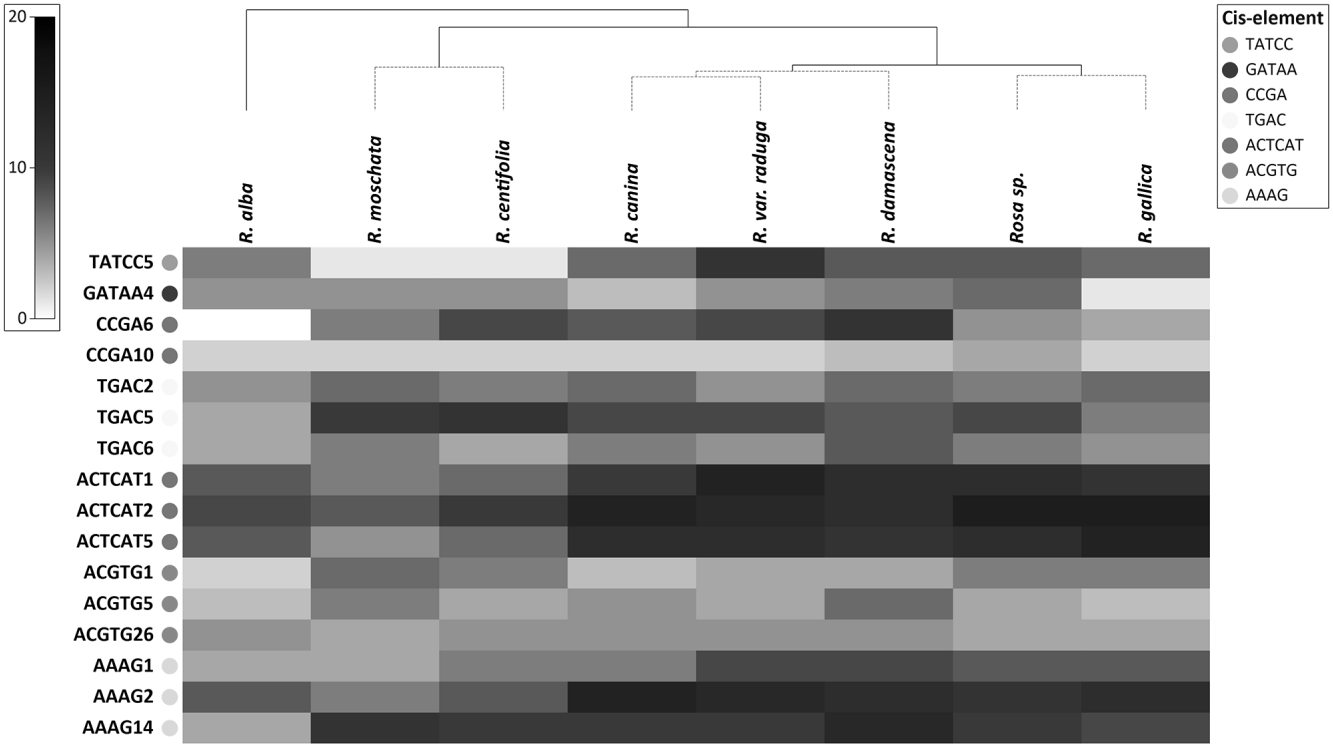

Fig. 3. Shade plot for molecular data obtained with sixteen CEAP primers

selection in studies involving these genotypes. The cis-elements AAAG and TGAC also proved effective in studying the genetic variation among selected rose genotypes.

Genetic similarity and relationships

The dendrogram, based on Jaccard genetic similary indices derived from the marker data for each cis-element, differentiated all *Rosa* genotypes into three distinct groups (figure not shown). Similarly, the PCoA analysis clearly distinguished the three groups along the first and second axes, explaining 61.9% of the variance (Figure 4). The highest genetic similarity was observed between *R. canina* and *R.* var. *raduga* (0.73), and they were closely related to *R. damascena* and *R. gallica*. *Rosa moschata*, *R. centifolia*, and *R. alba* were clearly separated, with the latter two species clustering together in a distinct group (similarity = 0.52). The *Rosa* sp. was slightly more similar to the genotype of *R. gallica* (0.60), *R.* var. *raduga* (0.63) and *R. canina* (0.64) than to the *R. damascena* (0.56) accession.

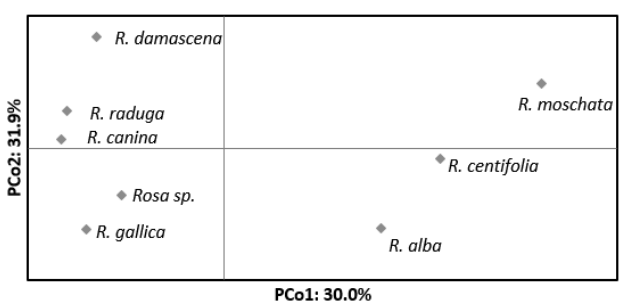

Fig. 4. CEAP-PCoA plot, based on the genetic distances among the studied rose genotypes

Discussion

Effectiveness of CEAP markers

The CEAP markers have been successfully amplified in various crops, including mango, citrus, rice, potato, wax gourd, longan, tomato and red beet (Chen et al., 2022a; Wang et al., 2024). In our study, we applied CEAP markers for the first time to assess the genetic diversity and relationships among *Rosa* genotypes. The individual efficiency of molecular markers was assessed using four parameters PIC, MI, EMR and Rp. All the average values of these parameters in our study confirm the ability of CEAP as an effective marker system in rose genotypes (PIC = 0.56, MI = 1.9, EMR = 3.3, Rp = 24.6). Among the 16 primers used, we observed a wide range of polymorphism, from low 0.12 (ACGTG26) to very

high 0.88 (ACGTG1), with a mean polymorphic information content (PIC) value of 0.56. Chen et al. (2022b) successfully distinguished germplasm from 48 mango accessions with an average PIC value of 0.47 using 20 CEAP primers. In another study, 17 CEAP primers were effectively applied to 32 red beet varieties (lines) to establish genetic relationships and assess genetic diversity, achieving a PIC value of 0.66 (Wang et al., 2024). Specific loci are essential diagnostic features for identifying genetic resources and have valuable applications as molecular markers in crop breeding. The tested CEAP primers generated a total of 25 specific loci across all eight *Rosa* genotypes. In the unknown genotype *Rosa* sp., specific loci were identified for primer CCGA10 at 500 bp, ACGTG5 at 200 and 250 bp, and for ACGTG1 at 200 bp. These findings could contribute to further selection-related research.

When comparing the use of multiple marker systems (ISSR, SRAP, CBDP, and CEAP) with a single-marker system (CEAP) for evaluating genetic relationships in mango germplasm, Chen et al. (2022b) observed highly similar clustering patterns across 24 accessions. In a follow-up study, Chen et al. (2022a) demonstrated the higher efficacy of CEAP compared to ISSR, CBDP, and iPBS markers.

Genetic similarity and relationships

Both the dendrogram and the PCoA analysis clearly distinguished three main groups, with the highest genetic similarity observed between *R. canina* and *R.* var. *raduga*. Both accessions were closely related to *R. damascena* and *R. gallica*. The *Rosa* sp. showed highest similarity to *R. gallica*, while *R. moschata*, *R. centifolia*, and *R. alba* were most distantly related. These results align with those of Rusanov et al. (2005), who studied the genetic diversity and relationships among rose accessions from the IRAP collection using 11 SSR markers. Their study revealed that *R. damascena* is genetically more similar to *R. gallica* and *R. canina* than to *R. moschata*.

Previous studies have suggested that *R. damascena* originated from a cross between *R. gallica* and *R. canina* (Wulf and Maleeva, 1969; Klimenko and Zubcova, 1986). A more recent study has identified *R. damascena* genotypes with greater genetic similarity to *R. canina* (Mirzaei et al. (2015). One of the five *R. damascena* genotypes they analysed clustered more closely with *R. canina*. The genetic variation in 23 *R. canina* genotypes using 15 ISSR markers revealed significant variability (Jamali et al., 2019), which supports the high outcrossing rates of this species (Debener et al., 2003; Wissemann, 2007). Its wild form is widespread and it was extensively used for budding most garden roses (Tutin et al., 1968; Wissemann, 2017; Trees and Shrubs Online, 2024).

Despite the advancement of the molecular methods, the ancestry of certain oil-bearing rose species remains controversial. Iwata et al. (2000) compared the ITS region of rDNA gene and the psbA–trnH spacer of the chloroplast genome in *R. damascena* '*Trigintipetala*' with its presumed parental forms, identifying (*R. moschata*× *R. gallica*) × *R. fedtschenkoana* as the likely progenitors. However, these findings contradict Rusanov's research (Rusanov et al., 2005), which demonstrated that *R. damascena* differs from *R. moschata* and *R. gallica* in all alleles at several microsatellite loci. Similarly, a more recent study by Ahmed (2019), based on the sequencing of two barcodes (matK and t-rnH), confirmed that *R. damascena* and *R. damascena* '*Trigintipetala*' from Taif cluster in a separate group from *R. moschata* and *R. gallica.* The proposed parental forms for *R. alba* are *R. arvensis*, *R. gallica*, and a white-flowered member of Sect. Caninae (Tutin et al., 1968). *Rosa moschata*, commonly grown as an ornamental plant in Southern and Western Europe, originates from Southwest Asia (Tutin et al., 1968).

Given these contradictions, further analysis of genotypes closely related to *R. damascena* and its presumed ancestors is necessary, using molecular markers that allow precise allele scoring. The taxonomy of roses remains complex, partly due to anthropogenic influence, which has led to the creation of new semi-wild or cultivated varieties. Moreover, many rose species are believed to have originated through hybridization, often accompanied by polyploidization (Smulders et al., 2011).

The application of modern biotechnological approaches, including advanced *in vitro* propagation techniques (Badzhelova, 2017; Badzhelova et al., 2018) combined with genetic evaluation of initial samples using DNA markers (Rusanov et al., 2005; 2013; 2022) could help to address key challenges in the selection of oil-bearing roses. Current market demands highlight the need for developing and introducing new high-yielding varieties that are resistant to drought and pests, while preserving the high quality of Bulgarian rose oil. The registration and preservation of initial genetic material require the use of modern methods for analysing and assessing the diversity of oil-bearing rose genotypes. The development and testing of new DNA marker systems, often used in combination, enable a more comprehensive understanding of genetic relationships and facilitate the study of genetic diversity in crops (Gogoi et al., 2020; Chen et al., 2022b).

Conclusion

Our study provides the first evidence of the utility and effectiveness of CEAP markers in studying different *Rosa*

genotypes. The obtained results confirm that CEAP markers are universal and applicable for assessing genetic diversity and genetic relationships in various plant species. In this study, the results for the cis-elements ACTCAT, AAAG, and TGAC represented by nine tested primers, demonstrate high polymorphism in all rose genotypes and can be effectively used for more in-depth studies of cultivars, lines, and hybrids. These markers can also be included in programs for the conservation and improvement of genetic resources of oil-bearing roses. Using 16 primers targeting 7 cis-elements, we successfully differentiated eight rose genotypes and assessed their genetic diversity and relationships.

Acknowledgements

This study was funded by the Bulgarian Ministry of Education and Science: the National Science Fund grant number КП-06-Н56/1 10.11.2021, the Bulgarian Ministry of Education and Science – National Roadmap for Scientific Research, project: "Distributed Research Infrastructure "Bulgarian Network for Long-Term Ecosystem Research" (LTER-BG)" grant agreement # Д01-320/30.11.2023 and National Program „Young Scientists and Posdoctoral Students – 2".

Conflict of interest

All authors declare no conflicts of interest in this paper.

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Received: **October, 09, 2024**; *Approved*: **October, 14, 2024;** *Published:* **December, 2024**