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MOLECULAR CHARACTERIZATION OF *Dittrichia viscosa* (L.) GREUTER (ASTERACEAE) POPULATIONS REVEALED BY ISSR MARKERS AND CHLOROPLAST (cpDNA) *trn*L INTRON SEQUENCES

Emre SEVINDIK[1*], Makbule BOZKURT[1], Mehmet YILMAZ[1], Ezginur ŞENYÜZ[1], Mehmet Yavuz PAKSOY[2]

[1*]Aydın Adnan Menderes University, Faculty of Agriculture, Department of Agricultural Biotechnology, Aydın, Turkey
[2]Department of Medical Services and Techniques, Tunceli Vocational School, Munzur University, Tunceli, Turkey

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In this study, molecular characterization of *Dittrichia viscosa* (L.) Greuter populations collected from Aydın province was carried out based on ISSR technique and chloroplast DNA *trn*L intron sequences. 10 ISSR primers were used to determine the molecular characterization among the populations. For cpDNA *trn*L intron amplification, *trn*C and *trn*D primers were used. In ISSR analysis, a total of 70 bands were obtained. The polymorphism rate was determined to be approximately 94.28%. According to the ISSR analysis, the UPGMA dendrogram consisted of three groups. For cpDNA *trn*L intron sequences, phylogenetic analyses were obtained along with genetic distances. For populations, cpDNA *trn*L intron sequences were determined between 454 and 472 bases. The maximum likelihood phylogenetic tree consist of two clades. In addition, cpDNA *trn*L intron sequences of some species (*Chrysophthalmum Pulicaria Inula Jasonia Stenachaenium Carpesium Blumea Iphiona Limbarda Rhanterium Lifago Duhaldea*

Corresponding author: Emre Sevindik, Department of Agricultural Biotechnology, College of Agriculture, Aydın Adnan Menderes University, South Campus, Cakmar-Aydin, Turkey, E-mail: ph.d-emre@hotmail.com

Chiliadenus) of Asteraceae family from NCBI with *Dittrichia viscosa* populations phylogenetic tree was constructed. As a result of the study, it was determined that the polymorphism rate of *Dittrichia viscosa* populations obtained by ISSR-PCR was higher than the results obtained from *trn*L intron sequences.

Keywords: Dittrichia viscosa, ISSR-PCR, cpDNA *trn*L intron, molecular

INTRODUCTION

The Asteraceae family worldwide is represented by 23.600 species and 1620 genera (KARLIOĞLU KILIÇ *et al.,* 2021). This family includes many important crops, many ornamental plants and many weeds, as well as several species containing molecules of medical interest (PASCUAL-DÍAZ *et al.,* 2021; DARQUI *et al.,* 2021). The genus *Dittrichia* Greuter belongs to the Asteraceae family. In *Dittrichia,* the presence of abruptly contracting cylindrical achenes under the pappus, the fusion of pappus hairs near the base, and the presence of simple and free pappus hairs compared to angled achenes that do not suddenly contract under the pappus; differ from the closely related genus *Inula* L. (BRULLO and DE MARCO, 2000; SANTILLI *et al.,* 2021). Its new name, *Dittrichia viscosa* (L.) Greuter, replaced the old name of Inula *viscosa* (L.) Aiton. *Dittrichia viscosa* has been included in the genus *Dittrichia* with a taxonomic revision (BRULLO and DE MARCO, 2000; GRAUSO *et al.,* 2020). *Dittrichia viscosa,* Stinkwort or False Yellowhead is a plant species common in southern Europe, the Mediterranean Basin, the Middle East and North Africa (BOARI *et al.,* 2021; SLADONJA *et al.,* 2021). This plant is an important natural food source for butterfly and moth caterpillars which feed on this plant's nectar for growth and development (PAROLIN *et al.,* 2014). Additionally, thanks to its high genetic plasticity, *Dittrichia viscosa* has the ability to adapt to a wide variety of environmental conditions including heavily polluted mining or industrial areas and arid or high saline lands (DE PAOLIS *et al.,* 2022). *Dittrichia viscosa* has various biological activity displaying compounds including those with antimicrobial, nematicidal and insecticidal activity (BLANC *et al.,* 2006; SASSI *et al.,* 2007; ALEXENIZER and DORN, 2007; OZKAN *et al.,* 2019; GRAUSO *et al.,* 2020), antioxidant (CHAHMI *et al.,* 2015; GHARRED *et al.,* 2019; OZKAN *et al.,* 2019), anticancer (HEPOKUR *et al.,* 2019; OZKAN *et al.,* 2019; SEVGI *et al.,* 2021), antifungal (RHIMI *et al.,* 2017; HAOUI *et al.,* 2016), intestinal disorders (MSSILLOU *et al.,* 2022).

DNA based molecular markers are used in phylogenetic, genetic diversity and breeding studies in plants (MONDINI *et al.,* 2009; FILIZ and KOÇ, 2011; MUKHERJEE *et al.,* 2013; NADEEM *et al.,* 2018). ISSR is a PCR-based marker that involves amplification of a DNA segment located at a reproducible distance between two identical microsatellite repeat regions oriented in opposite directions (REDDY *et al.,* 2002). ISSR technique is widely used for phylogenetics, population genetics, molecular characterization and diversity analyses (MEI *et al.,* 2017; ISMAIL *et al.,* 2019; YAN *et al.,* 2019; MIR *et al.,* 2021; PINAR *et al.,* 2021; BAGHERI *et al.,* 2022; KESKIN *et al.,* 2022). The chloroplast (cp) genome has a circular structure and is highly conserved in sequence and structure due to non-recombinant, haploid and uniparentally inherited structures (LI *et al.,* 2021; CHEN *et al.,* 2021). Chloroplast DNA sequences are widely used to identify interspecific relationships among plants (TABERLET *et al.,* 1991). Chloroplast *trn*L intron was first identified by TABERLET *et al* (1991) as a potential target for plant molecular studies. This

region is located between the group I intron of the *trn*L ^{UAA} gene and the *trn*L$^{5'}$ and *trn*L$^{3'}$ exons of the tRNA gene (LAMB *et al.*, 2016; NUZHDINA *et al.*, 2018).

In this study, molecular characterization among *Dittrichia viscosa* populations distributed through different provinces of the Aydın/Türkiye, was performed by using ISSR primers and chloroplast *trn*L intron sequences.

MATERIALS AND METHODS

Plant samples, genomic DNA isolation and PCR amplification

In the study, leaf samples belonging to *Dittrichia viscosa* populations were collected from different districts of Aydın province (Çakmar, İncirliova, Germencik, Koçarlı, Çine, Söke, Kuşadası, Aydın- Centre, Aydın-Danişment village, Aydın-Dalama, Aydın-Hamitler village). Among the samples, the genomic DNA isolation was performed by using a commercial kit (GeneMark). Table 1 and Table 2 list the primers used for the ISSR and cpDNA *trn*L intron region and PCR components used for the PCR amplifications. The PCR products were analyzed on 1.5% agarose gel via electrophoresis and the amplified products were detected after being dyed with ethidium bromide.

Table 1. ISSR primers and PCR components used for PCR amplification

ISSR Primers	DNA Sequences(5'-3')	Tm ºC	PCR components	PCR amplification (35 cycle)
UBC-807	AGAGAGAGAGAGAGAGT	50 ºC	1 µL genomic DNA 1 µL primer, 5 µL master mix (PCR buffer, 2 Mm $MgCl_2$, dNTP, 0.75 U Taq DNA polymerase) and 18 µL dH_2O	94 °C / 5 min 94 °C / 1 min 47-55 °C / 1 min 72 °C / 1 min 72 °C / 10 min.
UBC-808	AGAGAGAGAGAGAGAGC	52 ºC		
UBC-826	ACACACACACACACACC	52 ºC		
UBC-834	AGAGAGAGAGAGAGAYT	52 ºC		
UBC-836	AGAGAGAGAGAGAGAGYA	52 ºC		
UBC-840	GAGAGAGAGAGAGAGAAT	47 ºC		
UBC-856	ACACACACACACACACYA	52 ºC		
UBC-880	GGAGAGGAGAGGAGA	55 ºC		
UBC-892	TAGATCTGATATCTGAAT	52 ºC		
UBC-818	CACACACACACACACAG	47 ºC		

Table 2. cpDNA trnL intron primers and PCR components used for PCR amplification

Primer name	5' to 3' Primer sequence	References	PCR components	PCR amplification (35 cycle)
Forward *trn*C (F)	5'-CGAAATCGGTAGACGCTACG-3'	TABERLET *et al.*, 1991	1 µL genomic DNA 1 µL primer (*trn*C and *trn*D), 10 µL master mix (PCR buffer, 2 Mm $MgCl_2$, dNTP, 0.75 U Taq DNA polymerase) and 7 µL dH_2O	94 °C / 5 min 94 °C / 45 sec 50 °C / 45 sec 72 °C / 1 min 72 °C / 10 min.
Reverse *trn*D (R)	5'-GGGGATAGAGGGACTTGAAC-3'	TABERLET *et al.*, 1991		

ISSR Analyses

Following the ISSR-PCR analyses, the DNA bands were scored by giving the value "1" in the presence of DNA in the DNA bands, "0" in the absence of DNA and "?" for the unknown. The UPGMA phylogenetic tree was drawn using the program PAUP 4.0b10 (SWOFFORD, 2001). Using the same program, a genetic distance matrix was created between the populations.

cpDNA trnL intron Sequences

After amplifying the cpDNA *trn*L intron area via PCR, the resulting PCR products were sent to the biotechnology company TRIOGEN (İstanbul/Türkiye) for cycle sequencing. BioEdit 7.2.3 (HALL, 1999) and Finch TV 1.4.0 programs were used in processing the DNA sequences which came as files in the *ABI prism* format. *trn*L intron sequences were uploaded to NCBI. Genbank numbers: Çakmar population (OP961515), Çine population (OP961516), Dalama population (OP946323), Germencik population (OP961520), Hamitler population (OP961521), Incirliova population (OP961517), Koçarlı population (OP961518), Kuşadası population (OP961522), Aydın-Center population (OP961519), Söke population (OP961523) and Danişment population (OP961524). Using MEGA 6.0 (TAMURA *et al.,* 2013) program, maximum likelihood (Tamura-Nei model) phylogenetic tree was constructed. To evaluate the degree of support for given clades, a bootstrap analysis (1.000 replicates) was applied (FELSENSTEIN, 1985).

RESULTS AND DISCUSSION

With the improvement of molecular biology techniques and approaches, molecular markers have gained multiple utilization opportunities (PALIWAL *et al.,* 2022). Molecular markers are seen as a reliable tool for genetic identification among plant genotypes, identification of new species and breeding studies (KARIMI *et al.,* 2016; KIANI and SIAHCHEHREH, 2018). As a result of ISSR-PCR, total of 70 bands were obtained. Of these, 4 were monomorphic and 66 were polymorphic, and the polymorphism rate was found to be approximately 94.28%. Based on the ISSR data set, the UPGMA dendrogram created consisted of 3 groups (Figure 1).

Group A consists of Koçarlı and Çakmar populations. In Group B, İncirliova, Dalama, Center and Çine populations formed a subgroup, while Kuşadası, Germencik, Söke and Danişment Village populations formed a subgroup. Group C consisted of only the Hamitler village population. The maximum distance in the genetic distance matrix between the populations was found as 0.53333 between the Hamitler village population and Aydın-Centre population, and the minimum distance was 0.25714 between the Aydın-Centre and Dalama population (Table 3). cpDNA *trn*L intron region is widely used for phylogenetic inference at intraspecies and interspecies levels. In fact, noncoding regions provide the most practical data source for phylogenetic inference at lower taxonomic levels (SARRA *et al.,* 2015). In the cpDNA *trn*L analysis, the highest nucleotide sequence was detected in Kuşadası population (472 bp), and the lowest in Koçarlı population (454 bp). Average nucleotide ratio; A+T 64.5%, G+C 35.5% found.

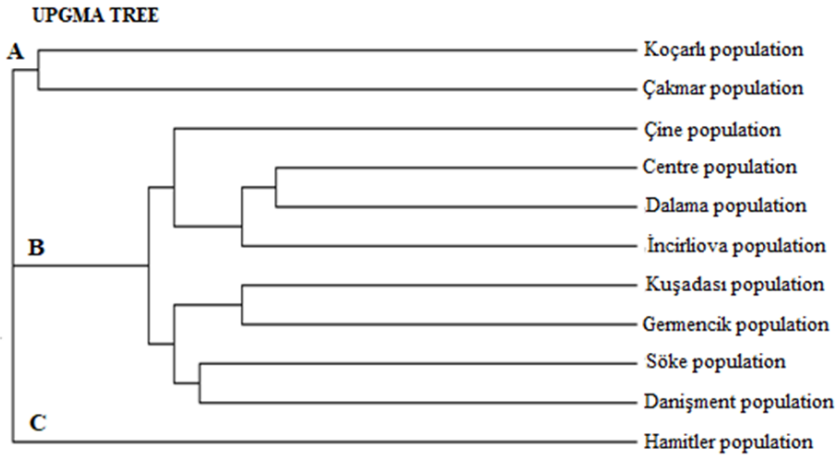

Figure 1. The UPGMA tree generated tree using ISSR data.

Table 3. Pairwise genetic distance matrix obtained from PCR with ISSR primers

Populations	1	2	3	4	5	6	7	8	9	10	11
Koçarlı	-	0.46552	0.50000	0.40000	0.43103	0.47143	0.44286	0.50000	0.44286	0.44286	0.45000
Çine	27	-	0.36207	0.31034	0.37931	0.31034	0.36207	0.43103	0.36207	0.32759	0.37500
Aydın-Centre	35	21	-	0.27143	0.43103	0.31429	0.34286	0.28571	0.34286	0.25714	0.53333
İncirliova	28	18	19	-	0.44828	0.32857	0.38571	0.35714	0.41429	0.30000	0.51667
Çakmar	25	22	25	26	-	0.44828	0.50000	0.46552	0.43103	0.43103	0.45833
Kuşadası	33	18	22	23	26	-	0.28571	0.37143	0.31429	0.31429	0.45000
Germencik	31	21	24	27	29	20	-	0.28571	0.37143	0.42857	0.46667
Söke	35	25	20	25	27	26	20	-	0.31429	0.34286	0.46667
Hamitler	31	21	14	29	25	22	26	22	-	0.28571	0.40000
Dalama	31	19	18	21	25	22	30	24	20	-	0.38333
Hamitler	27	18	32	31	22	27	28	28	24	23	-

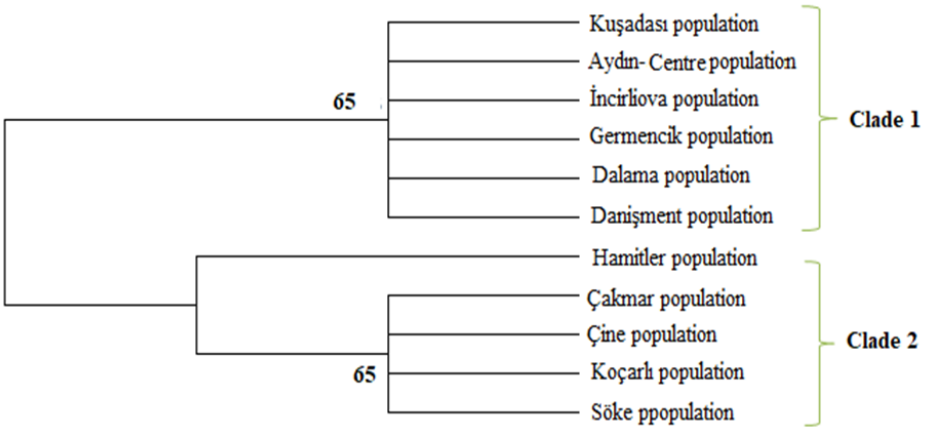

Figure 2. Phylogenetic tree of *Dittrichia viscosa* populations cpDNA *trn*L intron sequences constructed using maximum likelihood method

The genetic distance between *Dittrichia viscosa* populations was determined to be between 0.000 and 0.002. The phylogenetic tree based on maximum likelihood estimation is shown in Figure 2. Phylogenetic tree consist of two clades. Clade 1 consist of Kuşadası, Aydın-Centre, İncirliova, Germencik, Dalama and Danişment populations and this clade received 65% bootstrap value. Clade 2 consist of Hamitler, Çakmar, Çine, Koçarlı, and Söke populations.

In our study, cpDNA *trn*L intron sequences of *Chrysophthalmum montanum* (FM997843.1), *Chrysophthalmum gueneri* (FM997842.1), *Chrysophthalmum dichotomum* (FM997841.1), *Pulicaria dysenterica* (FM997870.1), *Pulicaria vulgaris* (FM997882.1), *Inula britannica* (AY216065.1), *Inula ensifolia* (KJ746382.1), *Inula salicina* (KU600396.1), *Jasonia tuberosa* (FM997859.1), *Stenachaenium campestre* (EF211026.1), *Carpesium glossophyllum* (MW137617.1), *Carpesium rosulatum* (MW137616.1), *Blumea virens* (EU195641.1), *Blumea sylvatica* (EU195638.1), *Iphiona scabra* (FM997857.1), *Iphiona pinnatifida* (FM997856.1), *Limbarda crithmoides* (FM997861.1), *Rhanterium epapposum* (EF211065.1), *Rhanterium suaveolens* (FM997884.1), *Lifago dielsii* (FM997860.1), *Duhaldea cappa* (EF211046.1), *Duhaldea rubricaulis* (FM997844.1), *Chiliadenus hesperius* (MG543223.1), *Chiliadenus rupestris* (MG543231.1), *Chiliadenus lopadusanus* (MG543238.1), and *Chiliadenus iphionoides* (MG543221.1) species were taken from NCBI (https://www.ncbi.nlm.nih.gov) nucleotide database, and combined with *Dittrichia viscosa* populations to form a maximum likelihood phylogenetic tree (Figure 3).

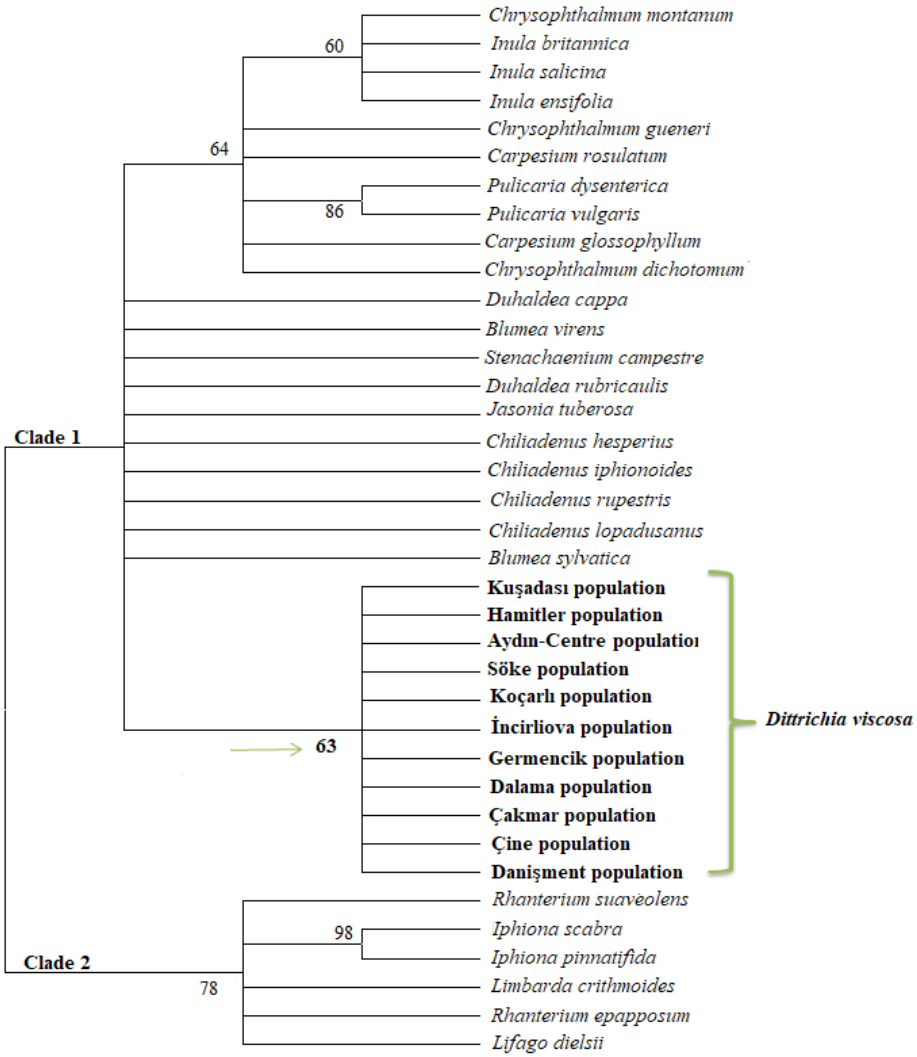

Figure 3. The maximum likelihood tree generated using cpDNA *tm*L intron *Dittrichia viscosa* populations and other species sequences retrieved from NCBI

The phylogenetic tree is divided into 2 major clades. In Clade 1; *Dittrichia viscosa* populations formed a separate group from other species and received 63% bootstrap value. Also, this clade consists of *Blumea*, *Chrysophthalmum*, *Pulicaria*, *Duhaldea*, *Stenachaenium*, *Carpesium*, *Jasonia*, *Inula* and *Chiliadenus* taxa. Clade 2 consists of *Lifago*, *Rhanterium*, *Limbarda*, *Iphiona* species. SEVINDIK *et al.* (2019), using RAPD teqnique 5 *Dittrichia viscosa*

populations polymorphism rate of 60% determined. In their study, the Koçarlı and Çine populations are one group, while the İncirliova, Çakmar and Merkez populations are a group. In our ISSR study, İncirliova and Merkez populations were related in group A, while other populations were detected in different groups. ISSR results were not very consistent with the RAPD study. In addition, the polymorphism rate determined in ISSR analysis was higher than that of the RAPD analysis. In *trn*L intron analysis, Aydın-Centre and İncirliova populations appeared in clade 1, while Çakmar, Çine and Koçarlı populations were in clade 2 (Figure 2). ELDENÄS *et al*. (1998) reported that *Dittrichia* was related to *Duhaldea* based on a strict consensus tree, while it is related to *Pulicaria* and *Jasonia* based on ITS sequence data. In a phylogenetic tree based on jackknife analysis of DNA sequences of *ndh*F (ANDERBERG *et al.*, 2005), *Pulicaria canariensis, Pulicaria dysenterica, Jasonia tuberosa* and *Dittrichia viscosa* species were identified in one group. This group received support with a bootsrapt value of 80%. In a combined cpDNA (*trn*L-F & *psb*A-*trn*H) and nrDNA (ITS) analysis, PORNPONGRUNGRUENG *et al.* (2007) determined that *Dittrichia viscosa* was associated with *Pulicaria paludosa, Anvillea garcini* ssp. *radiata, Pallenis spinosa* and *Rhanterium epapposum* which were placed in the same group. This group received support with a bootsrapt value of 64%. In another combined nrDNA ITS and cpDNA analysis, ENGLUND *et al.* (2009) identified *Dittrichia viscosa* species together with *Chiliadenus glutinosus Pulicaria dysenterica*, *Pulicaria vulgaris* and *Jasonia tuberosa*. Using ETS, ITS, *ndh*F, *trn*L-F and *trn*H-*psb*A analysis, NYLINDER and ANDERBERG (2015) detected *Dittrichia viscosa* and *Dittrichia graveolens* species together with *J. tuberosa, C.hesperius, C. rupestris, C. lopadusanus, C. iphionoides, C. saxatilis, P. glandulosa, P. dysenterica, P.auranitica, P. armena,* and *P. vulgaris*.

CONCLUSION

According to ISSR analysis, 94.28% polymorphism rate was detected among *Dittrichia viscosa* populations. Nevertheless, the phylogenetic position of *Dittrichia viscosa* with respect related taxa was documented using respective *trn*L intron sequences retrieved from NCBI. The results obtained support many previous studies in addition to presenting new insights about the phylogenetic relationship of *Dittrichia viscosa.*

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MOLEKULARNA KARAKTERIZACIJA POPULACIJA Dittrichia viscosa (L.) GREUTER (ASTERACEAE) POMOĆU ISSR MARKERA I HLOROPLAST (cpDNA) trnL INTRONSKIH SEKVENCI

Emre SEVINDIK[1*], Makbule BOZKURT[1], Mehmet YILMAZ[1], Ezginur ŞENYÜZ[1], Mehmet Yavuz PAKSOY[2]

[1*] Univerzitet Ajdin Adnan Menderes, Poljoprivredni fakultet, Odsek za poljoprivrednu biotehnologiju, Ajdin, Turska
[2]Odsek za medicinske usluge i tehnike, Tunceli strukovna škola, Univerzitet Munzur, 62000 Tunceli, Turska

Izvod

U ovoj studiji, molekularna karakterizacija populacija *Dittrichia viscosa* (L.) Greuter prikupljenih iz provincije Ajdin je sprovedena na osnovu ISSR tehnike i hloroplastne DNK *trnL* intronske sekvence. 10 ISSR prajmera je korišćeno za molekularnu karakterizaciju populacija. Za cpDNK *trnL* intron amplifikacije, korišćeni su *trnC* i *trnD* prajmeri. U ISSR analizi dobijeno je ukupno 70 traka. Utvrđeno je da je stopa polimorfizma približno 94,28%. Prema ISSR analizi, UPGMA dendrogram se sastojao od tri grupe. Za cpDNK *trnL* intronske sekvence, filogenetske analize su dobijene zajedno sa genetičkim distancama. Za populacije, cpDNK *trnL* intronske sekvence su određene između 454 i 472 baze. Filogenetsko stablo maksimalne verovatnoće se sastoji od dve klade. Pored toga, cpDNK *trnL* intronske sekvence nekih vrsta (*Chrisophthalmum Pulicaria Inula Jasonia Stenachaenium Carpesium Blumea Iphiona Limbarda Rhanterium Lifago Duhaldea Chiliadenus*) iz porodice Asteraceae i NCBI *Dittrichia viscosa* populacije upotrebljene su za konstruisanje filogenetskog stabla. Kao rezultat studije, utvrđeno je da je stopa polimorfizma populacija *Dittrichia viscosa* dobijenih ISSR-PCR-om veća od rezultata dobijenih iz *trnL* intronskih sekvenci.

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