Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 5, No. 1, p. 189-198, 2014
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Intra-specific variations of *Rubus* sp. (Rosaceae) in Northern Iran: morphometric analysis and microsatellite markers

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Article published on July 08, 2014

Key words: Iran, morphology, polyploidy, *Rubus*, simple sequence repeat marker.

Abstract

During the growing season, young raspberry leaf samples were collected from natural populations in northern Iran. A total of 65 *Rubus* samples were collected at altitudes ranging from 0 to 1021 meter above sea level. Morphometric and molecular studies were performed in seven *Rubus* species and four uncertain species from the sect. *Rubus* L. 7 species including *R. caecius*, *R. sanctus*, *R, discolor*, *R. dolichocarpus*, *R. hyrcanus*, *R. persicus* and *R. hirtus* are. In total, the delimitation of species of *Rubus* northern Iran, from 27 morphological characters and 13 SSR primer pairs were used. The dendrogram based on quantitative morphological traits, species are completely separated, and even species were differentiated on the basis of geographical pattern. A total of 12 loci and 29 alleles were detected. The number of alleles observed for each locus ranged from 1 to 6, with an average of 3.08 alleles per locus. The SSRs achieved cumulative polymorphism information content (PIC) of 0.9444. These PIC values demonstrated that, in spite of the low number of primers used, the SSR were sufficiently polymorphic and informative. Expected heterozygosities (He) were 0.021 to 0.286. The dendrogram based on microsatellite markers, no clear geographic pattern to interpret the results, we do not see. Status is unknown species and genotypes with high affinity and proximity to each other show. In general, the limits of species, the occurrence of hybridization between species with different ploidy levels between *Rubus* genotypes, is not subject to any specific geographic pattern.

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Introduction

Rubus belonging to the subfamily Rosoideae, including plants shrubs or rarely herbaceous perennial of the family Rosaceae (rose) is. Spiny stems, branches flowering stems, usually on long wooden occur prior years. Cluster compound inflorescences, flowers have five sepals and lateral sepals are absent. 5 pcs of petals, red, pink or white are. Stamens are too wor of them. Compound fruit, a few carpel, carpel and juicy meat. Seed in each carpel a numbe. *Rubus* global distribution of tropical lowlands and low altitudes, tropical upland (generally above 800 meters above sea level) is found to subartic areas (Thompson 1995). Growing in all climates except Antarctica climate (Focke 1910_a, 1911_b, 1914_c). Overall, *Rubus*, a range of latitude and altitude (0 to 4500 m above sea level) is to grow. *Rubus* can be an invasive weed in the primary succession can be seen in the forest (Thampson 1995; Hummer 1996; Howarth *et al*. 1997). The flowers on this plant are usually hermaphrodite (Nybom 1986; Richards 1986). Therefore the cross-fertilization can be seen in this genus (Antonius and Nybom 1995), its potential self-pollination and fertilization have (Nybom 1988). *Rubus* is the most complex known taxonomic groups. The complex taxonomic, a wide disagreements and contradictions in relation to number of species in this genus has been found. Boundaries of the species in the genus by the presence of biological processes such as polyploidy, apomixy and hybridization, complex and uncertain (Thompson 1995; Alice and Campbell 1999). Recent taxonomic perspective, *Rubus* contains about 429 species in 12 subgenera calls (Focke 1910a, 1914b).

The latest report on Iran are 8 species (Khatamsaz 1992). *R. saxatilis* L., a plant belonging to the Irano – Turanian region, this species is the only species in the subgenus *Cylactis* is a grass Raspberry Iran. Two species *R.caesius* L. and *R. sanctus* Schreber, belonging to the Caspian (or hyrcanian) and Irano – Turanian regions are. 5 species: *R. hirtus* Waldst. & Kit., *R. hyrcanus* Juz. , *R. dolichocarpus* Juz., *R. discolor* Weihe & Nees., and *R. persicus* Boiss. is owned by Caspian region. The past seven species in the subgenus *Rubus* are. Shoot structure and morphology of leaves of this plant are key characteristics. These two homoplasic features, the phylogenetic value are limited (Alice *et al*. 1999).

Genetic diversity among wild and cultivated *Rubus* species in Colombia using AFLP and SSR markers has been studied (Marulanda *et al*. 2007). DNA fingerprinting data using two PCR-based markers were determined for 51 samples of raspberry. Markers for genetic diversity by AFLP, a total of 229 bands were obtained with the three primer-enzyme combinations evaluated. The band size ranged between 30 to 330 base pairs and 91.6% polymorphism was obtained. Results from this study were consistent with those previously by Kollmanm *et al*. (2000) were reported. European *Rubus* species diversity using AFLP markers studied. kollmanm *et al*., argued that genetic variation in *Rubus* plants is determined by the replication system and proved that cross-pollination between polyploid species are found in raspberries. This type of cross-fertilized seeds and fruit quality are affected, in a manner that increases ploidy levels and proximity and taxonomic affinities. Hybridization in *Rubus* species are often close together and in some cases can be seen between the sexes. (Gustafsson 1942; Alice and Campell 1999). Assessment of genetic diversity of SSR markers, 20 loci was observed. SSR with high polymorphism. Polymorphic bands in *R. urticifolius* was high (16), *R. glaucus* with 14 bands, *R. robuscus*. 13, and continues *R. rosifolius* with 6 -band polymorphisms were introduced. Weight and number of bands observed was consistent with what Amsellem *et al*. (2001) study on Asian raspberry species was reported. SSR is also the possibility that different groups within *R. glaucus* (intraspecific diversity), as well the difference is between *Rubus* species (inter- species variation). Amsellem *et al*. (2000), 3-4 multiplication of individual alleles in *R. alceifolius* be seen, this observation confirmed the hypothesis that this species is a tetraploid. *R. robuscus*, *R. rosifolius* and *R. bogotensis* between 1-2 alleles generated, while *R. adenotrichos* 5 alleles can be reproduced. These results suggest that the *R. alceifolius* and *R.*

adenotrichos with higher ploidy levels than other species are listed. Based on morphological and molecular data presented by researchers in other species of raspberries, blackberries ploidy levels among genotypes could be quite different. Studies using SSR and AFLP markers, different ploidy levels and the high correlation between species can be confirmed. SSR markers, Polymorphism information content (PIC) showed that the rate for each locus between 0.273 to 0.499. In contrast, Ishiland Mecouch (2000), during his studies on rice using SSR markers, PIC amount equal to 0.267 to be reported. Cordeiro *et al.* (2000), PIC is a rate between 0.48 to 0.80 during the study were mentioned as sugarcane. Rates for the genus *Rubus* PIC proved that despite the low number of primers used during testing, SSR was highly polymorphic and informative. The results stated that SSR markers is a powerful tool to help *Rubus* species is classified (Amsellem *et al.* 2001). Each of the studied species are very special and unique banding patterns were produced that they can be separated from other species. SSR markers for the genus *Rubus* variance equal to 80.4 demonstrated, it was quite similar to what was reported by Saini *et al.* (2004) during a survey on rice societies, and a variance of 76.93 was reported. Amount of expected heterozygosity (He), and Amount of total heterozygosity (Ht), as a representative of the Amount of polymorphism was estimated. Amount of Ht 0.31286 were reported. Average He among populations of *R. robustus*, from 0.0000 to 0.3333 varied. This species compared with other species studied, showed the highest heterozygosity. *R. glaucus* (0.27863), *R. rosifolius* (0.2000) and *R. urticifolius* with heterozygosity 0.25333 showed similar values. Study on *Peuraria lobata* (Fabaceae), amount of expected heterozygosity between 0.290 to 0.213 showed (Pappert *et al.* 2000). Saini *et al.* (2004) stated that in order to perform a comprehensive molecular analysis, using a combination of marker systems is recommended. Two AFLP and SSR markers are complementary to each other, because of their composition, additional factors to explain the complex relationships between wild and cultivated *Rubus* species present in an area with a high genetic diversity provides. Currently approved and agreed to taxonomic species of *Rubus* plants is not known. Most taxonomists many different classification systems for this kind offer. Typical morphological studies as well as studies conducted phylogeny based on a limited number of cases, the boundaries between taxa failed and controversy in this area is still in place. The purpose of this study was to determine the Genetic variation and species limits in the genus *Rubus* northern Iran using morphological and molecular (Simple Sequence Repeat) markers.

Materials and methods

Plant material

During the growing season, young raspberry leaf samples were collected from natural populations in northern Iran. Samples from healthy plants that are free of any contamination by fungi and insects were collected and immediately freezing – 80° C moved. Work for morphological, herbarium specimens were prepared for each species. Agricultural Biotechnology Research Institute of the samples in the north of the country, Rasht, Iran exists. A total of 65 *Rubus* samples were collected at altitudes ranging from 0 to 1021 m above sea level (Tab. 1). The voucher specimens are deposited institute of agricultural biotechnology, Rasht, Iran.

Morphological analysis

Morphological studies were performed in seven *Rubus* species and four uncertain species from the section *Rubus* growing in northern Iran. In total, 27 morphological characters were used for morphometric, including quantitative and qualitative characters taken from published materials on *Rubus* and personal observation in the field. Quantitative morphological characters were randomly measured in at least 5 plants and the means were used in phonetic analysis. Quantitative characters were coded binary or multistate characters accordingly. Grouping of the species based on morphology characteristics was performed using neighbor-Joining (NJ) method, with

Darwin software (ver. 5.0.158). 10 quantitative traits studied are: Length thorn stem, Inter node length, width thorn stem, Stipule length, Stipule width, Leaflet number, Petiole length, Terminal leaflet width, Terminal leaflet length and Rachis length. Qualitative traits for each species studied are listed in Tab. 2.

Molecular techniques

DNA extraction

DNA extraction according to the protocol of Bousquet, *et al*., (1990) was performed with slight modifications. Approximately 100 mg of young leaves of plants using liquid Nitrogen to -196 °C in a mortar to powder. Leaf powder mixture into the microtube 2 mL. Extraction buffer (CTAB 2 %, EDTA 20 mM, Tris-HCl 10 mM, NaCl 1.4 M and PVP 1 %) for 30 min in a water bath heated to 60 ° C. Then 500 ml of the extraction buffer is added to each sample. Under the hood with 2 mL Mercaptoethanol were added and the tubes into a 60 °C water bath for 30 minutes to put. After 30 minutes of each tube were then poured into 5 ml Proteinase K bath temperature of 37 ° C for 30 min to maintain. Equal volumes of solution obtained, Chloroform – Iso amyl alcohol (ratio 1:24) and add the tubes are gently shake for 10 minutes. Then the samples for 10 min, and centrifuged at a speed of 13,000 rpm, supernatant separate solution and again added Chloroform – Iso amyl alcohol. The solution is centrifuged for 10 min at a speed of 13,000 rpm and the supernatant can be maintained. Than 3/2 Iso propanol solution float down to add cool, DNA coils can be seen at this stage. To achieve complete precipitation of DNA, the samples for 15 min at a temperature of - 20 °C can be transmitted. To view the white precipitate DNA at the bottom of the tubes, the samples were centrifuged for 10 min at a speed of 13,000 rpm and throw away the solution and float away. Wash the precipitate with cold 70 % Ethanol, centrifuge and dropping supernatant more work will be done. Order. At the end of the DNA deposited under the hood and dried, with 50 mL distilled water Precipitate dissolved, a temperature of - 20 °C can be maintained. To assess the quality of the product extracted DNA was transferred on to 2 % Agarose gel using a gel imaging system from Bio Doc Analyzer charges. Accepted samples, DNA band show typical (Fig.1). DNA quantity was determined using a Nano-Drop.

Analysis with SSR markers

A total of 13 microsatellite sequences from *Rubus* species were used (Tab. 3). The microsatellite was developed using following the protocol:

Amplification reactions were performed in a final volume of 15 µl, with 20 ng genomic.

DNA, 0.6 pmol of each primer, 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 0.2 mM of each dNTPs, 6.6 mM $MgCl_2$ and 1.0 unit of Taq DNA polymerase. The PCR was performed according to the following parameters:

94 °C for 3 min, 10 cycles of 94 °C for 30 s, 10 °C higher than annealing temperature (–1 °C/cycle) for 30 s and 72 °C for 30 s; 35 cycles of 94 °C for 30 s, annealing temperature (°C) for 30 s and 72 °C for 30 s; and 72 °C for 5 min, was followed by storage at 4 °C. PCR products were transferred onto a 6 % polyacrylamide gel which were silver-stained, according to the procedure described by Bassam *et al.* Imaging of polyacrylamide gel electrophoresis was performed using densitometer (Bio Rad, USA) (Fig. 2). Bands based on the presence (1) or absence (0) were scored. Dendrogram using the The resulting distance matrix data were used to construct the dendrogram using the neighbor joining (NJ) method, Darwin software (version 5.0.158).

Statistical analysis

The allelic diversity of the SSR was evaluated by determining the polymorphism information content (PIC) value, as described by Bonstein, *et al*., (1980) and cited and modified by Anderson, *et al*., (1993), as expressed below:

$$_{n}PIC_{i} = 1 - / \sum_{j=1} P_{ij^2}$$

Where Pij is the frequency of the jth pattern, i is the sum, and n are the patterns.

To measure the utility of the marker systems, the mean heterozygosity, expected heterozygosity (Weir and Cockerham 1984), percentage of polymorphic loci and number of effective alleles were calculated using GenAlEx software (version 6.4).

Results

Genetic variability detected by SSR markers

Positive amplification were obtained with the following SSR markers: *Rubus* $262b_2$, $Rubus98d_2$, $Rg\text{-}F3_4$, $Rubus76b_2$, $RhM011_3$, $RiM017_3$, $Rubus259f_2$, *Rubus* $105b_2$, $mRaCIRRIV2A8_1$, $mRaCIRRIV1E_8$, $mRaCIRRI12D_3$ and $mRaCIRRI1G3_1$. Amplification was not positive with the $mRaCIRRIV2F_4$ marker (Tab. 4).

Table 1. *Rubus* species studied and their localities.

Species	Location of sample collection
R. caecius	Guilan, Astara, Abasabad. 38º 046´ 405´´ N, 48º 890´ 467´´E, 280 m.
	Guilan, Langroud, Otaghvar. 37º 06´ N, 50º 06´ E, 80 m.
	Guilan, Langroud, Otaghvar. 37º 06´ N, 50º 06´E, 76 m.
R. discolor	Guilan, Talesh, Ghorogh. 37º 83´ N, 48º 96´ E, 92 m.
	Guilan, Astara, Khotbeh sara, 38º 046´ 445´´ N, 48º 890´ 427´´E, 272 m.
R. dolichocarpus	Guilan, Lahijan, Khokehab-e Loonak, 37º 00´ 30´´ N, 49º 51´ 48´´E, 1021 m.
	Guilan, Astara, Khalkhal, 37º 37´ N, 48º 32´ E, 778 m.
R. hirtus	Mazandaran, Noshahr, kheyroudkenar Forest, 36º 27´ N, 51º 33´ E, 603 m.
	Guilan, Astara, Khalkhal, 37º 37´ N, 48º 32´ E, 778 m.
	Guilan, Astara, Khalkhal, 37º 37´ N, 48º 32´ E, 545 m.
R. hyrcanus	Guilan, Astara, Khalkhal, 37º 37´ N, 48º 32´ E, 711 m.
	Guilan, Astara, Khalkhal, 37º 37´ N, 48º 32´ E, 634 m.
	Guilan, Astara, Khotbeh sara, 38º 046´ 445´´N, 48º 890´ 427´´E, 272 m.
	Guilan, Talesh, Chobar. 38º 1825´ 491´´N, 48º 892´ 4271´´E, 89 m.
	Guilan, Langroud, Otaghvar. 37º 06´ N, 50º 06´ E, 70 m.
R. persicus	Guilan, Astara, Khalkhal, 37º 37´ N, 48º 32´ E, 731 m.
	Guilan, Talesh, 37º 8055´ 108´´N, 48º 903´ 5071´´E, 80m.
	Mazandaran, Noshahr, kheyroudkenar Forest, 36º 27´ N, 51º 33´ E, 603 m.
	Guilan, Astara, Gardeneh-e Heyran, 38º 390´ 473´´ N, 48º 602´ 120´´ E, 317 m, 494 m and 541 m.
	Guilan, Astara, 25 Kilometer to Gardeneh-e Heyran, 38º 390´ 470´´ N, 48º 602´ 118´´ E, 89 m.
	Guilan, Talesh, Chobar. 38º 1825´ 491´´ N, 48º 892´ 4271´´ E, 89 m.
R. sanctus	Guilan, Talesh, 37º 8055´ 108´´ N, 48º 903´ 5071´´ E, 80 m.
	Mazandaran, Noshahr, kheyroudkenar Forest. 37º 34´ N, 52º 47´ E, 623 m.
	Guilan, Langroud, Leilakouh, 37º 011´ 72´´ N, 50º 09´ 09´´ E, 5 m.
	Guilan, Rasht, inside of Rasoul-e Akram Hospital, 38º 27´ N, 49º 27´ E, 0 m.
sp 1	Guilan, Lahijan, Siahkal, Bagh-e Vali, 37º 00´ 30´´ N, 49º 51´ 48´´ E, 1002 m.
sp 2	Guilan, Rasht, inside of Rasoul-e Akram Hospital, 38º 27´ N, 49º 27´ E, 0 m.
sp 3	Guilan, Rasht, inside of Rasoul-e Akram Hospital, 38º 27´ N, 49º 27´ E, 0 m.
sp 4	Guilan, Langroud, Leilakouh, 37º 011´ 72´´ N, 50º 09´ 09´´ E, 4 m.

R.: *Rubus*, SP: Uncertain species.

The bands obtained were similar in weight and number to those obtained by Amsellem *et al.* (2001) in Asian species. Seven private or exclusive alleles were detected for several subgenera, providing a molecular profile for genotypes based on their banding pattern. Amsellem *et al.* (2000) observed the amplification of three to four alleles per individual in *R. alceifolius*, which confirmed the hypothesis that this is a tetraploid species. In this paper, *R. sanctus, R. hirtus, R. dolicocarpus, R. hyrcanus* and *R. discolor* produced between one to four alleles, while *R. caesius* and *R. persicus* produced between one to five alleles. Most of *R. caesius* samples showed higher ploidy levels (tetraploid).

These results suggest that the last two species have higher ploidy levels than the others.

Alice and Campbell (1999) describe the ploidy level in the *Rubus* subgenus ranges from diploid to tetraploid, while the subgenus *Idaeobatus* presents both diploid and tetraploid species.

According to morphological and molecular

descriptions made by the abovementioned authors on *Rubus* species, in different regions and maybe in a region, the ploidy level among *Rubus* genotypes can vary greatly.

The results presented here, using 2 different markers, morphological and molecular markers, agree with the highly variable ploidy level found in *Rubus* species. With some SSR marker, for example, RhM011$_3$ *R. sanctus* amplified only 2 alleles, as in the case of the genotypes 1, 9, 10, 11, 17 and 18, while the *R. sanctus* genotypes 2, 3, 4, 5, 6, 7, 8, 12, 13, 14, 15, 16 and 19 amplified 3 and 4 alleles.

Some of *R. caesius* genotypes (2, 3 and 5) amplified more than 2 alleles (3 and 5 alleles) with these same SSR markers, suggesting that there are both diploid and tetraploid genotypes of *R. sanctus* and both diploid and pentaploid genotypes of *R. caesius* in the wild germplasm.

Table 3. Microsatellite sequences and characteristics of each SSR used to evaluate *Rubus* materials. T_a: Annealing temperature.

Rank	Locus	Primer sequences (5´- 3´)	Band about the size of the amplification products (bp)	T_a (°C)
1	*Rubus* 262b$_2$	F: 5´-TGCATGAAGGCGATATAAAGG-3´ R: 5´-AACAGTGCACAGGGGCTAAT-3´	200-250	55
2	*Rubus* 98d$_2$	F: 5´-GGCTTCTCAATTTGCTGTGTC-3´ R: 5´-TGATTTGAAATCGTGCGGTTA-3´	150-200	55
3	Rg-F34	F: 5´-GCCAATGAAACGGAAAGAC-3´ R: 5´-GCCTTCACTCATATCATTCTCC-3´	200-250	55
4	*Rubus* 76b$_2$	F: 5´-CTCACCCGAAATGTTCAACC-3´ R: 5´-GGCTAGGCCGAATGACTACA-3´	200-250	55
5	RhM011$_3$	F: 5´-AAAGACAAGGCGTCCACAAC-3´ R: 5´-GGTTATGCTTTGATTAGGCTGG-3´	250-350	60
6	RiM017$_3$	F: 5´-GAAACAGGTGGAAAGAAACCTG-3´ R: 5´-CATTGTGCTTATGATGGTTTCG-3´	150-200	55
7	*Rubus* 259f$_2$	F: 5´-TGGCACAAGAAGCCTGTAAC-3´ R: 5´-TCCCATATCCCTCAGCATTC-3´	200-300	55
8	*Rubus* 105b$_2$	F: 5´-GAAAATGCAAGGCGAATTGT-3´ R: 5´-TCCATCACCAACACCACCTA-3´	150-200	55
9	mRaCIRRIV2A8$_1$	F: 5´-TAAAAAGGCGCAACAGTC G-3´ R: 5´-AGACACAGAAACAGGCATCG-3´	150-200	58
10	mRaCIRRIV2F$_4$	F: 5´-CAG AGA TAT CAT TTG GTG TTTGG-3´ R: 5´-CGC AAG AAA CAT GCA AGC-3´	---	55
11	mRaCIRRIV1E$_8$	F: 5´-TCCTTAGTTTTTCCGGATTGG-3´ R: 5´-AGGCTTGAATGGAAAATTGG-3´	200-350	64
12	mRaCIRRI12D$_3$	F: 5´-CAAGCAGGGAATTAAGGTTCTG-3´ R: 5´-GTGCGGCTTCATAGCGTTAG-3´	150-300	56
13	mRaCIRRI1G3$_1$	F: 5´-CTCTACAAAAGGATCTGCATGA-3´ R: 5´-CAGCAA AAGTGAAATGGTTCA-3´	200-250	57

A total of 12 loci and 29 alleles were detected. The number of alleles observed for each locus ranged from 1 to 6, with an average of 3.08 alleles per locus. The SSRs achieved cumulative polymorphism information content (PIC) of 0.9444, and individual values for each locus between 0.3546 and 0.7656 (average 0.5571). The highest PIC value (0.7259) was found in the RhM011$_3$ locus, which presented a high number of alleles (6). In comparison, Ishii and McCouch (2000) obtained mean PIC values of 0.267 when evaluating rice SSRs and Cordeiro *et al.* (2000) obtained PIC values between 0.48 and 0.8 when evaluating sugarcane SSRs. These PIC values demonstrated that, in spite of the low number of primers used, the SSR were sufficiently polymorphic and informative (Tab. 4). Expected heterozygosities (He) were 0.021-0.286 (mean 0.154) (Tab. 5). The dendrogram based on microsatellite markers, no clear geographic pattern to interpret the results, we do not see. Status is unknown species and genotypes with high affinity and proximity to each other show (Fig. 4). Genotypes *R. hyrcanus*, (hir 1, 2, 3 and 4), a high affinity closer to

R. sanctus or *R. persicus*, and two sp ₂ and sp_3 genotypes with high affinity and proximity to *R. caesius* and *R. persicus* show. Perhaps, the last two genotypes, can be a hybrid of two species. Significant morphological affinities of the two genotypes showed the species *R. caesius*. Sp_1 genotype also significantly close proximity to two species *R. sanctus* and *R. discolor* with 50% bootstrap shows.The morphology of these genotypes, *R. sanctus* has a high affinity. Two genotypes cae 4, 5, similar to *R. persicus*, and cae 3, a high affinity to *R. discolor* show. Cae 1, 2, is located near *R. sanctus*. The results of the fact that, as much as in *R. caesius* is not clear. Genotypes the characteristics of interspecies show. Genotypes of *R. discolor*, (dis 2, 3, 5, 6), is close to *R. persicus* genotypes. It should be noted that the similarity between species in morphological traits with genotype dis3 and *R. persicus*, was confirmed.

Variations of Rubus with morphometric analysis

The dendrogram based on quantitative morphological traits, species are completely separated, and even species were differentiated on the basis of geographical pattern (Fig. 3). Genotypes *R. persicus* (per12, 13, 14, 15, 16, 17, 18, 19 and 20) of East Guilan, and genotypes (2, 3, 4, 5 and 6) the West Province have been collected, the tree shows each group of genotypes in a separate cluster with 100% bootstrap have been. Genotypes *R. sanctus* (san 13, 14, 15, 16, 17, 18 and 19) of the elevation range of 0 to 5 meters of East Guilan, (san 1, 11 and 12) from the elevation range of 89-80 m, and genotypes (san 2, 3, 4, 5, 6, 7, 8 and 9) the elevation range of 300 to 550 meters, West Guilan samples have been. 3 geographically mentioned quite a morphologically from each other distinctions obtained have. This result indicates that, in addition to geographic location, altitude can also affect the morphology of the species, and therefore, genotypes belonging to the same species can be observed morphological segregation.

Table 5. Genetic diversity in populations of *Rubus* species.

Rubus species	EA	PL	He
R. dolicocarpus	1	4.01	0.021
R. hirtus	1.157	24.14	0.092
R. caesius	1.429	68.97	0.249
R. hyrcanus	1.276	34.48	0.148
R. discolor	1.253	37.93	0.143
R. sanctus	1.432	82.76	0.246
R. persicus	1.492	93.10	0.286
R. sp.	1.334	48.28	0.189

*EA: number of effective allele, PL: percentage of polymorphic loci, He: expected heterozygosity.

4 genotype sp (1, 2, 3 and 4) based on morphological identification keys, a hybrid interspecies seemed. Based on quantitative morphological traits, genotype sp_1, close proximity to a number of significant genotype *R. sanctus* show, it was also observed qualitatively similar in most morphological characteristics and genotypes sp 2, 3 and 4, the high affinity of both qualitaatively and quantitatively genotypes R. caesius (cae 2, 3 and 5) showed (Tab. 2). Importantly, the majority of the samples belonging to the same species collected from different geographic areas, often qualitative morphological characters were not overwhelmed by environmental factors, and has remained unchanged (Tab. 1).

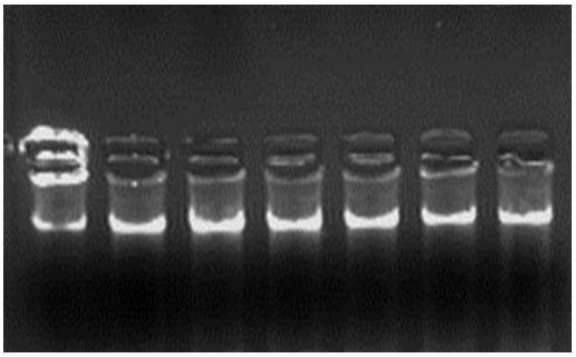

Fig. 1. Electrophoresis of gel agarose (2%) for DNA extraction product.

Maximum agreement sub tree (50%), for 65 genotypes were plotted (Fig. 5). Three distinct groups on the cluster can be seen. Genotypes in a group of at least 50% similarity in their morphological and microsatellite. For example, genotype group 1 (per 2, 3, 7, 8 and 11) showed 50% similar morphological and microsatellite. These individuals collected from the same geographical region and probably all belong to one species. Samples in Group 2 consists of (hir 1, hir 2 and hyr 3), the West Guilan, elevation 778 m were collected. Probably, the occurrence of interspecies hybridization, the similarity between the species the led. Similarly, genotype 3.

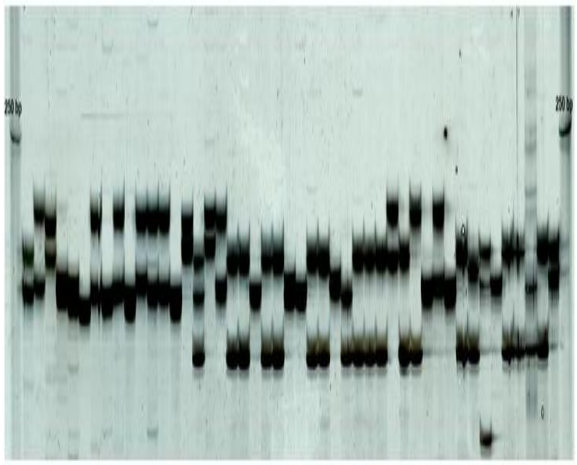

Fig. 2. Electrophoresis of Poly acrylamide gel: mRaCIRRI1G3$_1$ primer.

Discussion

Results from this study were quite similar to the results of a large number of researchers who had worked on other species of *Rubus*. Kollmanm, *et al*., (2000) stated, method of reproduction, genetic diversity *Rubus* is a factor. Cross -fertilization between polyploid species of blackberry factor is significant. This type of cross- fertilized seeds and fruit quality are affected, in a manner that increases ploidy levels and proximity and taxonomic affinities. Hybridization in *Rubus* species are often close together and in some cases can be seen between the genuses. (Gustafsson, 1942; Alice and Campell 1999) and ploidy levels among genotypes raspberries can be very different.

Marulanda *et al*. (2007) genetic diversity among wild and cultivated *Rubus* species (51 samples) in Colombia, AFLP and SSR markers using examined. Investigations by the two markers, different ploidy levels in the genus confirmed. SSR and AFLP - cadherin function results also confirm the high relevance between species. SSR data indicated the possibility that variation within a species as well is the difference between *Rubus* species (inter- species variation).

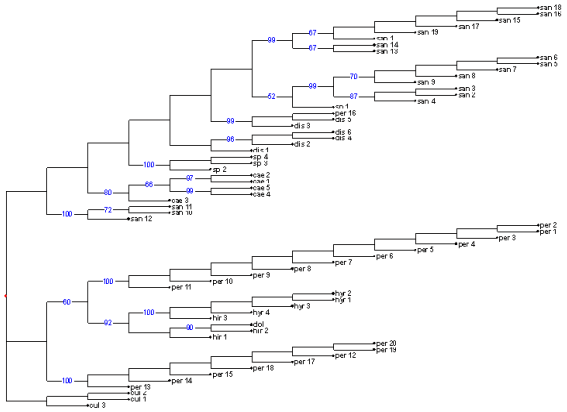

Fig. 3. Cluster analysis of 65 genotype of *Rubus* sp., based on the matrix of calculated based on morphological markers.

The neighbor joining method was the grouping criterion.

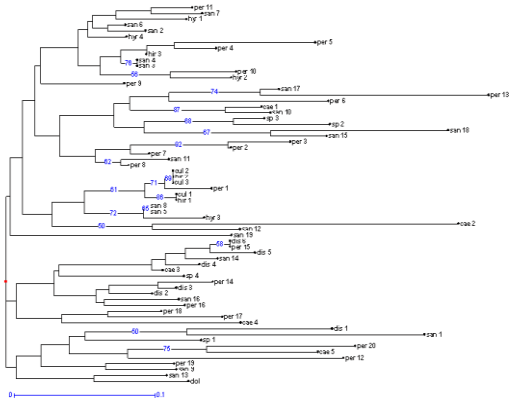

Fig. 4. Cluster analysis of 65 genotype of Rubus sp., based on the matrix of genetic similarity calculated based on SSR markers. The neighbor joining method was the grouping criterion.

Saini *et al*. (2004) stated that in order to perform a comprehensive molecular analysis, using a combination of marker systems is recommended.

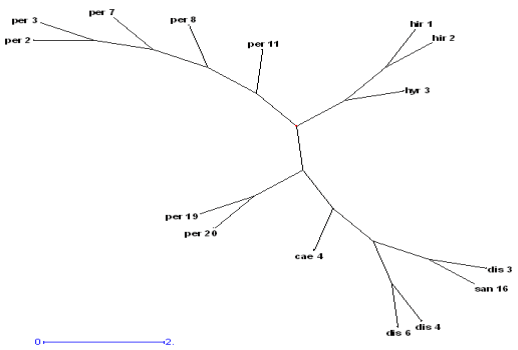

Fig. 5. Cluster analysis of 65 genotype of *Rubus* sp., based on the maximum agreement sub tree (50%) calculated based on SSR and morphological markers.

In general, the limits of species, the occurrence of hybridization between species with different ploidy levels between *Rubus* genotypes, is not subject to any specific geographic pattern. Nuclear markers such as microsatellite (Simple Sequence Repeat) also been overshadowed by hybridization between species of high and therefore will not be given. Therefore, it is proposed to define a species in the genus *Rubus* Iran, along with morphological and nuclear markers of the organelle markers (mtDNA and chDNA) should be used. Maternal inheritance of mitochondrial and chloroplast and would not affect hybridization, can be a powerful tool to help classify *Rubus* species.

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