Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

**Citation:** Fahimeh Koohdar, Neda Aram, Masoud Sheidai (2021) Biosystematics, fingerprinting and DNA barcoding study of the genus *Lallemantia* based on SCoT and REMAP markers. *Caryologia* 74(4): 77-83. doi: 10.36253/caryologia-1163

**Received:** December 14, 2021

**Accepted:** December 17, 2021

**Published:** March 08, 2022



**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Competing Interests:** The Author(s) declare(s) no conflict of interest.

# Biosystematics, fingerprinting and DNA barcoding study of the genus *Lallemantia* based on SCoT and REMAP markers

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**Abstract.** *Lallemantia* is a medicinally important plant in the world. Due to interspecific hybridization and horizontal gene transfer, spices relationship and delimitation on the genus *Lallemantia* is difficult based on different molecular markers. Therefore, selecting the appropriate marker can be important. Fingerprinting techniques continue to be used for genomic profiling for the characterization of germplasm and the establishment of the identity of varieties/hybrids/parental sources of aromatic and medicinal plants. For this, we need to produce detailed information on genetic diversity available in *Lallemantia* as well as investigate spices relationship and delimitation. Therefore, the present study was performed on Lallemantia species in Iran. We used the start codon targeted and retrotransposon-microsatellite amplified polymorphism molecular marker for our genetic investigation with the following aims: 1- To reveal the species delimitation and species relationship in *Lallemantia*, and 2- To investigate discriminating power of the start codon targeted and retrotransposon-microsatellite amplified polymorphism markers by Gst and NM analysis. The results obtained revealed that the start codon targeted marker is the best to show the relationships between species while the retrotransposon-microsatellite amplified polymorphism marker is the best for species delimitation. we found the loci with the high value of Gst (1.00) in start codon targeted and retrotransposon-microsatellite amplified polymorphism markers that can be used in barcoding and fingerprinting of *L. royleana*.

**Keyword:** *Lallemantia*, fingerprinting, ScoT, REMA, Iran.

## INTRODUCTION

The genus *Lallemantia* (Lamiaceae) is composed of 5 species (*Lallemantia royleana* (Benth.) Benth., *L. canescens* (L.) Fisch. & C.A.Mey.,*L. baldschuanica* Gontsch., *L. iberica* (M.Bieb.) Fisch. &C.A. Mey. and *L. peltata*(L.) Fisch. & C. A. Mey.) that are widely distributed in Afghanistan, China, India, Kazakhstan, kyrgyzstan, Iran, Russia, Tajikistan, Turkmenistan, Uzbekistan and Europe (Sheidai *et al.* 2018). There are all five species in Iran (Rechinger 1982).

*Lallemantia* species are herbaceous with simple leaves, interrupted inflorescence, aristate-toothed bracteoles, and oblong, trigonous, smooth, and mucilaginous nutlets (Harley *et al.* 2004). These species are well known

as a source of food and medicine plant. For example, *L.ibericai*s used as an oil seed plant in Iran and USSR (Rivera-Nunez and Obonde-Gastro 1992, Dinç *et al.* 2009), *L. royleana* Seeds have considerable anti - bacterial properties and is a suitable remedy for skin diseases and gastrointestinal diseases and also *L. peltata* that grows in limited area in Iran is known as medicinal plant that contains volatile and essential oil (Mahmood *et al.* 2013).

The molecular systematic study of plants is performed with different purposes like: species delimitation, population divergence, species relationships, date of divergence determination, etc (Broadhurst *et al.*, 2004; Millar *et al.*, 2011). Various molecular markers have been used to perform the above tasks such as, amplified fragments length polymorphism (AFLP), simple sequence repeats (SSRs), inter-simple sequence repeats (ISSRs), start codon targeted (SCoTs), retrotransposon-microsatellite amplified polymorphism (REMAPs) etc. (e.g., Sheidai *et al.* 2012, 2013, 2014, Minaeifar *et al.* 2015, Saboori *et al.* 2019).

DNA barcoding is a sequence of DNA that can help in rapid and accurate recognition of species. It has been used in the identification of medicinal plants and has been able to detect actual and original products from its fake type (Heubl *et al.* 2010, Sheidai *et al.* 2018). Nuclear and chloroplast DNA have been examined for their suitability as barcodes through DNA fingerprinting and DNA sequencing-based approaches. In principle, both approaches can be used to differentiate between individuals, species, and populations and to detect the presence of adulterants. Notwithstanding the increasing use of DNA sequence-based approaches, fingerprinting techniques continue to be used for genomic profiling for characterization of germplasm and establishment of the identity of varieties/hybrids/parental sources of aromatic and medicinal plants (Sheidai *et al.*, 2019).

Among DNA markers, there are ubiquitous retro elements in the plant genome like IRAP and REMAP (retrotransposon-microsatellite amplified polymorphism). The REMAP is produced by amplifying the fragments between a retrotransposon insertion site and a microsatellite site and employed in fingerprinting, linkage analysis, mapping, analysis of genome evaluation and genetic diversity. REMAP describe the profile of a population, discriminate between species or genotypes and analyze population diversity (Kumar *et al.* 2010).

Start Codon Targeted (SCoT) polymorphisms (Collard and Mackill 2009) are dominant and reproducible markers based on the short conserved region flanking the ATG start codon in plant genes and use a single 18-mer primer in the polymerase chain reaction (PCR) assays and high annealing temperature (50 °C). These markers could have potential in genotyping and to reveal polymorphisms that might be directly related to gene function. SCoT markers have been used to assess genetic diversity and structure, in bulked segregant analysis, and for quantitative trait loci (QTL) mapping and DNA fingerprinting (Collard and Mackill 2009, Luo *et al.* 2010).

Due to the morphological similarity of *Lallemantia* species and sell them in the market as seed, the present study was performed with the following aims: 1- To reveal the species delimitation and species relationship in *Lallemantia* by SCoT and REMAP markers, and 2- To investigate discriminating power of the SCoT and REMAP markers by Gst and NM analysis for barcoding and fingerprinting of medicinal spices in *Lallemantia*.

## MATERIAL AND METHODS

### *Plant materials*

Extensive field investigations and collections were under taken during 2013–2015. Forty-two specimens of five species, *Lallemantia royleana*, *L. canescens*, *L. baldschuanica* Gontsch., *L. iberica* and *L. peltata* were randomly collected from different geographic populations for molecular study.

### *SCoT and REMAP assay*

Fresh leaves were put to dry in silica gel powder. Cetyltrimethyl-ammonium bromide -activated charcoal protocol (CTAB) was applied to extract the genomic DNA. The extraction was done by activating charcoal and poly vinyl pyrrolidone (PVP) for binding of polyphenolics during extraction; for mild extraction and precipitation conditions, the high-molecular weight DNA isolation was boosted without the interference of impurities. The extracted DNA was examined in terms of quality by running on 0.8% agarose (Sheidai *et al.* 2013).

Three REMAP primer combinations, derived from one single IRAP primer (NIKITA) with 3 ISSR primers ((CA)7GT, (GA)9T, (GA)9C) were tested on plants samples. Using a 25 µL volume containing 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany); 50 mMKCl; 10 mMTris-HCl buffer at pH;8 1.5mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of each primer, polymerase chain reaction (PCR) was implemented.

The following program was used for amplification of nuclear region in a PCR reaction: 5 min initial denatura-

tion step 94°C, followed by 40 cycles of 1 min at 94°C; 1 min at 53.5°C and 2 min at 72°C. The reaction was completed by a final extension step of 7 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Four primers (SCOT1, SCOT2, SCOT36, and SCOT41) based on Collard and Mackill (2009) for monocotyledons plants were selected (Collard and Mackill 2009). These primer sequences are: SCoT1: CAACAATGGCTACCACCA, SCoT2: CAACAATGGCTACCACCC, SCoT36: GCAACAATGGCTACCACC and SCoT41: CAATGGCTACCACTGACA. PCR reaction mixture with total volume of 25 µl contained 10 mMTris-HCl buffer (pH = 8), 50 mMKCl, 1.5 mM MgCl2, 0.2 mM of dNTP (Bioron, Germany), 0.2 µM of primer, 20 ng genomic DNA and 1U of *Taq* DNA polymerase (Bioron, Germany).

The amplification reactions were performed in Techne thermocycler (Germany) with the following program: 5 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 49–58 °C (SCOT1 50 °C, SCOT2 49 °C, SCOT36 50 °C, SCOT41 58 °C) and 1 min at 72 °C and a final cycle of 7 min at 72 °C. The amplification products were visualized by running on 2% agarose gel, stained with syber green (Powerload, Kosar Co. Iran). The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

*Data analyses*

The SCoT and REMAP bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0). The number of private bands versus common bands and genetic diversity parameters like: The percentage of allelic polymorphism, allele diversity (Weising, 2005), Nei' gene diversity (He), and Shannon information index (I) (Weising 2005), were determined. We used GenAlex 6.4 for these analyses (Peakall and Smouse 2006). Discriminating power of REMAP and SCoT markers investigated by Gst and NM analysis as implemented in POPGENE32.

Grouping of the species was done by different clustering and ordination methods such as unweighted paired group using average (UPGMA), Multidimensional scaling (MDS), and Principal components analysis (PCA) (Podani 2000). PAST version 2.17 (Hammer *et al.*, 2012) was used for multivariate analysis.

## RESULTS

*SCoT results*

Almost all the SCoT primers produced bands were used and finally a data matrix of 70 × 42 was formed for further analysis. Based on band pattern in *Lallemantia* genus, the highest number of privet bands were observed in *L. iberica* and *L. baldschuanica* had the lowest value (Fig. 1).

Genetic variation parameters were investigated in 5 species of *Lallemantia* genus. Highest level of Shannon index (0.336), expected heterozygosity (0.218), and percentage of polymorphism (70.15) in *L. iberica* and lowest level of Shannon index (0.105), expected heterozygosity (0.072) and percentage of poly morphism (17.91) were observed in *L. baldschuanic* species (Table 1).

The AMOVA test showed significant genetic differences among *Lallemantia* species (P = 0.001). The results show that the species of this genus have been genetically distinguished from each other using SCoT marker.

Different ordination and clustering methods like PCA, MDS and UPGMA produced similar results; therefore, only UPGMA plot is presented here. UPGMA

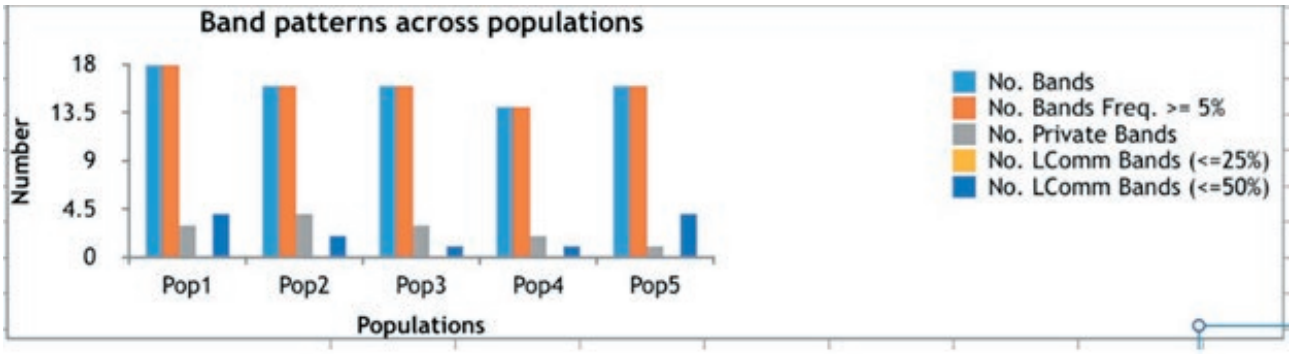


**Figure 1.** Band patterns of ScoT marker in *Lallemantia* spices. 1: *L. royleana*, 2: *L. iberica*, 3: *L. canescens*, 4: *L. peltata*, 5: *L. baldschuanica.*

**Table 1.** Genetic diversity parameters in *Lallemantia* species based on SCoT marker.

| Species | N | Na | Ne | I | He | uHe | P |
|---|---|---|---|---|---|---|---|
| *L. royleana* | 10.000 | 0.925 | 1.204 | 0.201 | 0.128 | 0.135 | 44.78% |
| *L. iberica* | 10.000 | 1.522 | 1.354 | 0.336 | 0.218 | 0.230 | 70.15% |
| *L. peltata* | 5.000 | 0.761 | 1.191 | 0.163 | 0.110 | 0.122 | 29.85% |
| *L. canescens* | 7.000 | 0.821 | 1.207 | 0.183 | 0.121 | 0.130 | 35.82% |
| *L. bahdchuanic* | 5.000 | 0.493 | 1.128 | 0.105 | 0.072 | 0.080 | 17.91% |

Abbreviations: N = No of plants studied; Na = No. of alleles; Ne = Effective No. of alleles; He = Gene diversity; uHe = Unbiased gene diversity; P = Polymorphism percentage.

plot of SCoT markers(Fig. 2) grouped the specimens of *L. platata*, *L. canescens* and *L. baldshuanica* together in a single cluster, separated from each other but in *L. iberica* and *L. royleana* the specimens were divided in two separate clades. In this plot, *L. royleana* and *L. baldshuanica* as well as *L. peltata* were placed close to each other while *L. iberica* and *L. canescens* was placed far from them.

*REMAP results*

Almost all the REMAP primers produced bands were used and finally a data matrix of 55 (number of bands) × 42 (number of samples) was formed for further analysis. Based on band pattern in *Lallemantia* genus, the highest number of privet bands were observed in *L. iberica* and *L. baldschuanica* had the lowest value (Fig. 3).

Genetic variation parameters and band patterns were investigated in 5 species of *Lallemantia* genus. Highest level of Shannon index (0.111), expected heterozygosity (0.073), and percentage of polymorphism (21.88%) in *L. baldschuanic* and lowest level of Shannon index (0.017), expected heterozygosity (0.011) and percentage of poly morphism (0.012) were observed in *L. royleana* species (Table 2).

AMOVA showed significant genetic differences among *Lallemantia* species (P = 0.001). The AMOVA test showed 45% inter-species diversity and 55% intra-species diversity. The results show that the species of this genus

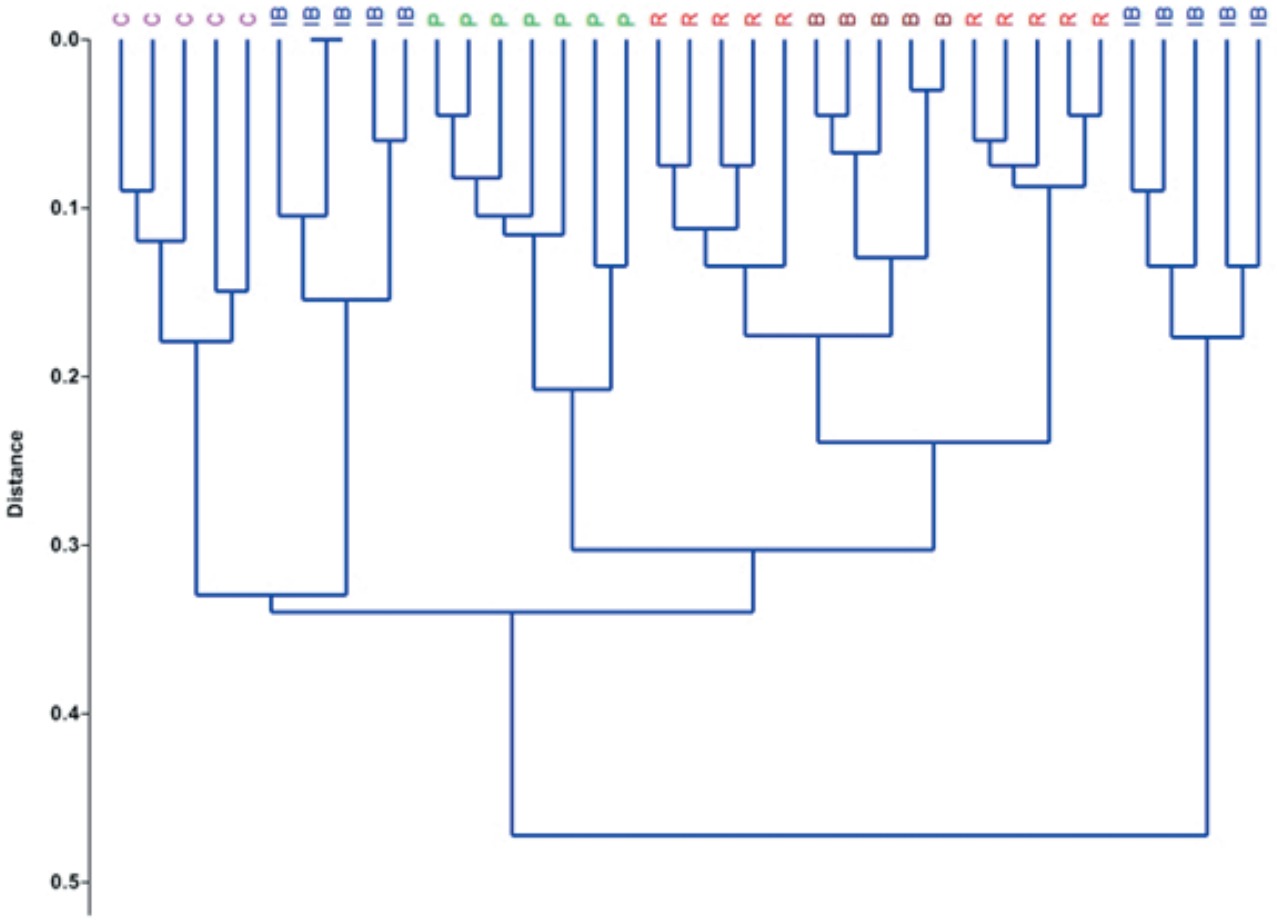


**Figure 2.** UPGMA plot of SCoT marker in *Lallemantia*. R: *L. royleana*, IB: *L.iberica*, C: *L. canescens*, P: *L. peltata*, B: *L. baldschuanica*.

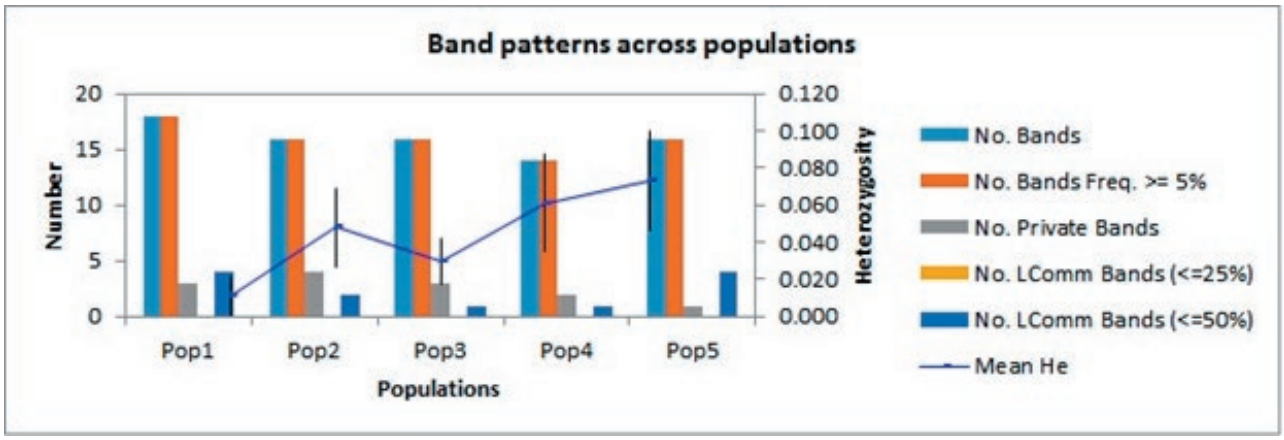


**Figure 3.** Band patterns of REMAP marker in *Lallemantia* Spices. 1: *L. royleana*, 2: *L.iberica*, 3: *L. canescens*, 4: *L. peltata*, 5: *L. baldschuanica.*

**Table 2.** Genetic diversity parameters in *Lallemantia* species based on REMAP marker.

| Species | N | Na | Ne | I | He | uHe | P |
|---|---|---|---|---|---|---|---|
| *L. royleana* | 5.000 | 0.594 | 1.017 | 0.017 | 0.011 | 0.012 | 3.13% |
| *L. iberica* | 5.000 | 0.656 | 1.075 | 0.075 | 0.048 | 0.054 | 15.63% |
| *L. canescens* | 5.000 | 0.656 | 1.036 | 0.053 | 0.030 | 0.033 | 15.63% |
| *L. Peltata* | 5.000 | 0.594 | 1.109 | 0.090 | 0.061 | 0.068 | 15.63% |
| *L. baldschuanic* | 5.000 | 0.719 | 1.123 | 0.111 | 0.073 | 0.081 | 21.88% |

Abbreviations: N = No of plants studied; Na = No. of alleles; Ne = Effective No. of alleles; He = Gene diversity; uHe = Unbiased gene diversity; P = Polymorphism percentage.

have been genetically distinguished from each other using SCoT marker.

Different ordination and clustering methods like PCA, MDS and UPGMA produced similar results; therefore, only UPGMA plot is presented here. UPGMA plot of molecular markers (Fig. 4) grouped the specimens of all species together in a single cluster, separated from the other species. This means that REMAP molecular markers are of taxonomic value and can delimit the *Lallemantia* species. In this plot, *L. royleana* and *L. baldschuanica* as well as *L. peltata* and *L. canescens* were placed close to each other while *L. iberica* was placed far from them.

### *Barcoding and fingerprinting*

Discriminating power analysis of molecular markers is important for fingerprinting and barcoding. for this purpose, Gst and Nm parameters was measured. The value of Gst in Seven loci in SCoT and 13loci in REMA marker in *Lallemantia* was 1 .00 while the mean Nm value was 0.00 which indicated that these markers can be used in *L. royleana* differentiation of other species.

### *Comparison of SCoT and REMAP markers*

In this study, SCoT marker was able to separate only three species of five species, while REMAP marker was able to demonstrate the boundary between any five species (Figs 1 and 2). According to AMOVA result, SCoT marker was more successful in showing intraspecific diversity.

## DISCUSSION

The present study revealed that species delimitation based on REMAP markers was more successful than SCoT marker. however, this marker was more successful in showing intraspecific diversity. These results are consisting with previous findings (Al-Qurainy *et al.* 2015, Saboori *et al.* 2019).

*L. baldschuanica* and *L. royleana* as well as *L. canescens* and *L. iberica* are similar to each other based on morphological and micro morphological (nutlet and pollen structure) studies (Talebi and Rezakhanlou 2010; Kamrani *et al.* 2018).

Lamiaceae family has many phylogenetically unresolved genera and therefore many species are of not determined relationship due to the conflict between molecular data and potential inter-specific hybridization as well as horizontal gene transfer.

Sheidai *et al.* 2018, revealed that the relationships between *Lallemantia* species based on cp- DNA, ITS and ISSR molecular markers differed from morphological and micromorphological as well as to each other due to inter-specific hybridization and horizontal gene transfer.

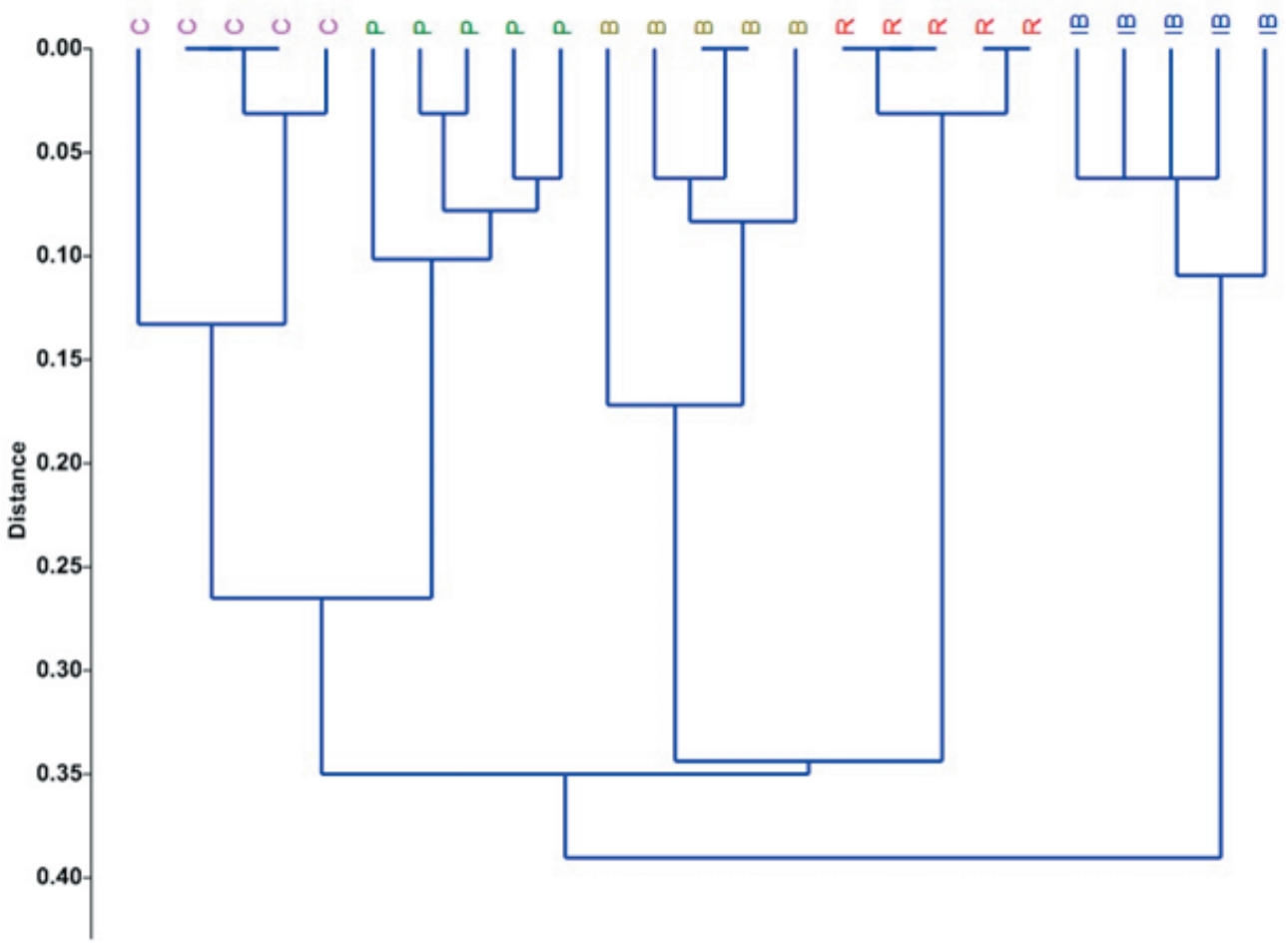


**Figure 4.** UPGMA plot of REMAP markers in *Lallemantia*. R: *L. royleana*, IB: *L.iberica*, C: *L. canescens*, P: *L. peltata*, B: *L. baldschuanica*

Our result suggested conflict between SCoT and REMAP marker in relationship of *Lallemantia* species but in both markers, *L. baldschuanica* and *L. royleana* were placed to each other. The SCoT result was more similar to the previous studies (Talebi and Rezakhanlou 2010; Kamrani *et al.* 2018), so this marker can be suggested to study the inter-species relationships in *Lallemantia*. In the present study, we found the loci with the high value of Gst (1.00) in SCoT and REMAP markers that can be used in barcoding and fingerprinting of *L. royleana*.

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