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Original Article

Identification of *Rubus* accessions in Romania and a comparison of their relatedness to European and North American cultivars

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Abstract

This study has arisen from the recent consumer interest in soft fruit crops, particularly *Rubus*, in Romania where currently demand has to be served by collecting the berries from wild populations. This study examined the species make up in two important regions by collecting a number of *Rubus* accessions from the wild flora and determined the species classification by morphological identification. From the 100 individuals, collected from 7 different locations within the two regions, 11 different species were identified. SSR markers were then applied to compare the genotypes represented by the Romanian accessions to commercial European and North American cultivars. Amongst the plants sampled two groupings were apparent, group A mainly consisting of Romanian species, and group B mainly the European and North American cultivars of the *Idaeobatus* genus.

Keywords: *Rubus*, accessions, SSR markers, genotypes, cultivars

1. Introduction

In the last few years there has been a radical change by consumers around the concept of nutrition and healthy eating in Romania. This has led to a growing demand for certain fruits, such as raspberries, blackberries, black currants and blueberries. With this renewed consumer interest in fruit and the promotion of the health and lifestyle benefits of small fruit consumption, an increased supply is essential as currently the demand is only partially served by collecting berries from the wild.

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According to the FAO, in Romania around two thousand tons annually of raspberries are harvested from wild flora. Few commercial growing operations currently exist.

Commercial raspberry operations decreased dramatically from 2007. This was due to the economic crisis coupled with un-favourable weather conditions. Previously raspberry was an important berry crops cultivated in Romania, but by 2008, the cultivated the area was restricted to only 4 ha according to FAOSTAT data (http://faostat3.fao.org).

In the last four years, the cultivated areas of raspberry and blackberry has begun to grow again due to the support provided by the European Community through the European Funds projects for farmers who are willing to set up "start-up farms".

Given the desire for raspberry and the popularity of wild raspberry fruit, this study

represents an evaluation of the *Rubus* material currently available in Romania and a morphological and molecular approach to understanding the diversity of, and classification of Romanian *Rubus* accessions and a comparison with other raspberry material cultivated around the world.

Rubus is one of the most diverse genera in the category of flowering plants. Taxonomic classification of the genus is a challenge due to difficulties caused by agamospermy, polyploidy and hybridization between species and also the lack of a widely accepted concept for classifying species in this genus. The number of species vary [24, 15, 11, 29, 4, 13]. In terms of classification into subgenera most authors agree with its division into 12 subgenera [13, 4].

Those containing the most numerous species are subgenus *Idaeobatus* (117 species of raspberry), *Malachobatus* (115 species, especially Asian species) and subgenus Rubus (or *Eubatus* Focke; about 132 species of blackberries).

Only three of the nine remaining subgenera include a larger number of species: subgenus *Cylactis* (14 species); *Lampobatus* subgenus (10 species) and subgenus *Orobatus* (19 species) [14]. *Rubus* displays an extraordinary morphological diversity including woody species, erect, semi-herbaceous species with creeping (crawling) stems (strains), and climbing species whose foliar limb is long and slender (thin) adapted to this feature [31].

The success of hybridization often cannot be predicted based only on the currently established taxonomic relationships [29]. The most relevant in terms of commercial importance is subgenus *Idaeobatus* (includes species of red raspberry), which is particularly well represented in the northern hemisphere and in other continents. This subgenus comprises about 200 species [13], which shows a significant differentiation.

Among these species, the European raspberry species (*R.* idaeus ssp. *Vulgatus Arrhen.*) and the North American raspberry (*R. idaeus* ssp. *Strigosus Mich*x.) are the most important in terms of economic value.

In Romania, morphological studies of the *Rubus* genus were first made by Nyarady, in 1956. Determining the taxonomic relationships among species, series and subgenres, has traditionally been based on morphological and phenotypic characters resulting in many species within this genus being incorrectly classified [21].

In the last 30 years, the development of molecular biology techniques based on DNA markers has led to new methods for identifying raspberry and blackberry cultivars, which in turn has led to a better understanding of the relationships between *Rubus* genus species [1].

SSR markers have been widely used for assessing the germplasm, genetic mapping and diagnosis of genetic diseases for *Rubus* genus [22, 30, 3, 28, 8, 26, 27, 34, 7, 33, 17, 19, 6, 32, 5]

This study set out to identify *Rubus* material available in Romania and classify based on a morphological and molecular approaches and compare with other raspberry material cultivated around the world as a first step in re-establishing a commercial industry that meets consumer expectations in Romania and is compatible with climatic and other factors.

2. Material and Method

Sample collection

Sampling of *Rubus* species was a challenging process which required the location of populations of *Rubus* to be identified based on Illustrated Flora of Romania [2].

Seven different locations were identified for sampling (Table 1) in Transylvania (counties: Cluj-Napoca, Alba-Iulia and Sibiu) and Muntenia (Pitesti) regions.

Cultivar 'Wilson' was collected from the research institute at Mărăcineni – Piteşti, little is known about the origin of this accession.

The European and North American cultivars were supplied by James Hutton Institute (Table 1). Biological material was collected during the period of July-October for both morphological and molecular analysis as follows: from each accession both a fertile shoots of the plant (leaves, flowers and fruit) and an infertile shoot were collected.

Each collected plant (sample) was included within a herbarium dossier for morphological analysis and individual identification. Every dossier contains the sampling location and an identification number.

For molecular analysis, from each individual plant a number of young leaves (from the top of the shoots) being on the optimal growing period were collected and stored at - 80 ^{0}C [25].

Table 1. *Rubus* species and cultivar analysed

No.	Species/Cultivars	Collecting area		Source
		Place (locality)	County	
1.	*R. idaeus*	Făget Forestry	Cluj-Napoca	Romania
		Mediaş „ După Stejari" Area	Sibiu	Romania
2.	*R. caesius*	Făget Forestry	Cluj-Napoca	Romania
		Mediaş „ După Stejari" Area	Sibiu	Romania
3.	*R. plicatus*	Făget Forestry	Cluj-Napoca	Romania
		Dângău	Cluj-Napoca	Romania
4.	*R. plicatus ssp. opacus*	Arieş Valley	Alba-Iulia	Romania
5.	*R. hirtus*	Arieş Valley	Alba-Iulia	Romania
		Dângău	Cluj-Napoca	Romania
6.	*R. discolor*	UASVM orchard	Cluj-Napoca	Romania
7.	*R. sulcatus*	Mediaş „ După Stejari" Area	Sibiu	Romania
8.	*R. saxatilis*	The Natural Reservation Scăriţa Belioara	Alba-Iulia	Romania
9.	*R. suberectus*	Mediaş „ După Stejari" Area	Sibiu	Romania
10.	*R. phoenicolasius*	Research Institute for Fruit Growing Mărăcineni - Piteşti		Romania
11.	*R. occidentalis*	Research Institute for Fruit Growing Mărăcineni - Piteşti		Romania
12.	*R. spectabilis*	JH Institute, Dundee, Scotland		Scotland
13.	Wilson	Research Institute for Fruit Growing Mărăcineni - Piteşti		Romania
14.	Latham	JH Institute, Dundee		Scotland
15.	Glen Moy	JH Institute, Dundee		Scotland
16.	Glen Coe	JH Institute, Dundee		Scotland
17.	Glen Garry	JH Institute, Dundee		Scotland
18.	Glen Lyon	JH Institute, Dundee		Scotland
19.	Glen Rosa	JH Institute, Dundee		Scotland
20.	Glen Prosen	JH Institute, Dundee		Scotland
21.	Glen Ample	JH Institute, Dundee		Scotland
22.	Cuthbert	JH Institute, Dundee		Scotland
23.	Malling Jewel	JH Institute, Dundee		Scotland
24.	Willamette	JH Institute, Dundee		Scotland

DNA extraction technique

From each species/cultivars, 1 g leaf material was ground in liquid nitrogen. Hexadecyltrimethylammoniumbromide (CTAB) solution (5 mls) with a spatula of insoluble polyvinylpyrrolidone (PVP) was added and incubated at 65^0C for 30 min prior to the addition of 7.5 ml chloroform/isoamyl alcohol (24:1). The mixture was agitated for 15 min followed by a spin of 15 min at 4000 rpm. The aqueous layer was filtered through sterile muslin, and an equal volume of ice cold propan-2-ol was added, mixed and incubated at room temperature for 15 min to precipitate the DNA. The DNA was pelleted by spinning at 4000 rpm for 15 min and resuspended in 1 ml SDW (sterile distilled water). Rnase 5-10 µl was added to the DNA, which was then incubated at 37 ^{0}C for 1 h, and stored at -20^0C until required.

DNA amplification (SSR)

The extracted DNA was diluted 20 µl DNA: 80 µl SDW. PCR reactions were carried out in 25 µl volumes containing per reaction, genomic DNA 10 µl, 2.5 µl dNTP solution, 1 µl primer forward +1 µl primer reverse, 2.5 µl of Taq buffer and 0.2 µl Taq DNA polymeras, 7.8 µl sterile distilled water. PCR reactions were carried out in a GeneAmp PCR System 9700 (Applied Biosystems) master cycler under the following programme conditions: 95°C for 5 min (1 hold), 35 cycles at 94°C for 1 min, 54°C for 1 min, 72°C for 1 min and 72°C for 8 min (2 holds).

Simple sequence repeat markers previously developed from genomic DNA of Glen Moy raspberry cultivar [8,9,10]; (Graham personal communication) were fluorescently labelled on the forward primer with HEX (yellow), FAM (blue) and TET (green) for germplasm genotyping and were prepared according to [16] for analysis on the ABI Prism 377.

The amplification products were subjected to a denaturation process at 95°C for 5 min before polyacrylamide gel run performed in a capillary sequencer ABI 3730 DNA.

Allele sizing and visualization was performed using Genescan software program (Applied Biosystems).

Data Analysis

A similarity matrix was calculated using the method of [18] and [23] using Genstat 14 statistical package and the data presented as a minimum spanning tree.

3. Results and discussion

Classification of accessions

100 *Rubus* plants from wild flora were collected from seven locations of the Transylvania and Muntenia regions and then morphologically identified to determine the number of *Rubus* species. From the 100 plants, 11 species were identified by morphological classification.

This was lower than expected probably due to the fact that *Rubus* shows great plasticity to environmental conditions so that even if they were harvested from different locations of Romania (who seemed distinct at first sight), after a detailed study of their morphology they were found to be just an adaptation of the same species to different environmental conditions, specific to each habitat.

In Transilvania region, samples were collected from three counties: Cluj, Alba-Iulia and Sibiu. In Cluj-Napoca, of the 3 locations (Făget Forestry, Dângău locality and UASMV orchard) 5 species were identified in total (Figure 1).

From Făget Forestry 3 species were identified (*R. idaeus, R.caesius, R. plicatus)*, from Dângău locality 2 species were identified (*R. plicatus, R. hirtus*) and from UASVM orchard, there was 1 species (*R. discolor).*

In Sibiu – Mediaş – "După Stejari" area 4 species were identified (*R. idaeus, R.caesius, R. sulcatus* and *R. suberectus).*

In Alba-Iulia, 2 locations were sampled: Arieş Valley and The Natural Reservation "Scărişa Belioara".

In Arieş Valley 2 species were identified (*R. plicatus ssp. opacus, R. hirtus) and in* The Natural Reservation "Scărişa Belioara" 1 species (*R. saxatilis)* was identified. In Muntenia (Greater Wallachia) region, the samples were collected from the Research Institute for Fruit Growing Mărăcineni – Piteşti. Here 2 species of were identified (*R. phoenicolasius, R. occidentalis*).

The Romanian species were subsequently genotyped with SSR markers alongside 12 European and North American cultivars as well as 'Wilson' a cultivar in Romania of which little is known.

All SSR primers used in the study revealed amplification products in all studied accessions.

Most accessions had one or two alleles for each primer, though some polyploids were identified *R. discolor*, *R. spectabilis* and an incorrectly classified accession named as Glen Coe which had 4 alleles for 2 of the primers tested.

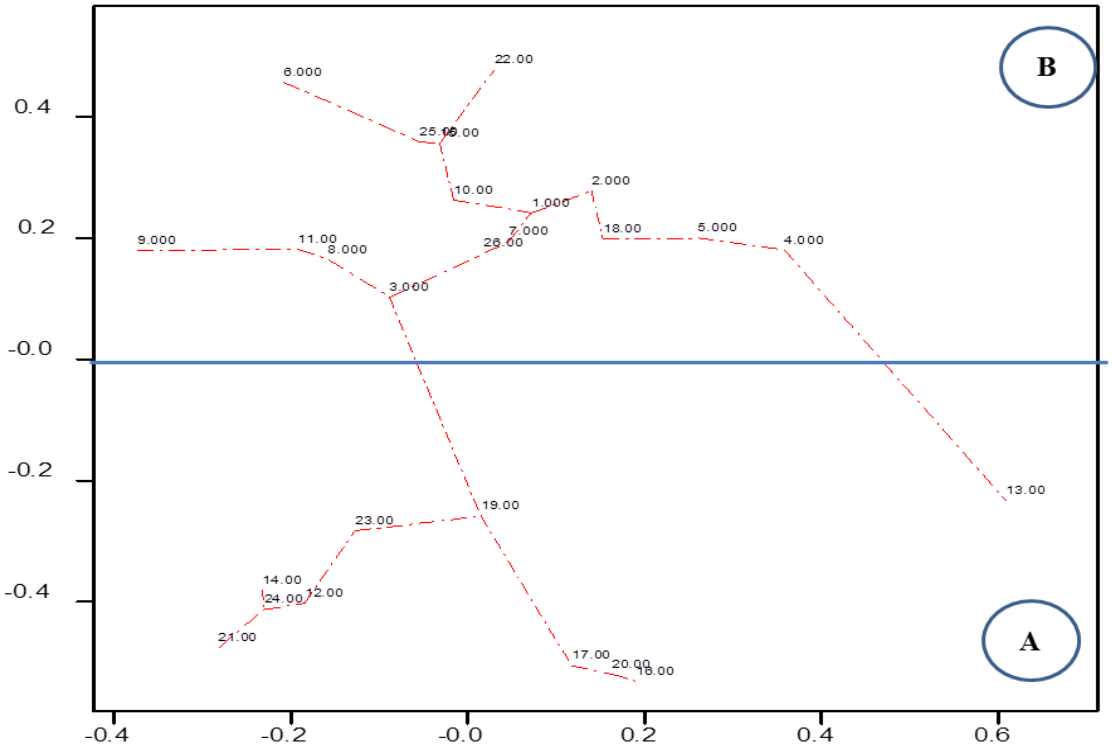
Figure 1. Eleven species identified across two Romanian regions (Transylvania & Muntenia) with accession number identified

From the allele identified a similarity index (Table 2) was estimated and the minimum spanning tree (Fig. 2) generated using the statistical package Genstat 14. The genetic distance between/among species and cultivars is illustrated with the size of the dotted line segments joining the minimum spanning tree components. The minimum spanning tree grouped the germplasm represented by Romanian *Rubus* species and European and North American *Rubus* cultivars mainly into two separated groups. The lower grouping (A) includes species of *Rubus*, *Idaeobatus* and *Cylacti*s subgenus. The species represented in this group *include R. caesius* (12*)*, *R. sulcatus* (24), *R. suberectus* (23), *R. hirtus* (14), *R. plicatus* (17) and *R.discolor* (13). The species of *Idaeobatus* subgenus are represented by: *R. occidentalis* (16) and two accessions of *R. phoenicolasius* (19 & 20) species. Also in this group is included *R. saxatilis* (21) belonging to *Cylacti*s subgenus (Figure 2).

The molecular characterisation of the Romanian species groups most of these species, in group A, although *R. discolor* (13) is located at a higher genetic distance from the group to which it belongs, and *R. plicatus* (17) is genetically closer to the group of *Idaeobatus* subgenus species. According to the tree (Figure 2) *R. plicatus* ssp. *opacus* (18) represents the exception of *Rubus* subgenus species group to which actually belongs and is genetically assigned into European and North - American cultivars group, which belong to subgenus *Idaeobatus*. Group A also include *R. occidentalis* (16) and *R. phoenicolasius* (19 and 20) belonging to the *Idaeobatus* subgenus and in this case it was expected to join the *R. spectabilis* (22) which grouped within the raspberry cultivars as belonging to the same subgenus. These species are genetically grouped together, which corresponds to the morphological classification and it can be seen that they are spaced apart from the majority group of *Rubus* subgenus species except the *R. plicatus* (17) species, which are closer in terms of genetic distance. However their ideal grouping, given the fact that they belong to the *Idaeobatus* subgenus, would have been within the second major group of accessions (B) that subscribing to *Idaeobatus* subgenus. *R. saxatilis* (21) species is the only representative of the *Cylactis* subgenera and it is genetic assigned at a longer distance from the species of the *Idaeobatus* subgenus than the species of *Rubus* subgenus.

Table 2. Estimated similarity of Rubus species and the European and North - American Rubus cultivars

No.	Accessions	Estimates of similarity	
1	Cuthbert	-0.1875	0.0814
2	Glen Ample	-0.2118	0.1341
3	Glen Coe 1	-0.1002	-0.0401
4	Glen Coe 2	-0.1587	0.3101
5	Glen Garry	-0.1679	0.2322
6	Glen Lyon	-0.3094	-0.1474
7	Glen Moy	-0.1593	0.0634
8	Glen Prosen	-0.1355	-0.097
9	Glen Rosa	-0.1394	-0.2685
10	Latham	-0.198	0.0115
11	Malling Jewel	-0.1449	-0.1247
12	*R. caesius*	0.2043	-0.0982
13	*R.discolor*	0.0821	0.5223
14	*R. hirtus*	0.1924	-0.1366
15	*R. idaeus*	-0.2535	-0.0041
16	*R.occidentalis*	0.2713	0.2018
17	*R. plicatus*	46 9	0.1435
18	*R. plicatus ssp.opacus*	-0.1638	0.1472
19	*R. phoenicolasius 1*	0.1129	0.0539
20	*R. phoenicolasius 2*	0.2655	0.18
21	*R. saxatilis*	0.2512	-0.1732
22	*R. spectabilis*	-0.3279	0.0402
23	*R. suberectus*	0.1313	-0.0575
24	*R.sulcatus*	0.2119	-0.1342
25	Willamette	-0.2545	-0.0222
26	Wilson	-0.1468	0.0415



Legend: 1. Cuthbery 2, Glen Ample, 3. Glen Coe 1, 4. Glen Coe 2, 5. Glen Garry, 6. Glen Lyon, 7. Glen Moy, 8. Glen Prosen, 9. Glen Rosa, 10. Latham, 11. Malling Jewel, 12. *R. caesius*, 13. *R discolor*, 14. *R. hirtus*, 15. *R. idaeus* 16. *R.occidentalis* 17. *R. plicatus* 18. *R. p. ssp.opacus* 19. *R. phoen. 1*, 20. *R. phoen. 2*, 21. *R. saxatilis*, 22. *Rspectabilis*, 23. *R. suberectus*, 24. *R.sulcatus* 25. Willamette 26. Wilson

Figure 2. Minimum spanning tree depicting Romanian *Rubus* species and European and North-American *Rubus* cultivars

The second group (B) within the minimum spanning tree consists of: European and North American cultivars, *R. idaeus* (15) and *R. spectabilis* (22), which are part of the *Idaeobatus* subgenus. The exception is the *R. plicatus ssp. opacus* (18), which is classified within *Rubus* subgenus. This genetic classification is ideal because the cultivars that were studied are raspberry cultivars with their genetic background including *R. idaeus* therefore their ranking alongside Romanian *R. idaeus* (15) and *R. spectabilis* corresponds to morphological classification. The reason why the genetic analysis places *R. plicatus ssp. opacus* (18) species of *Rubus* subgenus along with other species and cultivars pertaining to *Idaeobatus* subgenera remains is unexpected and may be due to miss classification..

This study included a cultivar (Wilson code 26) from the Research Institute for Fruit Growing Mărăcineni – Piteşti, whose origin was not known at the time of collecting. This grouped with the European and North American cultivars in group B (26), therefore it can be classified in the same *Idaeobatus* subgenus with them and that is a raspberry cultivar. *Rubus spectabilis* (22) is also genetically assigned in this group alongside raspberry cultivars, which means that is the most similar to them. Therefore this grouping can be considered as correct because both the raspberry species and cultivars belong to the same *Idaeobatus* subgenus thus confirming their morphological characterization.

4. Conclusions

This study has given an insight into the *Rubus* material available in Romania. Most of the fruit consumed is from wild flora and therefore distinct from commercially available material in Europe and the US. Given the interest in raspberry and other *Rubus* species due to consumer perceived health benefits there is a desire to re-establish a commercial Romanian soft fruit industry, rather than collecting from the wild. However the fact that 'Wilson' a cultivar of unknown origin grown in Romania grouped with the European and North American cultivars suggests these fruits would be acceptable to Romanian consumers.

This study highlighted the need to develop material possibly as hybrids between the wild material and cultivars like Wilson which are available and suitable for growing conditions in Romania

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