Molecular profiling of Rosa canina L. Greek native germplasm collection for enhanced fruit extract production: A comprehensive approach utilizing neutral, gene, and exon-based markers

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Abstract

The genus *Rosa* L. is globally distributed and encompasses the economically and ecologically important species *Rosa canina* L. Apart from the traditional uses of *R. canina* in folk medicine, food, cosmetics, and ornamental applications, it is renowned for the functional bioactive components found in rose hips. Identifying the genetic diversity within this species is crucial for any plant breeding project. This study employed three molecular markers (ISSR, SCoT, and EBAP) to conduct the first comprehensive genetic analysis of 12 *R. canina* genotypes. DNA extraction, marker selection, and PCR amplification were performed following established protocols. The resulting genetic data were analyzed for polymorphism, diversity indices, and population structure using various statistical methods, including PCA, UPGMA clustering, and STRUCTURE analysis. The ISSR analysis revealed a high level of polymorphism (81.82%) and identified two major clusters in the UPGMA dendrogram. SCoT and EBAP markers also exhibited substantial polymorphism (74.56% and 82.11%, respectively) and formed three distinct clusters. PCA indicated a consistent pattern across markers, suggesting reliable genetic grouping. STRUCTURE analysis supported the presence of three genetically uniform subpopulations (K = 3) within the studied *R. canina* germplasm collection. This study provides a comprehensive genetic characterization of the Greek native *R. canina* gene bank collection. The observed genetic diversity and population structure provided valuable insights for future breeding programs targeting specific genetic clusters within *R. canina* populations.

Introduction

The genus *Rosa* L. is one of the largest genera in the *Rosaceae* family, with 149 recognized species widespread in Asia, Middle East, Europe, and North America (www.theplantlist.org, accessed on 1 January 2024). One of the most important bush species in this genus with about 60 rose species of Eurasian distribution (Wisseman and Ritz, 2007) is the so-called “dog” rose (*Rosa canina* L.); the latter, apart from its ornamental use, it is also well known for its use in traditional medicine, food, cosmetics, owing to its aroma, and nutraceutical value (Ercisli 2007; Ayati et al. 2018). Rose hips are the fruits of *R. canina*, containing a broad number of high-value bioactive compounds, including vitamin C, flavonoids, tannins, anthocyanins, catechins, carotenoids, fatty acids, tocotrienols, and minerals (Rein et al. 2004; Ayati et al. 2018; Pehlivan et al. 2018; Maloupa et al. 2021). Due to its high nutraceutical importance, with only a few allergic reports and side effects, *R. canina* is regarded as an important berry-like fruit in the food industry, widely employed to treat inflammations, cold and cough, skin disorders and chronic pain, as well as to prevent from diabetes, hypertension, arthritis, aging, cardiovascular diseases etc. (Ahmad et al. 2016; Kerasioti et al. 2019).

-to its different reproductive strategies and its distinctive pattern of inheritance, the members of the genus of *Rosa* have a complex taxonomy and evolutionary history The genus *Rosa* exhibits an intricate taxonomy and evolutionary history because of its varied reproductive strategies and unique pattern of inheritance (Wisseman and Ritz 2007). DNA fingerprinting is being extensively used to analyze the variability that occurs in the genus *Rosa* (Gahlaut et al. 2021). This will help in plant breeding programs as well as in maintaining the genetic diversity of *Rosa* species. *Rosa canina* is generally considered as a pentaploid species (2n = 5x = 35, with base number of chromosomes x = 7), with most morphological characteristics as well as flower volatiles being maternally inherited (Wisseman and Ritz 2007). In this frame, most microsatellite loci show maternally inherited alleles, whereas most of RAPD bands are only passed through seeds to the next generation (Wisseman and Ritz 2007). A draft genome of the wild rose *R. multiflora* Thunb. has been recently sequenced, serving as a valuable genetic resource for breeding of cultivated roses (Nakamura et al. 2018). Additionally, the sequence of a double haploid rose line from *R. chinensis* Jacq. combining short- and long- reads, as well as the generation of a high-density SNP genetic map from a tetraploid *R. × polliniana* Spreng. (syn. *R.* x *hybrida* Schleich.) population (Raymond et al. 2018), have significantly broadened our current understanding of segmental allopolyploid patterns in *Rosaceae*. Furthermore, transcriptomic studies on diverse germplasm, tissues, and types of roses have enabled the development of a gene expression database underlying candidate genes of interest for various members of genus *Rosa*, i.e., those related to pathogen infection (Dubois et al. 2012; Liu et al. 2018; Neu et al. 2019).

Greece has a broad number of wild-growing *Rosa* species, with native *R. canina* germplasm being one of the most valuable ones as it is associated with high ornamental, pharmaceutical, cosmetic, and nutritional values (Maloupa et al. 2021). The identification of high-resolving DNA-based markers is of paramount importance to unlock the potential of neglected and underutilized germplasm across the Mediterranean basin. In this context, the present investigation aimed to present the first study on the genetic diversity and relationship of 12 *R. canina* genotypes, by combining molecular characterization strategies based on ISSR (Inter Simple Sequence Repeats) (Reddy et al. 2002), SCOT (Start codon-targeted) (Collard and Mackill 2009) and EBAP (Exon-based Amplified Polymorphism) (Xiong et al. 2022). In particular, the objectives of this study were: (i) to evaluate the genetic diversity and population structure of the *R. canina* genotypes using ISSR (as an arbitrary technique), SCoT (as a gene-targeting technique) markers and EBAP (as an exon-targeting technique, and (ii) to compare the level of information provided by ISSR, SCoT and EBAP markers to assess genetic similarities among the investigated germplasm. This study provides baseline data on the population structure and genetic information of *R. canina* wild-growing populations from Greece which could be useful and informative for further breeding programs.

Materials and Methods

Plant materials studied

In total, nine *R. canina* populations were collected from the wider area of the Balkan Botanic Garden of Kroussia in Northern Greece (41°05’27”N 23°06’36”E), where they are wild-growing at 600–650 m of altitude above sea level, including two within the botanic garden originating in the wild (Fig. 1; Table 1). The expeditions were conducted during fall 2021, when the rosehips were ripe and aimed at the selection of genotypes with specific

traits such as vigorous growth and strong fruiting potential. For DNA analysis, leaf samples from nine individuals were collected. Consequently, each genotype was given a unique IPEN (International Plant Exchange Network) accession number by the Institute of Plant Breeding and Genetic Resources (IPBGR) of the Hellenic Agricultural Organization – -DIMITRA (ELGO-DIMITRA). Additionally, another three wild-sourced *R. canina* population samples were exploited from previous research projects (Maloupa et al. 2021). All collections were performed using the institution's authorized special permit (Permit 26895/1527 of 21/4/2021). This permit is issued yearly by the Greek Ministry of Environment and Energy after detailed reporting of the applicant. The remaining genotypes were used from previous research projects (Maloupa et al. 2021).

Table 1
Selected *Rosa canina* Greek native genotypes sampled from various sites of Mt Kroussia and other regions in northern Greece assigned with different IPEN (International Plant Exchange Network) accession numbers.

IPEN accession number	Greek prefecture	Area	References
GR-1-BBGK-21,261	Central Macedonia	Mt Kroussia	This study
GR-1-BBGK-21,262	Central Macedonia	Mt Kroussia	This study
GR-1-BBGK-21,263	Central Macedonia	Mt Kroussia	This study
GR-1-BBGK-21,264	Central Macedonia	Mt Kroussia	This study
GR-1-BBGK-21,265	Central Macedonia	Mt Kroussia	This study
GR-1-BBGK-21,266	Central Macedonia	Mt Kroussia	This study
GR-1-BBGK-21,267	Central Macedonia	Mt Kroussia	This study
GR-1-BBGK-19,635	Central Macedonia	Mt Kroussia	This study
GR-1-BBGK-14,191	Central Macedonia	Mt Kroussia	This study
GR-1-BBGK-19,674	Western Macedonia	Ziaka	Maloupa et al. (2021)
GR-1-BBGK-03,2229	Epirus	Ioannina	Maloupa et al. (2021)
GR-1-BBGK-19,193	Epirus	Mt Xirovouni	Maloupa et al. (2021)

Genetic characterization using ISSR, SCoT and EBAP molecular markers

DNA was isolated from young leaves using the NucleoSpin® Plant II Kit (Macherey-Nagel, Nordrhein-Westfalen, Germany) according to the manufacturer's instructions. DNA concentration and quality were estimated spectrophotometrically at 260 and 280 nm using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany). The integrity of the DNA was determined using gel electrophoresis on a 0.8% (w/v) agarose gel.

After preliminary screening of 50 ISSR (University of British Columbia—UBC), ScoT primers (Collard and Mackill 2009) and EBAP (Xiong et al. 2022), 25 unambiguously scorable and reproducible markers were selected (7 ISSRs, 9 SCoTs and 7 EBAPs) based on percentage of polymorphism. Oligonucleotide primers complementary to simple sequence repeats (UBC807, UBC810, UBC811, UBC834, UBC835, UBC840 and UBC841) and others complementary to codon targeted polymorphisms (SCoT1, SCoT13, SCoT14, SCoT15, SCoT30, SCoT33, SCoT34, SCoT61 and SCoT66) and exon targeted polymorphisms (EBAP2, EBAP3, EBAP4, EBAP6, EBAP8, EBAP13 and EBAP21) were used for PCR amplification. The reaction mixture (total volume 25 µL) contained the following reagents: 0.5 µL dNTPs (10 mM), 2.5 µL 10×-buffer, 1 µL primer (10 mM), 0.5 µL template DNA, 0.1 µL Taq polymerase (5 U/µL) and 20.3 µL sterile dd H_2O. PCR amplifications were carried out according to Kadoglidou et al. (2023) using an Eppendorf Mastercycler EP Thermal Cycler Range (Eppendorf, Hamburg, Germany): DNA denaturation was initiated at 95°C for 5 min, followed by 35 cycles at 95°C of 30 s, for DNA annealing T at 48–56°C (all at 52°C except for UBC811 at 48°C), for 90 s for annealing of the primers and at 72°C for 90 s for chain extension. The temperature was held at 72°C for 5 min after the 35 cycles were completed.

The amplification products of the ISSR, SCoT and EBAP markers were separated by electrophoresis on a 1.5% (w/v) agarose gel and stained with ethidium bromide. The size marker was a 2-log DNA ladder (New England Biolabs, Ipswich, MA, USA). Gels were exposed to UV light in a UVItec Transilluminator (UVItec Limited, Cambridge, UK), and UVIDoc software UVIDocMw version 99.04 (UVItec Limited, Cambridge, UK) was used for analysis.

ISSRs, SCoTs and EBAPs alleles were scored based on whether specific fragments were present (1) or absent (0). The phenotypic data matrices for genetic (ISSR + SCoT + EBAP) information were also generated. All matrices were subsequently analyzed identically. Nei's coefficient (Lynch and Milligan 1994) was determined to assess the genetic variance within the groups of genotypes, while Nei's formula was used to determine genetic distance (Nei 1978). PCA was used as a graphical representation of a matrix to show how closely related the genotypes were. Analyses were carried out using GenAlex 6.5 (Peakall and Smouse 2012) and Microsoft® Excel 2010/XLSTAT©-Pro software (Version 2013.4.07; Addinsoft Inc., Brooklyn, NY, USA).

The Dice similarity coefficient (Dice, 1945) as implemented in the ade4 1.7–15 R package (Dray and Dufour, 2007) was used along with the UPGMA clustering algorithm to perform cluster analysis in R 4.0.2 (R Core Team 2020). The ape 5.4–1 package was used to compute bootstrap support

(1,000 bootstraps), and the phytools 0.7–47 package was used to display the resulting dendrograms (Revell 2012). The 'admixture' and 'independent allele frequencies' models were used to run STRUCTURE 2.3.4 (Pritchard, Stephens and Donnelly 2000). With a burn-in of 500,000 iterations and 1,000,000 MCMC repetitions for each run, 20 replicates from K = 1 to K = 6 were used. K was inferred using Evanno's method (Evanno Regnaut and Goudet 2005), which was run in the pophelper 2.3.0 R package (Francis 2017). The software Structure threader (Pina-Martins et al. 2017) was used to parallelize distinct runs of K.

Results

The utilization of ISSR, SCoT and EBAP markers in the genetic analysis of the germplasm collection of *R. canina* provided significant insights into the genetic diversity of the Greek populations maintained *ex-situ*. Regarding the ISSR markers, a comprehensive analysis of 99 genetic loci was conducted on a sample size of 12 individuals (one from each wild-growing population), yielding a substantial dataset suitable for evaluating their genetic diversity. The average number of distinct alleles (Na) in the *R. canina* germplasm collection was found to be 1.818, whereas the average number of effective alleles (Ne) was 1.524 (Table 2). The Shannon's Information Index (I) was found to be 0.448, suggesting a modest degree of genetic variation. The diversity (h) and unbiased diversity (uh) were determined to be 0.302 and 0.330, respectively, highlighting the comprehensive genetic abundance throughout the germplasm collection. The latter exhibited significant number of polymorphic loci, reaching 81.82%, which highlights the extant genetic diversity. The binary data exhibited a wide range of band patterns, with a total of 99 bands recorded, each with a frequency of 5% or higher. Significantly, no bands that were often found in 25% or 50% or less of the populations were detected, thus suggesting a lack of widespread prevalence in band frequencies.

Table 2
Genetic diversity statistics of *Rosa canina* Greek native germplasm collection based on ISSR, SCoT and EBAP markers.

Group		ISSR					SCOT					EBAP				
		N	*ne*	*I*	*uh*	*P (%)*	*N*	*ne*	*I*	*uh*	*P (%)*	*N*	*ne*	*I*	*uh*	*P (%)*
Rosa canina	**Mean**	12	1.524	0.448	0.33	81.82%	12	1.422	0.381	0.275	74.56%	12	1.537	0.457	0.342	82.11%
	SE		0.036	0.025	0.02			0.033	0.024	0.019			0.037	0.025	0.02	

N: sample size, I: Shannon's Information Index, uh: Nei's unbiased haploid gene diversity, P(%): Percent of polymorphism.

Accordingly, a comprehensive analysis using SCoT markers was conducted on 114 loci across 12 population samples consisting of the germplasm collection. The average number of distinct alleles (Na) was 1.746, whereas the average number of effective alleles (Ne) was 1.422 (Table 2). The Shannon's Information Index (I) was found to be 0.381, suggesting a moderate amount of genetic variation. The diversity (h) and unbiased diversity (uh) were determined to be 0.252 and 0.275, respectively. The population exhibited a significant proportion of polymorphic loci, amounting to 74.56%. This emphasizes the presence of genetic diversity within the germplasm collection. The data exhibited significant variation, with a total of 114 bands recorded, all having a frequency of 5% or above. Significantly, no bands that were often found in 25% or 50% or less of the populations were identified, hence suggesting a lack of widespread prevalence in band frequencies.

As for the EBAP markers, a total of 95 loci across 12 population samples were observed. The average number of distinct alleles (Na) was found to be 1.821, whereas the average number of effective alleles (Ne) was 1.537 (Table 2). The Shannon's Information Index (I) was assessed to be 0.457, suggesting a moderate to high amount of genetic variation. The diversity (h) and unbiased diversity (uh) were 0.309 and 0.342, respectively. The population revealed a significant proportion of polymorphic loci, reaching 82.11%, highlighting the presence of genetic diversity within the germplasm collection, like other molecular markers. In total, 95 bands were recorded, each with a frequency of 5% or above. Significantly, no bands that were prevalent in 25% or less, or 50% or less, of the populations were observed, suggesting a lack of general uniformity in band frequencies.

Additionally, UPGMA dendrograms based on Dice distance were constructed for each marker (Fig. 2). The ISSR data revealed the presence of two major clusters contributing to the observed grouping. The initial cluster comprised genotypes 21,261 to 21,266 (last five digits of IPEN codes), with the remaining accessions being grouped together. The SCoT markers resulted in the formation of three major clusters. The initial cluster included genotypes 21,261, 21,262, 21,264, and 3,2229 (last five digits of IPEN codes). The second cluster comprised the genotypes 21,263, 21,265, 21,266, and 21,267, while the final cluster encompassed the genotypes 19,674, 19,635, 14,191, and 19,193 (last five digits of IPEN codes). Concerning EBAP markers, three primary clades were once again identified. The first one, comprised of the 21,261, 21,262, 21,263 and 21,264, the second one consisted of the 19,635, 19,193, 19,674 and 14,191 genotypes, while the third one included the 21,265, 21,266, 21,267 and 3,2229 genotypes (last five digits of IPEN codes). These findings suggested marker-specific variations in the genetic structure of the studied *R.* germplasm collection. The consistent clustering patterns across markers implied reliability in the observed genetic grouping, and the distinct composition of genotypes within each cluster indicated potential genotypic associations and underlying genetic relationships.

The genetic structure was further elucidated by Principal Coordinates Analysis (PCA) using a covariance matrix and data normalization (Fig. 2). The initial two axes for ISSR, SCoT and EBAP markers accounted for a combined variance of 45.1%, 37.3% and 70.4%, respectively. The samples were evenly distributed over all three markers, showing a consistent pattern without any noticeable segregation. In the context of ISSR markers, a predominant concentration of samples was observed within the upper two quartiles, indicating a potential clustering tendency in the upper range. On the other hand, when considering SCoT and EBAP, a significant grouping of samples was depicted in the rightmost quartiles, indicating a tendency

towards higher values in these specific markers. This observation implied a potential correlation between the molecular markers utilized and the distribution pattern of the studied plant material.

To gain a deeper insight into the genetic composition of the ex-situ maintained *R. canina* germplasm gene pool (a snapshot of the species' Greek native gene pool), STRUCTURE analysis was employed (Fig. 2). The determination of the optimal number of genetic clusters (K) was based on the ΔK statistic of Evanno (Evanno et al. 2005)d = 3 was identified as the most fitting value across all molecular markers, including ISSR, SCoT, and EBAP. The latter suggested a consistent and robust genetic structuring pattern within the ex-situ maintained germplasm collection, indicating the representativeness of three distinct genetic subpopulations. The selection of K = 3 for all markers highlighted the consensus in determining the genetic composition of the ex-situ maintained germplasm and suggested that the *R. canina* collection can be effectively classified into three genetically uniform sections. This systematic method not only improved our understanding of genetic linkages throughout the germplasm collection but also offered useful insights for future breeding programs and conservation initiatives that focus on specific genetic clusters.

Discussion

The genetic diversity analysis of the *R. canina* germplasm collection using ISSR, SCoT, and EBAP markers has demonstrated substantial genetic variation within the species. This was consistent with findings from other studies using similar markers in *R. canina* and other species. For instance, Jamali et al. (2019) reported that ISSR markers can effectively separate *R. canina* genotypes based on geographic regions, which is crucial for genetic preservation and breeding purposes (Jamali et al. 2019). Similarly, the high genetic diversity observed in our study aligned with findings from Joshi et al. (2021) who has emphasized the potential of ISSR markers in the improvement of the ornamental and perfume industries in roses (Joshi et al. 2021). The SCoT markers have been shown to be informative in evaluating genetic diversity and relationships in various species. Previous studies have underlined that SCoT markers were reliable in assessing genetic relationships among dill genotypes (Kadoglidou et al. 2023). This was also true for the herein study, where SCoT markers contributed significantly to understanding the genetic makeup of *R. canina* germplasm collection. Xanthopoulou et al. (2015) have also found that SCoT markers were more informative and useful for identifying and analyzing summer squash genetic diversity compared to ISSR markers.

Furthermore, the consistent clustering patterns across the three markers (ISSR, SCoT, and EBAP) indicated robust and reliable genetic grouping within the *R. canina* germplasm collection. These findings have significant implications for sustainable exploitation and future breeding programs. The identification of distinct genetic clusters within the studied germplasm collection represents a valuable resource for breeding programs aimed at enhancing specific traits in *R. canina*.

Declarations

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Conflict of interest

The authors declare no conflict of interest.

Author Contribution

Conceptualization, Aliki Xanthopoulou, Ioannis Ganopoulos; methodology, Anastasia Boutsika, Ifigeneia Mellidou; investigation, Nikos Krigas, Katerina Papanastasi, Eleni Maloupa, Katerina Grigoriadou; data analysis, Anastasia Boutsika; writing—original draft preparation, Aliki Xanthopoulou; writing—review and editing, Anastasia Boutsika, Ifigeneia Mellidou, Nikos Krigas, Katerina Grigoriadou, Ioannis Ganopoulos, Aliki Xanthopoulou. All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1

Ex-situ maintained *Rosa canina* germplasm originating from wild-growing Greek native populations employed in this study.

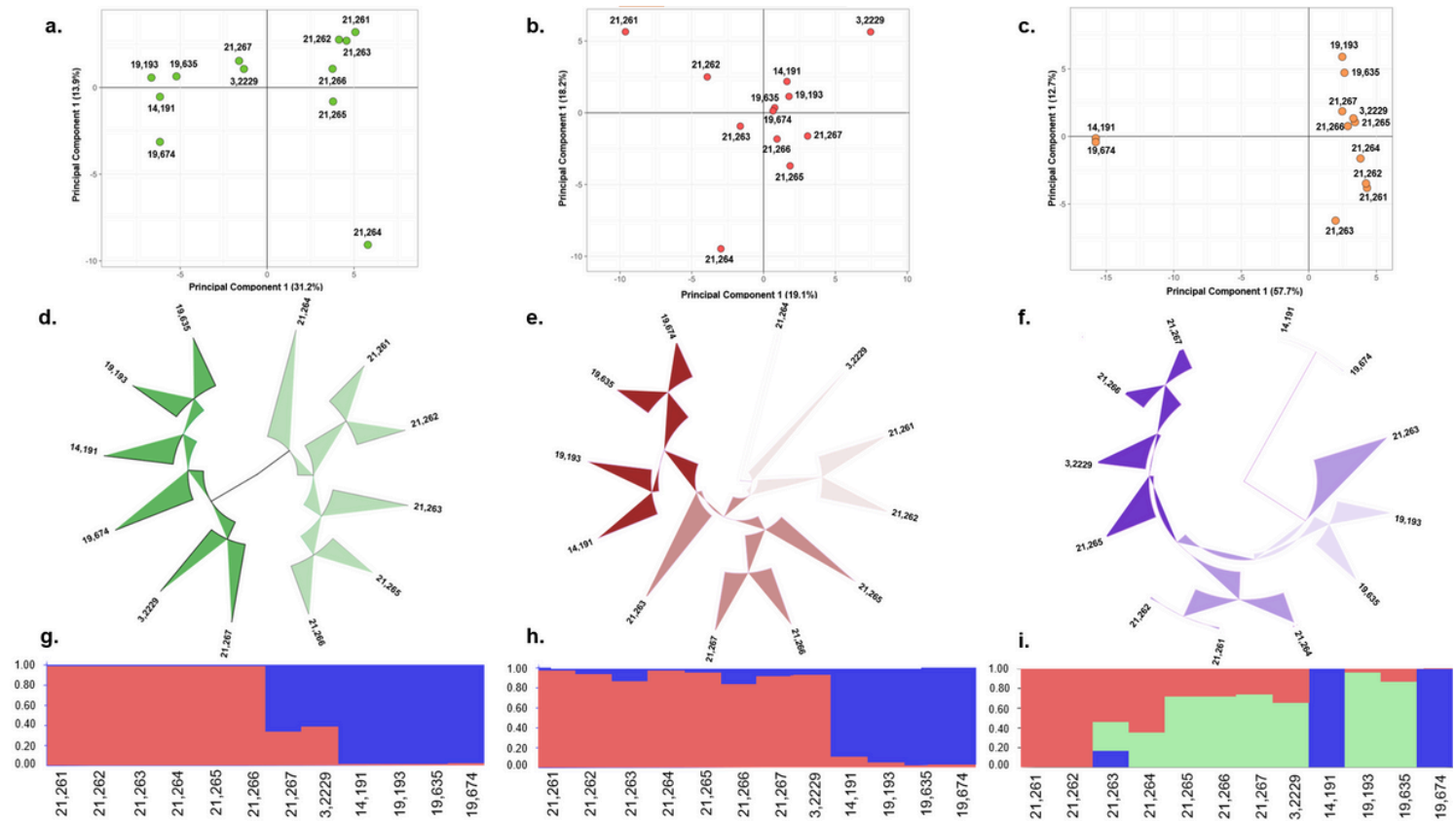

Figure 2

Cluster analysis and Bayesian inference of genetic differentiation between ex-situ maintained accessions of wild-growing Rosa canina(shown with last five digits of their IPEN codes). The results are presented for ISSR, SCoT and EBAP analysis from the left to the right. **a. b. c.** Two-D PCoA plot of the first two components for the 12 R. canina genotypes, **d. e. f.** UPGMA dendrograms using Dice distance, **g. h. i.** Genetic assignment based on STRUCTURE using the Values of Evanno's ΔK statistic for the most probable genetic structure model.