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Phylogenetic relationship analysis of *Genista* L. (Fabaceae) species from Turkey as revealed by inter-simple sequence repeat amplification

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Genista L. (Fabaceae) is distributed in Europe, South Africa, and West Asia and consists of almost 90 species in the world. Thirteen species of the genus are distributed in Turkey. Taxonomic problems of the species started to be resolved with recently developed DNA-based molecular methods. These methods, in contrast to phenotypical analyses, are free from the effects of environmental conditions. In this study, DNA of the species which belong to the *Genista* genus, grown naturally in Turkey and collected from the different localities, were isolated with a commercial kit. Inter-simple sequence repeat (ISSR) is a simple molecular marker system that provides reliable results. Based on ISSR data, genetic similarities and dendrogram demonstrating the phylogenetic relationships among the *Genista* taxa were prepared by the NTSYSpC 2.0 software. In this study, infrageneric classifications of the *Genista* taxa belonging to the Flora of Turkey were conducted based on molecular data. ISSR analysis strongly supported the hypothesis that *G. aucheri* is accepted as the synonym of *G. sessilifolia*.

Key words: Leguminosae, papilionoideae, *Genista*, ISSR, phylogeny, Turkey.

INTRODUCTION

Fabaceae is the third largest family within flowering plants and is constituted of 650 genera that include about 18 thousand species (Kass and Wink, 1997). The family is represented with 69 genera and 974 species in Turkey (Davis et al., 1988) and is divided into 3 subfamilies, namely *Mimosoideae*, *Caesalpinioideae* and *Papilionoideae*. *Genista* with about 90 species belongs to *Papilionoideae* (Hickey and King, 1997; Duran and Dural, 2003). *Genista* is mainly distributed at the Mediterranean phytogeographic region that is related to the European region as well as North Africa and West Asia. The genus

is generally accepted to originate in the Mediterranean region. The species within *Genista* are all perennial shrubby plants. In Turkey, the genus is specifically widespread in the Mediterranean part of the country. Additionally, it is prevalent at the transition zones between both Mediterranean and Irano-Turanian regions and Mediterranean and Euro-Siberian regions. Specimens that belong to this genus are scarcely found at the Eastern and South-Eastern Anatolia (Gibbs, 1970; Davis et al., 1988; Duran and Dural, 2003). *Genista* is represented with 13 species in Anatolia. Among them five species are endemic to Turkey, with an endemism ratio of 33.3%. The endemic species are as follows; *Genista burdurensis* P.Gibbs, *Genista involucrata* Spach, *Genista aucheri* Boiss., *Genista sandrasica* Hartwig and Strid, *Genista vuralii* A. Duran and H. Dural (Gibbs, 1970; Davis et al., 1988; Duran and Dural, 2003). Important taxonomic problems exist among the species *Genista tinctoria* L., *Genista*

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Abbreviations: ISSR, Inter-simple sequence repeat; PCR, polymerase chain reaction; CTAB, cetyl trimethyl ammonium bromide; RAPD, random amplified polymorphic DNA.

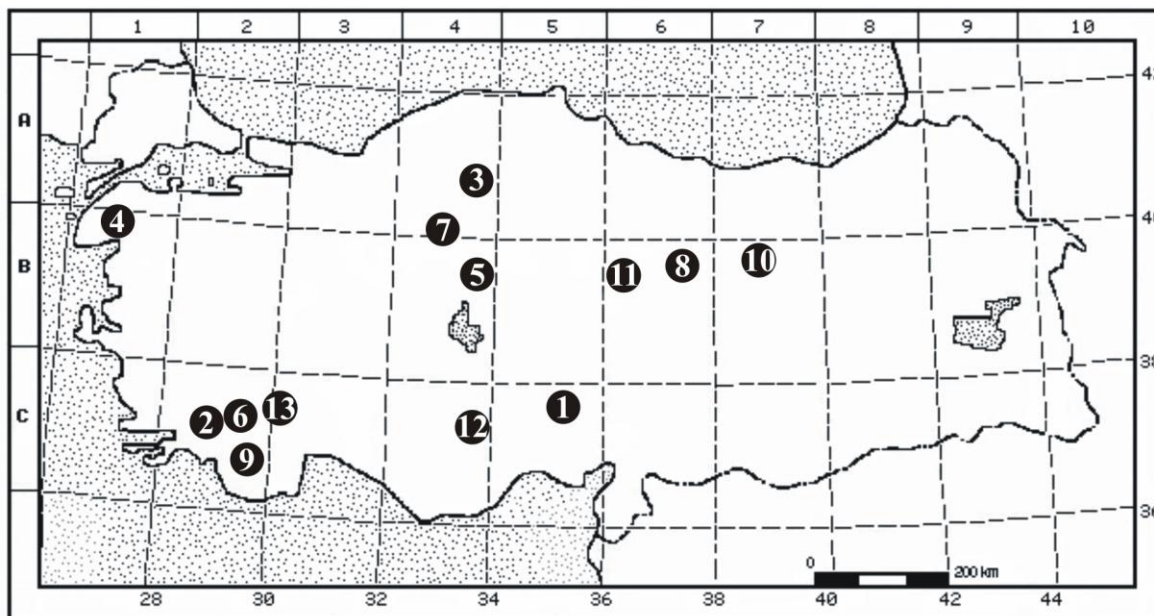


Figure 1. Distribution map of the examined specimens of the genus *Genista*. 1- *G. albida*, 2- *G. sandrasica*, 3- *G. vuralii*, 4- *G. carinalis*, 5- *G. sessilifolia*, 6- *G. anatolica*, 7- *G. januensis*, 8- *G. aucheri*, 9- *G. acanthoclada*, 10- *G. tinctoria*, 11- *G. involucrata*, 12- *Genista* sp., 13- *G. burdurensis*.

januensis Viv, *G. vuralii*, *G. sandrasica*, *G. burdurensis*, *Genista albida* Willd. and *G. involucrata* Spach. that constitutes a complex with apparent confusions. Specifically, *G. tinctoria*, *G. januensis*, *G. albida* and *G. involucrata* show high variation. Based on changing habitat leaf shape and size, plant height, hairiness of stem, leaf and fruit are significantly varying characters. Complications in the nomenclature of the herbaria specimens of *G. tinctoria* and *G. januensis* species as well as their sub-taxa are evident. Numerous specimens belonging to the genus *Genista* were collected in Turkey but unfortunately remained nameless due to the lack of adequate material, ambiguity of its variation limits and lack of comprehensive taxonomical studies. Literature records denote that uncertainty exists on the taxonomical status, hence nomenclature, of certain *G. tinctoria*, *G. januensis* and *G. sandrasica* specimens. Beyond the specimens of just mentioned complex, the species named as *Genista aucheri* is morphologically indistinguishable from the species named *Genista sessilifolia* DC, a situation frequently leading to misnaming. The objectives of the present study are: 1) To determine the genetic relationships among the selected Mediterranean phytogeographic region originated *Genista* species in Turkey, 2) to resolve the unclear and controversial status, based on conventional morphological studies, of the Turkish *Genista* species, 3) to determine the utility of inter-simple sequence repeat (ISSR) as a DNA based molecular marker system where a simple sequence repeat sequence is applied as the primer in polymerase

chain reaction (PCR).

MATERIALS AND METHODS

Plant materials

Genista specimens were collected from different localities in Turkey during vegetation seasons of 2004 - 2007. The collected specimens were marked with research's number and dried according to standard herbarium methods. The Flora of Turkey (Gibbs, 1970; Davis et al., 1988) was used to identify the plant specimens. The collected plant specimens were kept in Selçuk University Education Faculty Herbarium, Konya. The specimens' localities (Figure 1) and examined representative specimens are given in appendix.

DNA isolation

Leaves of specimens dried within silica gel or herbarium material were used for nuclear DNA isolations. DNA extractions were first tried with a standard 2x cetyl trimethyl ammonium bromide (CTAB) method. Due to the very poor DNA quality produced, a commercial kit (Nucleon Phytopure, Amersham Pharmacia) was used in all isolations and extractions or nucleic acid purifications were repeated whenever necessary. DNAs were isolated for every genotype and concentrations were determined by the use of a Nanodrop spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific). Sample DNAs were diluted to 25 ng/μl working concentrations and kept at -20°C. Stock DNAs were kept at -86°C.

ISSR amplifications

ISSRs are the molecular tools utilized in DNA fingerprinting. Out of

Table 1. ISSR primers used in this study and their specifications.

Primer	Primer sequence	T _m (°C)	SIZE (bp)	GC%	T _{an}
ISSR M1	(AGC) ⁶ -G	63.1	19	68.4	63
ISSR M2	(ACC) ⁶ -G	63.1	19	68.4	63
ISSR M3	(AGC) ⁶ -C	63.1	19	68.4	63
ISSR M5	(GA) ⁹ -C	56.7	19	56.7	56
ISSR F1	GAG-(CAA) ⁵	49.1	18	38.9	49
ISSR F2	CTC-(GT) ⁸	56.7	19	52.6	56
ISSR F3	(AG) ⁸ -CG	56	18	55.6	56
ISSR F8	(GCC) ⁵	67	15	100	67

50 primers used in preliminary tests, 8 most suitable ones (in terms of repeatability, scorability and the ability to distinguish between species) were selected for identification. The characteristics of the primers used are given in Table 1.

Each reaction contained 2 mM MgCl₂; 10 mM Tris-HCl (pH 8.8); 50 mM KCl; 0.8% Nonidet P40; 200 mM of each of the dNTPs; 0.5 μM primer; 20 ng DNA template and 0.3 units of *Taq* DNA Polymerase (Dream *Taq*, Fermentas) in a final reaction volume of 25 μl. After a pre-denaturation step of 3 min at 94°C, amplification reactions were optimized for every individual primer and optimization was started by cycling the reaction 35 times at 94°C for 1 min, at annealing temperature (Table 1) for 50 sec and 72°C for 1 minute in Eppendorf Mastercycler gradient thermocycler. A final extension was allowed for 10 min at 72°C. Amplifications were repeated at least twice (in different time periods) for each primer using the same reagents and procedure. Upon completion of the reaction, amplified products were loaded onto a 1.5% agarose/1x Tris-Borate EDTA gel run at 4 V/cm for gel electrophoresis.

Data collection and cluster phylogenetic analysis

Amplified fragments were visualized under a UV transilluminator and photographed using a gel documentation system (Vilbert Lourmat, Infinity model). All the fragments amplified were treated as dominant genetic markers. Each DNA band generated was visually scored as an independent character or locus (1 for presence and 0 for absence). Qualitative differences in band intensities were not considered. Every gel was scored in triplicate (independent scorings) and only the fragments consistently scored were considered for analysis. A rectangular binary data matrix was prepared and all the data analysis was performed using the Numerical Taxonomy System, NTSYS-pc version 2.0 (Applied Biostatistics, Exeter Software, Setauket, New York, USA). Cluster analysis of the specimens was performed with the unweighted pair-group method with the arithmetic mean (UPGMA) (Rohlf, 1992).

RESULTS AND DISCUSSION

Plants were collected from different locations, from the natural Flora of Turkey, and transferred to the laboratory on silica gel. DNA isolations from the silica-gel dried leaves of these shrubby plants were highly problematic. The first method applied was a standard 2x CTAB procedure. A very viscous solution was unavoidable with this procedure, even after a number of modifications.

These impurities were probably attributed to the rich phenolic contents of these plants. Phytoestrogens that are of pharmaceutical value due to their estrogen-like biological activities were previously reported within the genus *Genista* (Erdemoglu et al., 2006). Genistein is of utmost value among other nutraceutical isoflavonoids produced in plants including *Genista* (Tosun et al., 2003). DNA quality from this woody plant was enhanced by use of a commercial DNA extraction kit, Nucleon Phytopure. Viscosity due to phenolic compounds was eliminated in most of the specimens. DNA isolates were further purified by applying the solution to a Qiagen DNeasy plant mini kit column. Although this was in expense of DNA concentration, impurities that inhibit PCR amplifications were avoided and still, adequate amount of DNA were obtained. From an initial screening of 50 ISSR primers, eight primers revealed high levels of polymorphism while most of the eliminated primers either produced inconsistent fragments in only one or two specimens or did not amplify any fragment at all. The selected 8 reliable primers generated 83 highly polymorphic bands that were consistently amplified in repeated experiments conducted in separate dates. The guanine-cytosine (GC) percentages of the selected primers were all above 52% (three of them being 68.4% and one 100%) except for one (ISSR F1, 38.9%). Five of the primers used in the analyses were 19 bp long and the two primers were 18 bp long, while the remaining one (ISSR F8) was 15 bp in size. Annealing temperatures of the oligos were about the same as the melting temperatures. The characteristics as well as the sequences of the primers revealing a polymorphism is shown in Table 1. In total, the average number of polymorphic fragments per primer used was roughly 10. A representative figure containing ISSR M3 and ISSR F8 banding patterns is given (Figure 2).

An acceptable and reliable infrageneric classification of the *Genista* taxa naturally grown in Turkey is a necessity. This should obviously be achieved *via* currently available molecular phylogenetics methods. It is almost impossible to make a sound and convenient classification solely based on morphological data. Within this study, the phylogenetic relationships of the taxa belonging to *Genista*

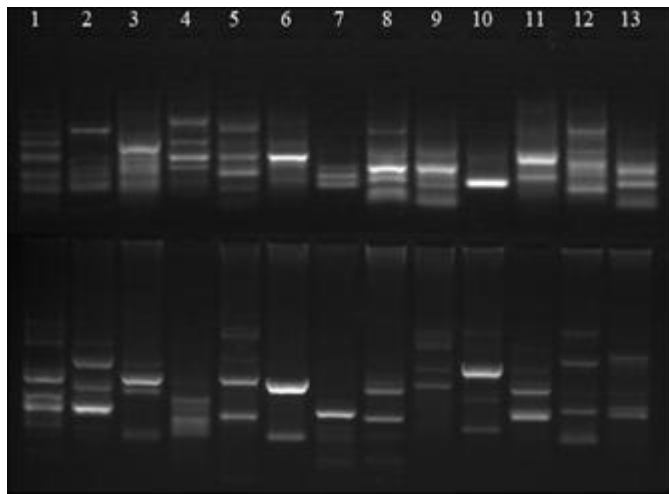


Figure 2. Representative agarose gel where PCR products were amplified with the primers ISSR M3 (top) and ISSR F8 (bottom). 1- *Genista albida*, 2- *G. sandrasica*, 3- *G. vuralii*, 4- *G. carinalis*, 5- *G. sessilifolia*, 6- *G. anatolica*, 7- *G. januensis*, 8- *G. aucheri*, 9- *G. acanthoclada*, 10- *G. tinctoria*, 11- *G. involucrata*, 12- *Genista* sp., 13- *G. burdurensis*.

were faithfully determined by use of a simple but effective molecular method named ISSR. ISSR is a technique offering the simplicity of random amplified polymorphic DNA (RAPD) but as a dominant marker it is much more reliable. It involves amplification of genomic regions between adjacent, inversely oriented microsatellites using a single simple sequence repeat primer. The use of the tandem repeat motifs of di-, tri-, or tetranucleotides that are abundant in all eukaryotic genomes produces a high number of polymorphic fragments, especially in plants (Hamada et al. 1982). Since it is simple, fast, cost-effective, highly discriminating and highly reliable, in recent studies, it is widely applied in plant genetic analyses (Liu and Wendel, 2001; Arnau et al. 2003; Vijayan and Chatterjee, 2003; Dogan et al., 2007; Hakki et al., 2007). Sections belonging to the taxa of the genus *Genista* in Turkey are as follows:

- Section *Genista*: *G. tinctoria*, *G. januensis*, *G. vuralii*, *G. burdurensis*, *G. sandrasica*
- Section *Spartioides* Spach.: *G. albida*, *G. involucrata*
- Section *Voglera* (P.Gaertner, B.Meyer and Scherb.) Spach: *Genista carinalis*, *Genista anatolica*
- Section *Asterospartum* Spach.: *G. sessilifolia*, *G. aucheri*
- Section *Acanthospartum* Spach.: *Genista acanthoclada*

General characteristics of the sections that include the above taxa are given in Table 2. Sectional classification of the taxa revealed congruence with the dendrogram based on the evaluation of the molecular data (Figure 3, Table 2). *Genista* and *Spartioides* are the sections gathered in one clade while the remaining three sections were

grouped into the second clade. It is worthy of mention that the taxa belonging to the section *Genista* were clearly separated as a distinct subgroup. On the other hand, *G. albida* and *G. involucrata* were the taxa that belong to *Spartioides* and constituted a different subgroup on the same clade. With the exception of their common hair character, the sections *Genista* and *Spartioides* were similar in terms of their remaining morphological characteristics, a feature that is also strongly supported by molecular evidence (Figure 3, Table 2).

The sections *Voglera*, *Asterospartum* and *Acanthospartum* constitute another clade on the dendrogram. Shared characters of the sections within this clade include the fruits that are ovoid-acuminate and the number of their seeds that are one or two. Taxa of the section *Asterospartum* in Turkey are thornless and their leaves are 3 - foliolate. This section was found to form another subgroup on the dendrogram. The section named as *Acanthospartum* was distinguished from the section *Asterospartum* due to its spiny character, hence it is found as a distinct subgroup under the same clade with *Asterospartum* (Figure 3, Table 2). *Voglera* is the section that is represented with *G. anatolica* and *G. carinalis* in Turkey. Both of these species have simple leaves, but *G. carinalis* is the species that is thornless while *G. anatolica* is a spiny species. On the dendrogram, the species belonging to the section *Voglera* were found in different subgroups. *G. anatolica* was part of a subgroup with *G. acanthoclada*, a species included in the section *Acanthospartum*. These two species are morphologically similar due to their common spiny characters, a special feature that seems to be the most decisive character in sectional assortment. On the dendrogram, the remaining species of the section *Voglera*, named as *G. carinalis*, was clearly separated from the two other sections (sect. *Asterospartum* and *Acanthospartum*) of the clade (Figure 3, Table 2). The specimen collected from Eregli (B.Doğan 1547, Konya province) has some resemblance to the species *G. sessilifolia*, but distinguishing morphological characters suggested it as a different subspecies (submitted for publication). It was named as *Genista* sp. It is also similar to *G. sessilifolia*. It mainly differs from *G. sessilifolia* because it has low shrubs, stems 5 - 8 (-13) cm tall, ending in leafy (not tall shrubs, stems 15 -100 cm tall, ending in an inflorescence), flowers solitary or two per stem (not inflorescence, a raceme or sometimes axillaries cluster), bracts foliaceous, equaling the cauline leaves (not bracts usually reduced, smaller than the cauline leaves).

The species *G. sessilifolia*, *G. aucheri* and *Genista* sp. have remarkable morphological characteristics in common with an overall similarity, based on simple matching similarity index of about 87%. In this study, it was not possible to discriminate *G. aucheri* from *G. sessilifolia*. Thus, ISSR analysis strongly supported the hypothesis.

Table 2. Sectional characteristics of the taxa belonging to the genus *Genista* in Turkey (Gibbs, 1970).

Section	Taxa	Characters			
		Plant	Leaves	Corolla	Fruits
<i>Genista</i>	<i>G. tinctoria</i> <i>G. januensis</i> <i>G. vuralii</i> <i>G. burdurensis</i> <i>G. sandrasica</i>	unarmed	simple, mostly glabrous	glabrous, standard broadly ovate, equaling the wings and keel	narrowly oblong, seeds 3-10
<i>Spartioides</i>	<i>G. albida</i> <i>G. involucrata</i>	unarmed	simple	glabrous, standard broadly ovate, equaling the wings and keel, usually sericeous	sericeous to semi-patent hairs, narrowly oblong, seeds 2 or more
<i>Voglera</i>	<i>G. carinalis</i> <i>G. anatolica</i>	unarmed or spiny	simple or 3-foliolate	standard usually triangular or ovate with an acute apex, usually shorter than keel	ovoid-acuminate, seeds 1-2
<i>Asterospartum</i>	<i>G. sessilifolia</i> <i>G. aucheri</i>	unarmed shrubs	simple or 3-foliolate, leaves usually opposite or subopposite	standard broadly or angular-ovate, equaling or shorter than keel	ovoid-acuminate to falcate, seeds 1-2
<i>Acanthospartum</i>	<i>G. acanthoclada</i>	Shrubs with opposite branches, spiny	3-foliolate, opposite or alternate	standard rhombic, shorter than the keel	ovoid-acuminate, seeds 1-2

that *G. aucheri* is accepted as the synonym of *G. sessilifolia*. Additionally, our hypothesis that *G. sp.* is the new subspecies that is most closely related to *G. sessilifolia* was also supported with the molecular data. Within the context of this study, infrageneric classifications of the *Genista* taxa belonging to the Flora of Turkey were conducted based on molecular data. According to the cluster analysis, the species with similar morphological characters constituted the first clade. The remaining taxa constituted the second clade with a remarkably low resemblance to the first one. Their similarity coefficient, based on simple matching similarity index, was only 0.28 (Figure 3). A remarkable correlation was detected between the places of the taxa that are obtained with the use of morphological characteristics and that of the dendrogram generated by use of

molecular data (Figure 3, Table 2).

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APPENDIX

Examined representative specimens

G. albida. Adana: Aladağ, 672 m, 09.06.2007, under forest, M.Dinc 2930, A. Duran and B. Bilgili; *G. involucrata*. Sivas: between Akdağmadeni-

Yıldızeli, 25th km, 1275 m, 26.07.2006, open *Quercus* forest, M.Dinc 2808 and A.Duran; *G. burdurensis*. Burdur: Tefenni, road Yeşilova, Karamanlı damp vicinity 1220 m, 12.06.2006, open *Quercus* forest, M.Dinc 2733. *G. sandrasica*. Muğla: Köyceğiz, Sandras Mountain, 1620 m, 11.06.2006, open *Pinus nigra* forest, M.Dinc 2694; *G. januensis*. Ankara: Yenimahalle, SW of Yuva village, 1100 m, 30.05.2004, A.Duran 6408; *G. vuralii*. Çankırı: Ilgaz Mountain, transmitter vicinity, 2050 m, 29.07.2006, open *Abies* forest, M.Dinc 2879 and A.Duran; *G. tinctoria*. Erzincan: between Refahiye-İliç, 7th km, 1740 m, 27.07.2006, steppe, M.Dinc 2844 and A.Duran; *G. carinalis*. Balıkesir: Edremit, Kaz Dağı, 550 m, 09.06.2006, open forest, M.Dinc 2654 and B.Dogan; *G. sessilifolia*. Kırşehir: between Şereflikoçhisar-Karaman, 5th km to Karaman, 1000 m, 26.07.2006, steppe, M.Dinc

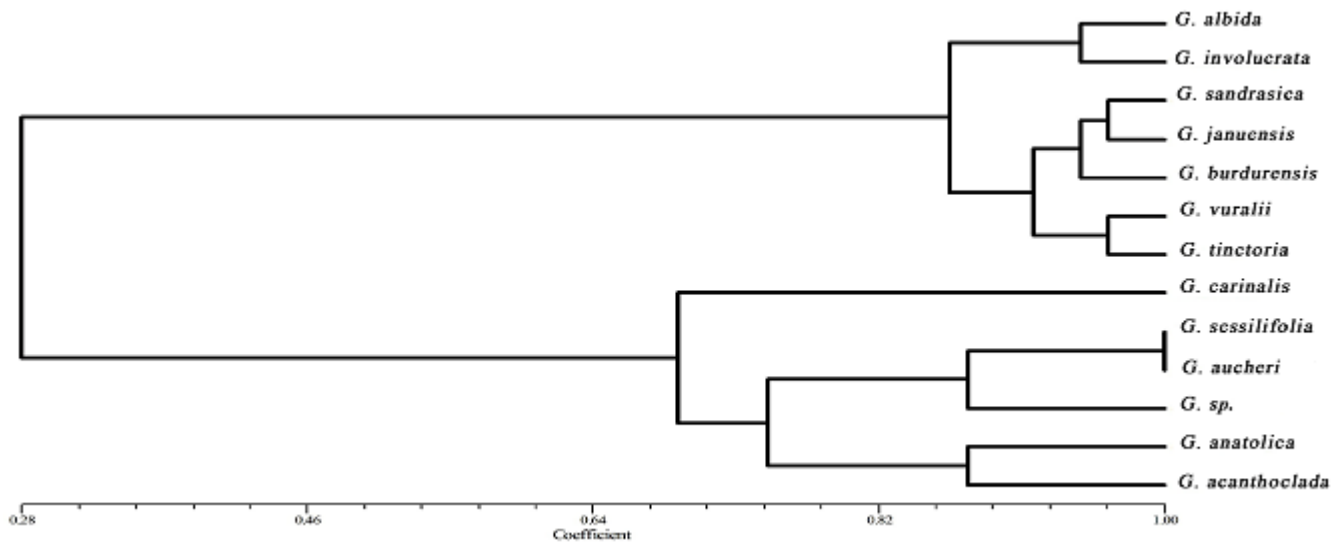


Figure 3. Dendrogram showing the genetic relationships of *Genista* taxa generated by using inter simple sequence repeats.

2810 and A.Duran; *G. aucheri*. Sivas: between Sivas-Zara, 5th to Zara, 1350 m, 26.07.2006, steppe, M.Dinc 2811 and A.Duran; *Genista* sp. Karaman: between Ayrancı-Çatköy, 4th km to Çatköy, 1600 m, 03.08.2007, slopes, B.Doğan 1547; *G. anatolica*. C2 Muğla: Köyceğiz Sandras Mountain, 1180 m, 11.06.2006 open forest, M.Dinc 2682 and B.Dogan; *G. acanthoclada*. C2 Muğla: between Fethiye-Korkuteli, 900 m, 13.06.2006, open forest, M.Dinc 2736 and A.Duran.

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