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# Assessment of genetic diversity of oil-bearing rose (*Rosa damascena* Mill.) using ISSR markers

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## Abstract

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Bulgaria has long-standing traditions of cultivating oil-bearing roses and is one of the leading producers of rose essential oil and rose water worldwide. We studied the genetic diversity of twenty-two *R. damascena* accessions from Bulgaria and Saudi Arabia using 10 inter-simple sequence repeat (ISSR) primers. In total, 212 bands were generated, of which 204 were polymorphic and eight monomorphic. High mean values of polymorphic information content (0.75), effective multiplex ratio (8.90), resolving power (32.40), marker index (6.59), and percentage of polymorphic bands (96.26) were obtained, implying significant genetic variability between the studied genotypes. The ISSR-based analyses showed that the level of polymorphism in this crop is appreciably high (Ne = 1.28, I = 0.24, He = 0.16). The coefficient of genetic differentiation among pre-defined groups was 0.46, indicating that 46% of total genetic variability was between groups and 54% was within groups. The method was appropriate for distinguishing individuals with a high level of similarity. The genetic relationships analysed with PCoA and hierarchical clustering analyses clearly separated the group of Bulgarian *R. damascena* accessions collected from local industrial plantations from the other two pre-defined groups. The clustering of Bulgarian rose cultivars and accessions from Saudi Arabia was independent of their geographical origin. The reported data could further facilitate the management of genetic resources and the development of rose breeding programs. Our study provides the first data on the genetic diversity of *R. damascena* accessions from Bulgaria using ISSR markers.

*Keywords:* DNA markers; genetic diversity; genotype; ISSR; polymorphism; *Rosa damascena;* rose

## Introduction

Culturing oil-bearing roses and producing rose oil in Bulgaria is a tradition with a long history of over 300 years (Van de Pol, 2003). Among oil-bearing rose species, *Rosa damascena* Mill. is the most important species for essential oil production, with broad applications in various industries (food, perfumery, cosmetics, and pharmacy) as well as in traditional and modern therapy (Pal, 2013; Venkatesha et al., 2022). Its economic and cultural importance form a significant part of the modern economy of Bulgaria and in other countries of the Middle East and eastern Mediterranean region (Kovacheva et

al., 2010). Although the number of rose plantations in Bulgaria is currently decreasing, producing rose oil and rose water is a national tradition and a livelihood for family farms, especially in the region known as the Rose Valley (Atanasova et al., 2016; Shishkova et al., 2022). Industrial rose cultivation exclusively involves the species *R.* x *damascena* f. *trigintipetala* Dieck (i.e., thirty-petalled rose) due to its high essential oil content and superior quality (Kovacheva et al., 2010). This is a typical summer-flowering Damask rose, also known as the Kazanlik rose in its botanical characterstics. It was introduced in Western Europe by the German botanist George Dieck in 1889 from the famous rose fields of Bulgaria, situated on the southern side of the Balkan Mountains near the town of Kazanlik and named it *R. gallica* var. *damascena* f. *trigintipetala* (Trees and Shrubs Online, 2023). In 1941, Hurst proposed two wild rose species (*R. gallica* L. and *R. phoenicia* Boiss.) as putative ancestors of the Summer Damask Rose group (i.e. 'Kazanlik' and 'York and Lancaster'). However, a recent DNA-based study that explored the relationship between the Damask varieties of *Rosa* spp. and their putative ancestors 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). Currently, in Bulgaria, the oil rose plantations are mainly established by the planting of intraclonal lines of *R. damascena* f. *trigintipetala*: 'Population 5', and four cultivars 'Iskra', 'Svejen', 'Eleina' and 'Janina'. 'Population 5' and 'Iskra' were created by Astadzhov (1978; 1988), and 'Svejen' by Staykov & Astadzhov (1975) selection of clones with higher flower yield, oiliness and the specific for this species aroma. 'Eleina' and 'Janina' are products of radiation and chemical mutagenesis by Raev (1984).

Since the beginning of modern plant breeding and genetics, genetic markers have been used for the indirect selection of desirable traits and against undesirable traits, as well as for taxonomic, phylogenetic, and genetic diversity analyses (Godwin et al., 1997). The applications include DNA fingerprinting for cultivar identification and plant variety rights, phylogenetic and genetic diversity analysis, hybrid confirmation, genome mapping, and gene tagging for marker-assisted selection and map-based cloning (Duan et al., 2019; Rahman et al., 2022). Studies on crops' genetic diversity and variability are also crucial for biodiversity protection and conservation planning (Govindaraj et al., 2015). A variety of molecular markers have been used to study the genetic diversity of *R. damascena*, such as randomly amplified polymorphic DNA (Kiani et al., 2008; Tabaei et al., 2006), amplified fragment length polymorphisms (Baydar et al., 2004; Pirseyedi et al., 2005), simple sequence repeats (Rusanov et al., 2005; Farooq et al., 2013; Akhtar et al., 2019), inter-sequence simple repeats (Mirali et al., 2012; Redwan et al., 2018; Aldhebiani et al., 2018) universal rice primers and start codon target (Mostafavi et al., 2021), DNA barcoding (Ahmed, 2019). Previous findings related to the genetic diversity and genome selections of essential oil roses in Bulgaria are mainly related to the works of Rusanov et al. (2005; 2019; 2022).

The inter-simple sequence repeat (ISSR)-PCR is a technique that uses microsatellite sequences as primers in a polymerase chain reaction to generate multilocus markers (Pradeep et al., 2002). ISSR markers are effective multilocus markers for various applications such as diversity analysis, finger-printing, and genome mapping (Ramesh et al., 2020; Juibary et al., 2021). As no prior sequence knowledge is required, they are more rapidly applied than SSR markers. They are also more reliable and robust than RAPD markers because of the detection method and possibly because primers are longer, hence PCR conditions – more stringent (Chen et al., 2011; Costa et al., 2016; Kumar et al., 2016).

In the present study, we have assessed genetic diversity among accessions of *R. damascena* collected from two countries, Bulgaria and Saudi Arabia, using ISSR markers. Both countries have long-standing traditions of cultivating damask rose as an essential oil crop. They represent different bioclimatic zones due to their distant geographic locations and contrasting altitude levels.

## Material and Methods

Leaf samples of *R. damascena* were collected in 2022 from eleven different plantations in Southern Bulgaria (Table 1). Two accessions (DG and DGw) were collected from Gabarevo locality only, due to the observed deviation in the flower color of one rose plant that was pink-white. Additionally, four accessions from Bulgarian intraclonal lines ('Population 5', incl. cultivars 'Svejen', 'Eleina' and 'Janina') were collected from the experimental fields of the Institute for Roses and Aromatic Plants, Agricultural Academy, Kazanlak (IRAP). Leaves were stored at -20°C until DNA extraction. Six leaf samples from *R. damascena* plantations from the Taif area, Saudi Arabia, were provided and stored at -20°C. Detailed information for all 22 accessions is presented in Table 1. Samples (or accessions) were divided into three groups: D, Bulgarian accessions; DS, Bulgarian intraclonal lines (incl. cultivars); and DSA, accessions from Saudi Arabia.

### *DNA extraction*

Total genome DNA from young *R. damascena* leaves was extracted using optimized protocols of Plant DNA Preparation Kit (Jena Bioscience) and purified with Zymo-Spin™ II-μHRC Filter (Zymo Research). The quality and yield of

DNA samples were checked by NanoVue Plus spectrophotometer and separated on 1% agarose gel electrophoresis visualised on the Transilluminator (Bio-Imaging System).

**Table 1. Details of *Rosa damascena* Mill. accessions used in the study**

| Accession name | Cultivar name | Locality | Altitude, m |
|---|---|---|---|
| Group D – Bulgarian accessions | | | |
| DA | | Asen | 487 |
| DB | | Biyaga | 268 |
| DBr | | Bracigovo | 402 |
| DG | | Gabarevo | 441 |
| DGw | | Gabarevo | 441 |
| DI | | Isperihovo | 225 |
| DM | | Mirkovo | 721 |
| DMg | | Maglish | 360 |
| DK | | Karnare 1 | 624 |
| DK2 | | Karnare 2 | 645 |
| DY | | Yasenovo | 513 |
| DZ | | Zimnica | 157 |
| Group DS – intraclonal lines/cultivars | | | |
| DSP | 'Population 5' | IRAP, Kazanlak | 396 |
| DSE | 'Eleina' | IRAP, Kazanlak | 396 |
| DSY | 'Janina' | IRAP, Kazanlak | 396 |
| DSS | 'Svejen' | IRAP, Kazanlak | 396 |
| Group DSA – Accessions from Saudi Arabia | | | |
| DSA1 | | Al Shafa | 2000 |
| DSA2 | | Al Shafa | 2000 |
| DSA3 | | Al Shafa | 2000 |
| DSA4 | | Al Hada | 1359 |
| DSA5 | | Al Shafa | 2000 |
| DSA6 | | Al Shafa | 2000 |

*Abbreviation:* IRAP, Institute for Roses and Aromatic Plants

***ISSR analysis***

A set of forty ISSR primers was tested, and 10 of them, the most reproducible, were used for amplification with all 22 genotypes (Table 2). Each primer annealing temperature was optimized to a specific annealing temperature (sTa°). The PCR amplifications were performed in a total volume of 16 µl, containing 1.5 µl genomic DNA, 8 µl Red Taq DNA Polymerase 2×Master Mix, 20 pmol Primer (Invitrogen, Germany), and 5.5 µl nuclease-free ddH2O. Amplification was performed on Doppio Gradient 2 × 48 well thermal cycler (VWR®, Germany), following program described by Jabbarzadeh et al. (2010): initial denaturation at 94ºC/5 min, followed by 35 cycles at 94°C/30 s denaturation, 51 – 58°C specific primer annealing for 45 s, extension at 72ºC/2 min; and final extension at 72°C/10 min. Gels were stained with GelRed® (Biotium, USA) and comprised 12.5 µl of product mixed with 2.5 µl loading buffer and 100+ DNA-ladder (100 – 3000 bp). ISSR-PCR amplified products were detected through horizontal electrophoresis on 1.7% agarose gel with 1×TBE buffer for 90 min at 80 V/cm. Image acquisition and band detection were carried out using an electrophoresis gel imaging analysis system (Bio-Imaging System, Israel).

All experiments were conducted in the DNA laboratory of the Agriculture Faculty, Trakia University, Bulgaria.

***Data analysis***

Primer efficiency was assessed by the following parameters: polymorphic information content (PIC) by Roldan-Ruiz et al. (2000), effective multiplex ratio (EMR) by Nagaraju et al. (2001), marker index (MI) by Varshney et al. (2007) and resolving power (RP) by Prevost & Wilkinson (1999).

The genetic relationships between the 22 accessions of *R. damascena* were analysed using GenAlEx ver. 6.5 (Peakall

**Table 2. The ISSR primers used to assess genetic variation among *Rosa damascena* accessions. Specific annealing temperature (sTa°), Total bands (TB), Polymorphic bands (PB), Monomorphic bands (MB), Effective multiplex ratio (EMR), Polymorphic information content (PIC), Resolving power (Rp), Marker index (MI)**

| Primer /Sequence | sTa° | TB | PB | MB | % PB | EMR | PIC | Rp | MI |
|---|---|---|---|---|---|---|---|---|---|
| (AGC)4M AGCAGCAGCAGCM | 58 | 25 | 24 | 1 | 96 | 8.52 | 0.78 | 35.59 | 6.62 |
| (AGC)4Y AGCAGCAGCAGCY | 58 | 25 | 25 | 0 | 100 | 9.28 | 0.75 | 40.07 | 6.98 |
| (AGC)4R AGCAGCAGCAGCR | 58 | 22 | 22 | 0 | 100 | 9.68 | 0.75 | 31.27 | 7.26 |
| (CA)6R CACACACACACAR | 52 | 21 | 19 | 2 | 90.5 | 10.43 | 0.67 | 27.00 | 6.98 |
| (CA)6K CACACACACACAK | 53 | 21 | 21 | 0 | 100 | 8.43 | 0.80 | 33.01 | 6.78 |
| (AG)8YC AGA GAGAGAGAGAGAGYC | 51 | 19 | 19 | 0 | 100 | 6.42 | 0.88 | 31.19 | 5.65 |
| (CAA)5 CAACAACAACAACAA | 53 | 32 | 30 | 2 | 93.9 | 7.44 | 0.80 | 54.01 | 5.94 |
| (ATG)6AC ATGATGATGATGATGATGAC | 56 | 22 | 21 | 1 | 95.5 | 6.33 | 0.85 | 38.23 | 5.41 |
| (CA)6RM CACACACACACARM | 54 | 15 | 13 | 2 | 86.7 | 8.96 | 0.72 | 20.25 | 6.41 |
| (CA)6RY CACACACACACARY | 54 | 10 | 10 | 0 | 100 | 13.5 | 0.53 | 13.37 | 7.17 |
| Total | | 212 | 204 | 8 | | | | | |
| Mean | | | | | 96.2 | 8.90 | 0.75 | 32.4 | 6.59 |

& Smouse, 2006) and MEGA version 4 (Tamura et al., 2007). Different number of alleles (Na), effective number of alleles (Ne), Shannon's Information Index (I), expected (He) and unbiased (uHe) expected heterozygosity, percentage of polymorphic bands (PPB) and Nei Genetic distance and identity were calculated based on binary matrix with presence (1) and absence (0) of bands in GenAlEx v. 6.5. Unclear bands were noted as missing value (-1). Analysis of molecular variance (AMOVA), Principal coordinate analysis (PCoA), and Mantel test were carried out with GenAlEx v. 6.5. Hierarchical clustering based on the unweighted pair-group method with arithmetic averages (UPGMA) algorithm was done using MEGA v.4 (Tamura et al., 2007).

## Results

***ISSR polymorphism***

Ten primers were screened for their ability to generate consistently amplified band patterns and to assess polymorphism between accessions within and among studied groups. A total of 212 bands were detected; 204 were polymorphic, and eight were monomorphic, varying in size range from 100 to 3000 bp and number from 10 (CA)6RY to 32 (CAA)5. The percentage of polymorphism ranged from 86.7% [(CA)6RM] to 100% [(AGC)4Y, (AGC)4R, (CA)6K, (AG)8YC, and (CA)6RY] with an average of 96.2% (Table 2). An example of a photograph illustrating the ISSR fingerprinting of all *R. damascena* accessions by primer (CA)6R is visualised in Figure 1. The polymorphic information content (PIC), measured as the presence of polymorphic fragments for all primers, was high (0.75) and varied from 0.53 [(CA)6RY] to 0.88 [(AG)8YC], showing that ISSR markers are informative and relevant for discriminating the evaluated genotypes. The mean value of the Effective multiplex ratio (EMR) for the primers was 8.90, with the highest value (13.5) at (CA)6RY and the lowest (6.33) at (ATG)6AC. The mean value of Resolving power (Rp) was 32.4, which was the lowest (13.37) for (CA)6RY primer and highest (54.01) for (CAA)5. The highest Marker index (MI) was recorded for primer (AGC)4R – 7.26, and the lowest was with primer (ATG)6AC – 5.41 (Table 2).

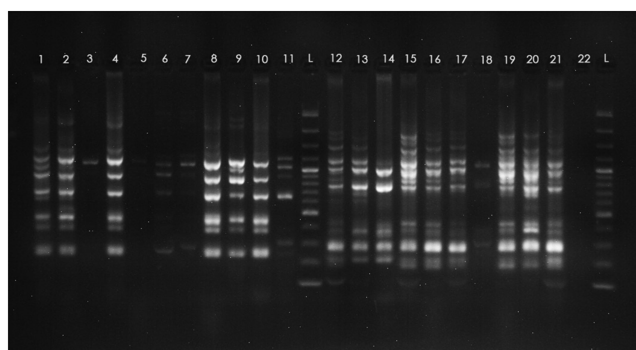


**Fig. 1. ISSR-PCR polymorphism in 22 *Rosa damascena* accessions**

***Genetic diversity within and among pre-defined groups***

The mean frequency of loci for all twenty-two *R. damascena* accessions was 0.43, with mean allele frequencies of 0.35 and 0.65, respectively. Effective (Ne) number alleles were calculated within the range from 1.13 at (AG)8YC to 1.42 at (CA)6RY, averaging 1.28. The highest value of Shannon's information index was 0.32 at (CA)6RY, and the lowest was 0.13 at (CA)6RM, with a mean value for all loci and accessions of 0.24. In the studied genotypes, the expected heterozygosity ranged from 0.09 at primers (AG)8YC and (CA)6RM to 0.23 at (CA)6RY, with a total mean of 0.16 (Table 3).

**Table 3. Genetic diversity in studied *Rosa damascena* accessions revealed by ISSR markers, p and q (allele frequency), different (Na) and effective (Ne) number alleles, Shannon's Information Index (I), expected (He) and unbiased expected heterozygosity (uHe)**

| | Size range, bp | Band Freq. | p | q | Na mean | Ne mean | I mean | He mean | uHe mean |
|---|---|---|---|---|---|---|---|---|---|
| (AGC)4M | 100–2000 | 0.38 | 0.30 | 0.70 | 1.15 | 1.29 | 0.26 | 0.17 | 0.19 |
| (AGC)4Y | 100–3000 | 0.42 | 0.34 | 0.66 | 1.05 | 1.28 | 0.24 | 0.16 | 0.17 |
| (AGC)4R | 100–1600 | 0.48 | 0.42 | 0.58 | 1.03 | 1.22 | 0.19 | 0.13 | 0.14 |
| (CA)6R | 100–2000 | 0.53 | 0.43 | 0.57 | 1.19 | 1.37 | 0.29 | 0.20 | 0.22 |
| (CA)6K | 100–1300 | 0.36 | 0.27 | 0.73 | 1.14 | 1.30 | 0.27 | 0.18 | 0.19 |
| (AG)8YC | 100–1200 | 0.30 | 0.25 | 0.75 | 0.79 | 1.13 | 0.14 | 0.09 | 0.09 |
| (CAA)5 | 150–3000 | 0.33 | 0.24 | 0.76 | 1.16 | 1.30 | 0.27 | 0.18 | 0.20 |
| (ATG)6AC | 100–3000 | 0.31 | 0.22 | 0.78 | 1.26 | 1.31 | 0.29 | 0.19 | 0.21 |
| (CA)6RM | 100–1500 | 0.56 | 0.51 | 0.49 | 0.93 | 1.15 | 0.13 | 0.09 | 0.10 |
| (CA)6RY | 100–400 | 0.62 | 0.50 | 0.50 | 1.33 | 1.42 | 0.32 | 0.23 | 0.24 |
| Grand mean | | 0.43 | 0.35 | 0.65 | 1.10 | 1.28 | 0.24 | 0.16 | 0.17 |

**Table 4. Distribution of the band by groups and accessions obtained in the *Rosa damascena* accessions by ISSR markers**

| | Group D | | | | | | | | | | | | Group DS | | | | Group DSA | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Accession name | DA | DB | DBr | DG | DI | DK | DK2 | DM | DMg | DY | DZ | DGw | DSE | DSP | DSS | DSY | DSA1 | DSA2 | DSA3 | DSA4 | DSA5 | DSA6 |
| Total Band | 81 | 51 | 72 | 126 | 61 | 115 | 61 | 72 | 83 | 94 | 88 | 96 | 79 | 88 | 89 | 92 | 101 | 99 | 94 | 99 | 85 | 82 |
| Total Band per group | 162 | | | | | | | | | | | | 117 | | | | 133 | | | | | |
| Private bands | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Private bands per group | 68 | | | | | | | | | | | | 3 | | | | 9 | | | | | |
| PB (%) | 56.8 | 43.2 | 56.8 | 64.9 | 43.2 | 78.4 | 54 | 67.6 | 62.2 | 94 | 88 | 73 | 79 | 89 | 78 | 92 | 78.4 | 78.4 | 81 | 81 | 81 | 78.4 |
| PB (%) per group | 69.81 | | | | | | | | | | | | 29.25 | | | | 37.26 | | | | | |

*Abbreviations:* D, Bulgarian accessions; DS, Bulgarian intraclonal lines (incl. cultivars); DSA, accessions from Saudi Arabia. For labels descriptions – see Table 1

The highest values of the effective number of alleles, Shannon's information index, and expected heterozygosity were observed in *R. damascena* accessions Group D (Ne = 1.40, I = 0.36, He = 0.24), with a total of 162 bands, of which 68 bands are missing in the other groups. On the other hand, for all accessions in the group, only three specific inter-simple sequence repeats were noticed (by one per each of the following accessions: DA, DG, and DY). The percentage of polymorphic bands in the group (69.8%) varied from 43.2% at accessions DB and DI to 94% at accession DY, and the total number of bands from 51 at accessions DB to 126 at accession DG, respectively (Tables 4 and 5).

For the group consisting of *R. damascena* intraclonal lines (DS), a total of 117 bands were noticed, with three of them absent in the other groups, and only one at 'Population 5' (DSP) was observed as a specific ISSR band. The group's total percentage of polymorphic bands was 29.25%, with the lowest value in cultivar DSE (79%) and the highest value in DSY (92%). The mean values of the effective number of alleles, Shannon's information index, and expected heterozygosity were 1.19, 0.16, and 0.10, respectively, and were lowest compared with the other groups. 'Janina' and 'Eleina' showed the highest (92%) and lowest (79%) polymorphic bands, respectively (Tables 4 and 5).

A total of 133 bands were recorded for accessions of Saudi Arabia, i.e., for the DSA group (Ne = 1.24, I = 0.21, He = 0.14), of which nine were specific for the whole group, and one was specific for accession DSA3. A higher number of bands (101) was detected for accession DSA1 and the lowest (82) – for DSA6. The percentage of polymorphic bands specific to this group was 37.26 and varied from 78.4% (DSA1, DSA2, and DSA6) to 81% (DSA3, DSA4, and DSA5).

**Table 5. Genetic diversity in studied *Rosa damascena* accessions per groups: different (Na) and effective (Ne) number alleles, Shannon's Information Index (I), expected (He) and unbiased expected heterozygosity (uHe)**

| Groups | Na | Ne | I | He | uHe |
|---|---|---|---|---|---|
| DSA | 1.00 | 1.24 | 0.21 | 0.14 | 0.15 |
| DS | 0.84 | 1.19 | 0.16 | 0.10 | 0.12 |
| D | 1.46 | 1.40 | 0.36 | 0.24 | 0.25 |
| Grand mean | 1.10 | 1.28 | 0.24 | 0.16 | 0.17 |

**Table 6. ANOVA analysis for studied pre-defined groups (DSA, DS and D) *Rosa damascena* accessions**

| Source | df | SS | MS | Est. Var. | % variation |
|---|---|---|---|---|---|
| Among Group | 2 | 332.242 | 166.121 | 21.499 | 46 |
| Within Group | 19 | 482.667 | 25.404 | 25.404 | 54 |
| Total | 21 | 814.909 | 191.525 | 46.902 | 100 |

**Table 7. Pairwise Population Matrix of Nei Genetic Distance (below diagonal) and Identity (above diagonal) for studied *Rosa damascena* genotypes**

| | DA | DB | DBr | DG | DI | DK | DK2 | DM | DMg | DY | DZ | DGw | DSE | DSP | DSS | DSY | DSA1 | DSA2 | DSA3 | DSA4 | DSA5 | DSA6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DA | *** | 0.783 | 0.797 | 0.618 | 0.755 | 0.623 | 0.736 | 0.712 | 0.811 | 0.599 | 0.693 | 0.627 | 0.547 | 0.528 | 0.552 | 0.505 | 0.500 | 0.491 | 0.524 | 0.491 | 0.519 | 0.524 |
| DB | 0.245 | *** | 0.825 | 0.608 | 0.802 | 0.604 | 0.736 | 0.731 | 0.783 | 0.618 | 0.712 | 0.646 | 0.604 | 0.585 | 0.580 | 0.552 | 0.538 | 0.519 | 0.542 | 0.528 | 0.594 | 0.590 |
| DBr | 0.227 | 0.192 | *** | 0.651 | 0.769 | 0.627 | 0.750 | 0.783 | 0.778 | 0.575 | 0.689 | 0.642 | 0.571 | 0.524 | 0.566 | 0.528 | 0.533 | 0.505 | 0.528 | 0.524 | 0.552 | 0.557 |
| DG | 0.481 | 0.497 | 0.429 | *** | 0.684 | 0.797 | 0.608 | 0.660 | 0.675 | 0.717 | 0.764 | 0.811 | 0.505 | 0.458 | 0.472 | 0.433 | 0.495 | 0.467 | 0.491 | 0.458 | 0.505 | 0.543 |
| DI | 0.281 | 0.221 | 0.263 | 0.380 | *** | 0.651 | 0.755 | 0.759 | 0.774 | 0.655 | 0.797 | 0.731 | 0.613 | 0.575 | 0.590 | 0.542 | 0.547 | 0.519 | 0.561 | 0.509 | 0.566 | 0.571 |
| DK | 0.474 | 0.505 | 0.466 | 0.227 | 0.429 | *** | 0.651 | 0.665 | 0.670 | 0.750 | 0.788 | 0.816 | 0.500 | 0.472 | 0.505 | 0.467 | 0.500 | 0.462 | 0.467 | 0.462 | 0.491 | 0.495 |
| DK2 | 0.307 | 0.307 | 0.288 | 0.497 | 0.281 | 0.429 | *** | 0.778 | 0.726 | 0.637 | 0.722 | 0.665 | 0.623 | 0.557 | 0.637 | 0.552 | 0.575 | 0.557 | 0.561 | 0.538 | 0.594 | 0.599 |
| DM | 0.339 | 0.313 | 0.245 | 0.415 | 0.275 | 0.408 | 0.251 | *** | 0.722 | 0.613 | 0.726 | 0.698 | 0.571 | 0.524 | 0.585 | 0.528 | 0.542 | 0.542 | 0.538 | 0.514 | 0.580 | 0.585 |
| DMg | 0.209 | 0.245 | 0.251 | 0.394 | 0.257 | 0.401 | 0.320 | 0.326 | *** | 0.599 | 0.731 | 0.684 | 0.519 | 0.519 | 0.524 | 0.467 | 0.519 | 0.500 | 0.514 | 0.519 | 0.538 | 0.524 |
| DY | 0.512 | 0.481 | 0.553 | 0.333 | 0.408 | 0.288 | 0.451 | 0.489 | 0.512 | *** | 0.783 | 0.783 | 0.542 | 0.524 | 0.528 | 0.509 | 0.524 | 0.514 | 0.519 | 0.476 | 0.524 | 0.500 |
| DZ | 0.366 | 0.339 | 0.373 | 0.269 | 0.227 | 0.239 | 0.326 | 0.320 | 0.313 | 0.245 | *** | 0.811 | 0.599 | 0.561 | 0.585 | 0.557 | 0.580 | 0.524 | 0.538 | 0.542 | 0.580 | 0.566 |
| DGw | 0.466 | 0.437 | 0.444 | 0.209 | 0.313 | 0.203 | 0.408 | 0.359 | 0.380 | 0.245 | 0.209 | *** | 0.524 | 0.486 | 0.528 | 0.462 | 0.524 | 0.505 | 0.491 | 0.467 | 0.514 | 0.500 |
| DSE | 0.603 | 0.505 | 0.561 | 0.684 | 0.489 | 0.693 | 0.474 | 0.561 | 0.656 | 0.612 | 0.512 | 0.647 | *** | 0.811 | 0.892 | 0.873 | 0.792 | 0.745 | 0.731 | 0.774 | 0.830 | 0.807 |
| DSP | 0.638 | 0.536 | 0.612 | 0.782 | 0.553 | 0.751 | 0.586 | 0.647 | 0.656 | 0.647 | 0.577 | 0.722 | 0.209 | *** | 0.788 | 0.807 | 0.755 | 0.708 | 0.759 | 0.802 | 0.792 | 0.835 |
| DSS | 0.594 | 0.544 | 0.569 | 0.751 | 0.528 | 0.684 | 0.451 | 0.536 | 0.647 | 0.638 | 0.536 | 0.638 | 0.115 | 0.239 | *** | 0.877 | 0.759 | 0.722 | 0.708 | 0.741 | 0.807 | 0.792 |
| DSY | 0.684 | 0.594 | 0.638 | 0.813 | 0.612 | 0.761 | 0.594 | 0.638 | 0.761 | 0.674 | 0.586 | 0.772 | 0.136 | 0.215 | 0.131 | *** | 0.797 | 0.759 | 0.745 | 0.797 | 0.797 | 0.774 |
| DSA1 | 0.693 | 0.620 | 0.629 | 0.703 | 0.603 | 0.693 | 0.553 | 0.612 | 0.656 | 0.647 | 0.544 | 0.647 | 0.233 | 0.281 | 0.275 | 0.227 | *** | 0.896 | 0.788 | 0.840 | 0.830 | 0.778 |
| DSA2 | 0.712 | 0.656 | 0.684 | 0.761 | 0.656 | 0.772 | 0.586 | 0.612 | 0.693 | 0.665 | 0.647 | 0.684 | 0.294 | 0.346 | 0.326 | 0.275 | 0.110 | *** | 0.854 | 0.811 | 0.811 | 0.741 |
| DSA3 | 0.647 | 0.612 | 0.638 | 0.712 | 0.577 | 0.761 | 0.577 | 0.620 | 0.665 | 0.656 | 0.620 | 0.712 | 0.313 | 0.275 | 0.346 | 0.294 | 0.239 | 0.158 | *** | 0.844 | 0.797 | 0.736 |
| DSA4 | 0.712 | 0.638 | 0.647 | 0.782 | 0.674 | 0.772 | 0.620 | 0.665 | 0.656 | 0.741 | 0.612 | 0.761 | 0.257 | 0.221 | 0.300 | 0.227 | 0.175 | 0.209 | 0.169 | *** | 0.838 | 0.835 |
| DSA5 | 0.656 | 0.520 | 0.594 | 0.684 | 0.569 | 0.712 | 0.520 | 0.544 | 0.620 | 0.647 | 0.544 | 0.665 | 0.186 | 0.233 | 0.215 | 0.227 | 0.186 | 0.209 | 0.227 | 0.186 | *** | 0.901 |
| DSA6 | 0.647 | 0.528 | 0.586 | 0.792 | 0.561 | 0.703 | 0.512 | 0.536 | 0.647 | 0.693 | 0.569 | 0.693 | 0.215 | 0.180 | 0.233 | 0.257 | 0.251 | 0.300 | 0.307 | 0.180 | 0.104 | *** |

Subsequently, we performed an analysis of molecular variance (AMOVA) to quantify the level of genetic differentiation between the pre-defined groups (Table 6). The coefficient of genetic differentiation among pre-defined groups was 0.46, indicating that 46% of total genetic variability was between groups and 54% was within groups (p = 0.001).

The pair-wise group comparisons of Nei genetic distance between DS and DSA (0.180-0.326) was significantly lower than between DS and D (0.451-0.813) and DSA and D (0.512-0.782) (Table 7). The highest genetic distance (0.813) was observed between Gabarevo accession (DG) and cultivar 'Janina' (DSY). Within group D, a significant genetic distance (0.553) was noticed between accessions from Bracigovo (DBr) and Yasenovo (DY). Between the groups DSA and D, the lowest genetic distance was detected between accessions DSA6 and DK2 (0.512), while the highest was between DK and DSA3 (0.761). Within the group of Bulgarian intraclonal lines (DS), the highest genetic distance (0.239) was observed between 'Svejen' (DSS) and 'Population 5' (DSP). The highest levels of genetic identities were revealed in DSA5 *vs.* DSA6 (0.901), DSS *vs.* DSE (0.892), and DB *vs.* DBr (0.825). All pair-wise comparisons between accessions are presented in Table 7.

The genetic relationships among all 22 *R. damascena* accessions were analysed and visualised with a PCoA plot and UPGMA dendrogram (Figures 2 and 3). The PCoA analysis clearly distinguished three groups along the first and second axes, explaining 54.7% of the variance. Along the first axis, group D (the Bulgarian accessions) was clearly distinguished from the other two groups (DS and DSA); along the second axis – group D was divided into two sub-groups (Figure 2). A slight deviation from the PCoA-based grouping was obtained when the UPGMA clustering was applied (Figure 3). Cluster I consisted of all Bulgarian cultivars, the accessions from Saudi Arabia and one Bulgarian accession from Karnare (DS, DSA and DK). Cluster II comprised all other Bulgarian accessions with the exception of DG (Gabarevo), which comprised most bands (126) that revealed the most specific ISSR profile. The Mantel test analysis showed no correlation between geographic (in km) and genetic distance (GD) among studied Bulgarian accessions, group D (r = 0.05, Figure 4).

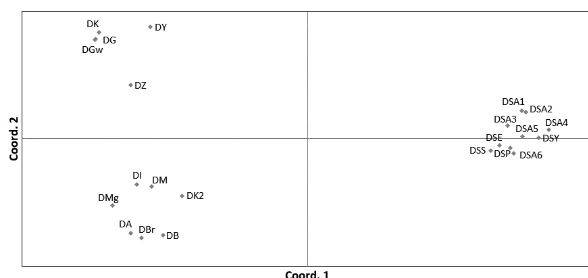


**Fig. 2. PCoA plot of patterns within *R. damascena* accessions and cultivars based of 10 ISSR primers**

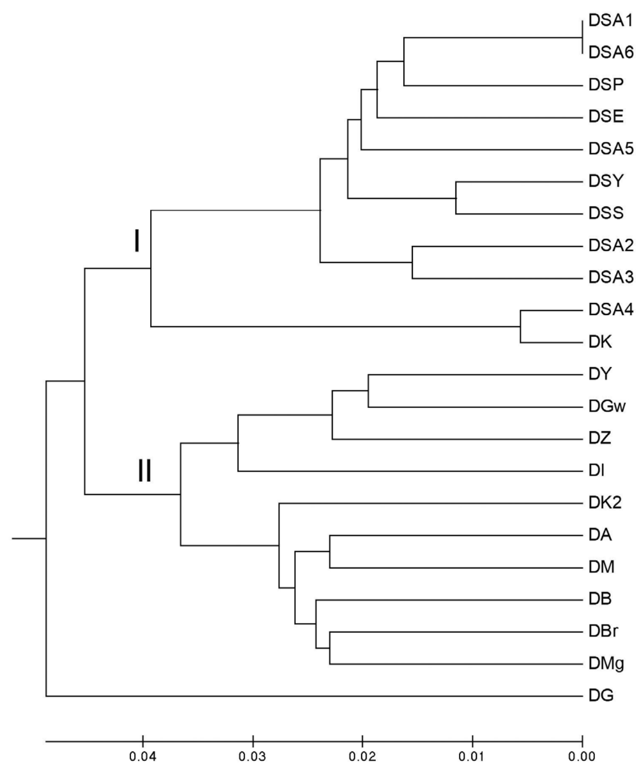


**Fig. 3. UPGMA dendrogram for studied 22 *Rosa damascena* accessions based on 10 ISSR markers**

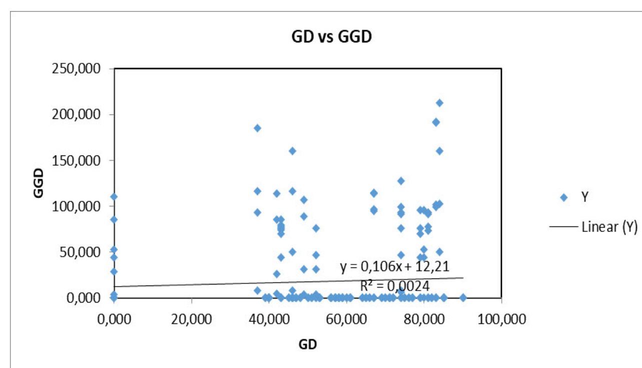


**Fig. 4. Mantel test analysis for correlation between geographic (in km) and genetic distance (GD) among studied Bulgarian accessions, Group D**

## Discussion

In this study, we have applied the ISSR-PCR technique as an efficient procedure to fingerprint a set of *R. damascena* accessions from Bulgaria and Saudi Arabia, including three Bulgarian cultivars ('Svejen', 'Eleina' and 'Janina') and one intraclonal line ('Population 5'). The reliability of ISSR markers to detect DNA polymorphisms in this essential oil crop is confirmed. The 10 primers used in this study generated 212 amplification products with an overall percentage of polymorphism of 96.2%. This average value is the highest compared to previously reported data for *R. damascena* accessions originating from other countries; however, values obtained for our pre-defined groups were lower (Table 8). Oğraş et al. (2017) used 15 ISSR primers to study 19 rosa genotypes and reported that they could detect more polymorphism than using mtDNA, cpDNA, RAPD, and isoenzyme markers in closely related plants. A high level of polymorphism (66.7–100%) using ISSR markers was revealed in similar studies involving *R. damascena* and other rose species in Syria, Pakistan, Iran, and Turkey (Mirali et al., 2012; Ogras et al., 2017; Redwan et al., 2018). The authors explain the high genetic diversity in *R. damascena* accessions with evolution through genetic drift, mutation, and recombination caused by geographical differences and climatic conditions (Farooq et al., 2011; Ahmed, 2019).

A recent study based on simple sequence repeats (SSR) analysis found that twenty-four accessions of *R. damascena* f. *trigintipetala* maintained in the IRAP experimental fields and selected during the period 1924–1980 from different regions of the Rose Valley possessed identical microsatellite profiles (Rusanov et al., 2005). Our results for the Bulgarian cultivars group (DS) confirmed these findings, for which low values of Nei genetic distances were obtained. The highest genetic distance (0.115–0.239) was between 'Svejen' and 'Population 5'. Simultaneously, we observed a high genetic similarity (Nei I, 0.462–0.599) between accessions from Bulgaria and Saudi Arabia and very high (0.708–0.835) between Saudi Arabian accessions and Bulgarian cultivars from the IRAP experimental fields. Similarly, identical microsatellite profiles for all accessions from Bulgaria, India, Iran, France, and Turkey, and old European Damask rose varieties were obtained by Rusanov et al. (2005).

On the other hand, our study demonstrated 68 unique bands distinguishing group D (the Bulgarian accessions) from both other studied groups (DS and DSA). High polymorphism levels in industrial plantations in Bulgaria have been registered for all main morphological traits (Topalov, 1978; Georgiev & Stoyanova, 2006). The influence of landscape heterogeneity and variability in local environmental conditions, in conjunction with long-term cultivation, might have induced significant plant changes that led to morphological differences between various accessions in the country (Topalov, 1978). However, the performed Mantel test showed no correlations between genetic and geographic distances, which corresponds with data reported from Iran (Mostafavi et al., 2021). The authors highlithed the primary role of humans in developing *R. damascena* 'populations'. Due to its adaptability to different soil and climatic conditions and its high tolerance to drought, farmers tend to spread it to different regions of the country (Mostafavi et al., 2021).

Various parameters for assessing genetic diversity in ISSR-based studies have been used (Table 8). Ogras et al. (2017) investigated the genetic diversity in *R. damascena* in Turkey and other rose species using 15 ISSR primers and reported that genetic distances between six *R. damascena* genotypes ranged from 0.112 to 0.336. A similar range of dissimilarity values (0.00 to 0.38) detected with Jaccard's similarity index was found between 20 *R. damascena* accessions in Latakia province, Turkey (Redwan et al., 2018). The higher value of genetic distance obtained between DS and D groups (Nei D 0.451–0.813) as compared to DS and DSA

**Table 8. Summary of genetic diversity data in studies of *Rosa damascena* Mill. genotypes**

| Authors | Country | NG | NP | MP% | Private bands | Genetic similarity/dissimilarity |
|---|---|---|---|---|---|---|
| Mirali et al. (2012) | Syria | 7 | 21 | 81.7 | 7 | – |
| Mirzaei et al. (2015) | Iran | 5 | 6 | 86 | – | 0.33–0.67 Jacard similarity |
| Ahmed et al. (2017) | Saudi Arabia | 5 | 8 | 91.7 | 5 | – |
| Ogras et al. (2017) | Turkey | 5 | 15 | – | – | 0.05–0.177 Nei distance |
| Aldhebiani et al. (2018) | Saudi Arabia | 6 | 7 | 62.30 | 43 | – |
| Redwan et al. (2018) | Syria | 20 | 10 | 73.02 | – | 0.00–0.380 Jacard dissimilarity |
| Mostafavi et al. (2022) | Iran | 40 | 12 | 0.41 (PIC) | – | 0.141–0.837 Jacard dissimilarity |
| Group D (this study) | Bulgaria | 12 | 10 | 69.8 | 68 | 0.192–0.512 |
| Group DS (this study) | Bulgaria | 4 | 10 | 29.2 | 3 | 0.115–0.239 |
| Group DSA (this study) | Saudi Arabia | 6 | 10 | 37.3 | 9 | 0.104–0.251 |

*Note:* NG – number of *R. damascena* genotypes, NP – number of primers used in each study, MP% – mean polymorphism

groups (0.186–0.346) could be explained by the fact that despite their good characteristics, high yield, and resistance, the Bulgarian cultivars ('Janina', 'Eleina' and 'Svejen') were not imposed into the practice of local rose production. Considering also the finding that a relatively high number of specific bands (68) was found for group D, we can assume that the unregulated cultivation of *R. damascena* plants in different parts of the country can be a source of increased genetic diversity, known in science as genotype-environment interaction.

A clear separation of the Bulgarian accessions (group D) from all other accessions was obtained by the PCoA and partly by the clustering analysis. This group consisted of accessions collected from rose plantations from southern regions of the country. Because these plants should have a common genetic origin, evident in our study and several other studies, the genetic diversity could be explained by the climatic differences and factors variation among localities. Moreover, it is well-known that *Rosa* species owe their intra- and inter-genetic diversity to cross-pollination (Cole & Melton, 1986; Ueda Akimoto, 2001). Obtaining more data will aid breeding programs and genetic resources management and help understand genetic variation among cultivars (Mirzaei et al., 2015; Shahbazi et al., 2020; Aldhebiani et al., 2018). Assessing the genetic diversity of planting material is one of the most essential activities in breeding programs. The best choice of parents is to use genotypes with high genetic distances, which was the goal of studies focused on the *Rosa* genome in Turkey, Syria, and Iran (Oğraş et al., 2017; Redwan et al., 2018; Jamali et al., 2019). The pairwise genetic distances among four cultivars and 12 *R. damascena* accessions from Bulgaria (Table 7), provide useful information for managing and protecting our genetic resources.

## Conclusion

The present study provides the first findings on the genetic diversity of *R. damascena* accessions from Bulgaria using ISSR markers. It demonstrates that ISSR markers are powerful tool for quantifying genetic diversity in *R. damascena* genotypes from various locations, thus providing valuable information for its polymorphism within and among regions. The analyses showed that the level of polymorphism in this crop is appreciably high. It could even distinguish individuals with a high level of similarity. Moreover, the clustering of rose cultivars and Saudi Arabian accessions was independent of their geographical origin.

The results from the present study confirmed the importance of genetic studies for designing collections and developing germplasm conservation strategies. The obtained data could facilitate the management of genetic resources and the development of rose breeding programs. Further studies for analysing genetic diversity and variability within *R. damascena* will assist in selecting plants of interest. Comparative studies with different molecular and morphological markers will help to extend our knowledge and develop adequate germplasm conservation strategies.

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***Conflict of interest***

All authors declare no conflicts of interest in this paper.

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