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Phylogenetic and cladistic analyses of the enigmatic genera *Bituminaria* and *Cullen* (Fabaceae) in Turkey

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Abstract: Three taxa of the genus *Psoralea* L. growing naturally in Turkey and the outgroup taxa belonging to the closest genera *Vicia* L., *Cicer* L., and *Astragalus* L. were subjected to molecular and quantitative morphological analyses in order to characterize their phylogenetic relationships. Both the taxonomical and the molecular characteristics of the tribes Psoraleeae (*Bituminaria* Heist. ex Fabr., *Cullen* Medik.), Vicieae (*Vicia*), Astragalae (*Astragalus*), and Cicereae (*Cicer*) were clearly revealed for the first time with the inter-simple sequence repeat (ISSR) method. The phylogenetic relationships were determined with 253 ISSR band scores and 125 quantitative morphological character measurements. The numerical and molecular data sets were analyzed with NTSYSpc and Minitab software. Based on these data, the current circumscription of the genus *Psoralea* was reevaluated and compared with outgroups in Turkey. Consequently, the closely related genera of *Bituminaria* and *Cullen* clearly occur in Turkey instead of the genus *Psoralea*.

Key words: *Psoralea*, Papilionoideae, Leguminosae, numeric, ISSR, Turkey

1. Introduction

Fabaceae, the legumes, is the third largest plant family on earth after the orchids (Orchidaceae) and daisies (Asteraceae or Compositae), with an estimated 19,000 species (Lewis et al., 2005). Within this family, the subfamily Papilionoideae comprises the majority of the species (~14,000) as compared to Mimosoideae and Caesalpinioideae (Egan and Crandall, 2008). In the most recent overview of the complete family, Polhill (1994) increased the recognized total number of genera from 671 to 727. This recent increase in the number of genera was largely due to the genus-level subdivision of several large paraphyletic (unnatural) genera (Lewis et al., 2005).

In Turkey, Fabaceae is the second largest flowering plant family with 1013 species belonging to 71 genera. Among these species, 400 are endemic to Anatolia, representing a 40% endemism rate for the family, the second highest rate of endemism according to the *Flora of Turkey* (Erik and Tarıkahya, 2004).

Barneby (1977) combined Daleae Hutch. and the genera *Parryella* Torr. & A.Gray ex A.Gray, *Eysenhardtia* Kunth, *Psorobatus* Torr. & A.Gray ex A.Gray, *Psorodendron* Nutt., *Psorothamnus* Rydb., *Apoplanesia* C.Presl, *Marina*, and *Amorpha* L. of Psoraleeae Hutch. into the tribe Amorpheae. In Barneby's view, Amorpheae and Psoraleeae differ in branching patterns, total anthotaxy, and, to a lesser extent, petal insertion, foliage, and geographical distribution. Stirton (1981a) used cotyledons, the arrangements of the embryo and radicle in seeds, seed shape, fruit structure, and pollen to support Barneby's separation of Psoraleeae and Amorpheae.

After a critical inspection of the remaining 10 genera accepted by Hutchinson (1964), with 8 transferred to Amorpheae, it became necessary to reallocate species into 6 genera: *Psoralea* L., *Hallia* Thunb., *Cullen* Medik., *Bituminaria* Heist. ex Fabr., *Otholobium* C.H.Stirt., and *Orbexilum* Raf. This redelineation was based on detailed dissections of flowers, inflorescence, fruits, seeds and study of leaf arrangement with leaf morphology. These considerations are implicit in the reorganization of Psoraleeae by Stirton (1981a). The tribe Psoraleeae is a monophyletic group of 9 genera and 185 species (Lewis et al., 2005).

The genus *Psoralea* is restricted to only 20 species, mostly endemic to Mediterranean regions or southern Africa. The remaining species are assigned to other genera. The genus *Cullen* is considerably expanded to include the remaining 6 species of *Psoralea* from Africa. In total, 35 *Cullen* species are recognized, most of which extend through India, Sri Lanka to Burma, the Philippines, Papua

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New Guinea, and Australia. The essentially European genus *Bituminaria* comprises 2 species endemic to Mediterranean Europe, North Africa, and Euxine areas (Stirton, 1981a).

The genus *Psoralea* is represented by 3 species in the *Flora of Turkey* (Davis, 1970; Davis et al., 1988; Özhatay et al., 2009). According to Stirton (1981b), *Psoralea bituminosa* and *P. acaulis* were transferred into the genus *Bituminaria* Heist. ex Fabr. as *B. bituminosa* (L.) C.H.Stirt. and *B. acaulis* (Hoffm.) C.H.Stirt., respectively, and *Psoralea jaubertiana* Fenzl was transferred into the genus *Cullen* Medik. as *Cullen jaubertianum* (Fenzl) C.H.Stirt. According to the latest classification of Stirton, 1 *Cullen* taxon and 2 *Bituminaria* taxa occur in Turkey (Güner et al., 2012).

Previous phylogenetic analyses of Fabales using molecular methods revealed conflicting interfamilial relationships, failing to provide adequate and convincing support for these relationships (Doyle et al., 2000; Persson, 2001; Bello et al., 2009; Bandara et al., 2013). Tucker exploited previous explorations of floral evolution within Leguminosae for phylogeny reconstruction several times (e.g., Tucker, 1987, 1997, 2003; Tucker and Stirton, 1991; Tucker and Douglas, 1994; Prenner and Klitgaard, 2008).

The *matK* and *rbcL* coding genes revealed elevated nucleotide substitution rates for the tribe Psoraleeae, showing rapid evolution or diversification (Lavin et al., 2005). Age estimation demonstrates the recent diversification of 2 genera, *Cullen* of Australia and *Rupertia* in tribe Psoraleeae, endemic to the western United States, at approximately 6.3 million years ago and especially as the North American clade of Psoraleeae diversified after the transcontinental split (Lavin et al., 2005; Egan and Crandall, 2008).

The inter-simple sequence repeat (ISSR) method targets particular sequences that are abundant in the eukaryotic genome; it overcomes the technical difficulties of restriction fragment length polymorphism and random amplified polymorphic DNA. As a commonly preferred marker system, it generates reproducible results and is a highly polymorphic DNA-level characterization method (Rafalski et al., 1996; Bornet and Branchard, 2001).

The aim of this study was to determine the genetic relationships among the *Bituminaria* and *Cullen* species and to resolve controversial inter- and intraspecific statuses using a combination of morphological and reliable molecular marker analyses. Such a comprehensive study covering all *Psoralea* genera in Turkey would be necessary to make a more thorough classification to decide the current circumstance of the genera *Bituminaria* and *Cullen*. Although this study contributes new conclusions to the literature, it is limited to the known *Bituminaria* and *Cullen* genera. It would be very useful for further studies to use numerical and molecular data.

2. Materials and methods

2.1. Specimen collection

During field trips in Anatolia between the 2011 and 2012 vegetation periods, specimens from 3 *Psoralea* L. taxa were collected. Leaf samples belonging to 4 *Psoralea* and 3 outgroup specimens from different localities were dried in silica gel. A list of the taxa used in this study, specimens of which are currently kept in the KNYA herbarium, is given in the Appendix.

In total, 125 morphological characters were determined from the 3 taxa of the genus *Psoralea* and compared with species of *Cicer*, *Astragalus*, and *Vicia*. These characters were placed under 5 headings of habit, stem characteristics, leaf characteristics, flower structure, and pod characteristics (Table 1). Character state transformations were selected as unordered. The polarity of characters was determined using the outgroup method (Maddison et al., 1984; Meher et al., 2012).

2.2. DNA isolation

Nuclear DNA was isolated from leaves of both herbarium specimens and fresh materials using the CTAB method as given in the Appendix by asterisks (Sambrook et al., 1989). Total DNA was obtained from 50 to 75 mg of dried leaf tissue from 9 different individual samples. DNA concentrations were determined with NanoDrop. DNA samples were diluted to 25 ng/µL. Stock DNA solutions were kept at –86 °C.

2.3. ISSR amplifications

PCR reactions with ISSR primers were amplified in a thermal cycler (Eppendorf Mastercycler Gradient Thermocycler). The characteristics of the primers used are given in Table 2. Each 25-µL PCR reaction contained 2.5 µL of PCR buffer (10 mM TRIS/50 mM KCl buffer, pH 8.0), 3 µL of 25 mM $MgCl_2$, 0.5 µL of primer solution (25 pmol total), 0.5 µL of 100 mM dNTP mix, 0.4 µL of 5 U/µL Taq DNA polymerase, 4 µL of template DNA, and 14.1 µL of distilled water. After a 3-min predenaturation step at 94 °C, reactions were cycled 40 times at 94 °C for 1 min, at annealing temperature (Table 2) for 1 min, and at 72 °C for 1 min with a final extension for 10 min at 72 °C. Upon completion of the reaction, 15-µL aliquots of the PCR products were mixed with 3 µL of loading dye (50% glycerol, 0.25% bromophenol blue, and 0.15% xylene cyanol) and loaded onto a 2% agarose 1X Tris-borate-EDTA gel and subjected to electrophoresis at 4 V cm^{-1}. Amplified fragments were visualized under a UV transilluminator and photographed using a gel documentation system (Vilber Lourmat, Infinity model) (Figure 1).

2.4. Data collection and cluster analysis

All fragments amplified were treated as dominant genetic markers. Each DNA band was visually scored as an independent character or locus ('1' for presence and '0' for

Table 1. List of characters used in numerical taxonomic analyses.

GENERAL	**LEAVES**
	Leaf arrangement: 0 = imparipinnate; 1 = paripinnate; 2 = trifoliate
Growth cycle: 0 = annual; 1 = perennial	Rachis length (mm)
Root radius (mm)	Rachis apex: 0 = leaflet; 1 = tendrils; 2 = tendrilous
Nodules: 0 = absent; 1 = present	Leaf shape outline: 0 = oblong; 1 = ovate; 2 = obovate-linear
Vegetative shoots: 0 = absent; 1 = present	
Form: 0 = erect; 1 = procumbent; 2 = ascending	Midrib tooth: 0 = toothless; 1 = prominent; 2 = distinct
Phenology: 0 = April; 1 = May; 2 = June; 3 = July; 4 = August	Veins of leaflet surface: 0 = none; 1 = prominent; 2 = distinct
Altitudinal range min (m)	Leaflet color: 0 = discolor; 1 = bicolor
Altitudinal range max (m)	Leaflet hair type: 0 = eglandular; 1 = glandular; 2 = mixed; 3 = glabrous
Distribution: 0 = broad; 1 = province; 2 = local	Leaflet hairiness density: 0 = sparse; 1 = dense; 2 = glabrous
Habitat: 0 = cultivation; 1 = fallow fields; 2 = steppe; 3 = forest; 4 = screes; 5 = rubble; 6 = variable	Petiolulate length (mm)
Soil type – Geology: 0 = calcareous; 1 = serpentine; 2 = basalt, 3 = volcanic	Leaflet petioles: 0 = sessile; 1 = subsessile; 2 = petiolate
Endemic: 0 = no; 1 = yes	Stipule outline: 0 = subulate; 1 = ovate; 2 = obovate; 3 = lanceolate; 4 = triangular
Phytogeographical element: 0 = cosmopolite; 1 = Ir.-Tur.; 2 = Medit.; 3 = Eu.-Sib.; 4 = multiregional	Stipule teeth shape: 0 = incised; 1 = dentate; 2 = incised-dentate; 3 = incised- laciniate; 4 = serrate; 5 = toothless
IUCN category	Stipule length (mm)
STEMS	Number of teeth on stipule
Woodiness: 0 = woody; 1 = not woody	Stipule length/leaflet length: 0 = longer; 1 = shorter; 2 = equal
Stem strength: 0 = weak; 1 = strong	Terminal leaflet: 0 = smaller; 1 = equal; 2 = bigger
Tufted: 0 = yes; 1 = no	Tendril structure: 0 = none; 1 = simple; 2 = branched
Branching: 0 = simple; 1 = branched; 2 = simple+branched	Rachis apex: 0 = absent; 1 = slightly; 2 = strongly
Flowering: 0 = completely; 1 = flowering part; 2 = at base; 3 = absent	Number of pairs of leaflets min
Secondary branches' length (cm)	Number of pairs of leaflets max
Stem length min (cm)	Leaflet shape: 0 = oblong; 1 = linear; 2 = elliptic; 3 = rounded; 4 = broadly ovate; 5 = lanceolate-broadly ovate; 6 = obovate
Stem length max (cm)	Leaflet length min (cm)
Cross-section of stem: 0 = slender; 1 = quadrangular; 2 = circular	Leaflet length max (cm)
Stem surface ribbed: 0 = faintly; 1 = ribbed; 2 = prominently	Leaflet width min (cm)
Stem hairiness density: 0 = sparse; 1 = dense; 2 = glabrous	Leaflet width max (cm)
Stem hair type: 0 = eglandular; 1 = glandular; 2 = mixed; 3 = glabrous	Leaflet margin: 0 = entire; 1 = incised; 2 = incised-serrate; 3 = serrate; 4 = dentate; 5 = crenate-dentate
Internode length (cm)	Leaflet teeth on margin: 0 = entire; 1 = 0-2\3; 2 = 1\2-2|\3; 3 = apex-5\6; 4 = absent
Flowering part on stem: 0 = horizontal; 1 = erect; 2 = decumbent	Leaflet apex: 0 = truncate; 1 = acute; 2 = cuspidate; 3 = acuminate; 4 = rounded; 5 = cirrose; 6 = apiculate; 7 = obtuse; 8 = aristate
Stem orientation: 0 = straight; 1 = flexuous; 2 = both; 3 = dichotomic	Corolla length (mm)
	Corolla color: 0 = white; 1 = purple; 2 = pale smoky blue; 3 = yellow; 4 = fuchsia

Table 1. (continued).

Petiole length (mm)	Calyx/corolla length (mm): 0 = longer; 1 = shorter; 2 = equal
End of leaflets' veins: 0 = toothless; 1 = teeth; 2 = spinulose	Vexillum width (mm)
Leaflet base: 0 = truncate; 1 = cuneate; 2 = rounded-cuneate; 3 = rounded; 4 =obtuse	Vexillum limb length (mm)
Leaflet teeth shape: 0 = mucronate; 1 = acuminate; 2 = triangular; 3 = acute; 4 = absent	Vexillum claw length (mm)
Leaflet teeth apices: 0 = spine; 1 = mucro; 2 = none	Vexillum auricle length (mm)
Number of teeth per leaflet	Vexillum length (mm)
FLOWERS	Vexillum auricle secretory: 0 = glabrous; 1 = glandular
Flowering part: 0 = erect; 1 = erect-ascending; 2 = procumbent	Vexillum claw/limb ratio: 0 = longer; 1 = shorter; 2 = equal
Flowering part length (cm)	Vexillum hairiness: 0 = glabrous, 1 = hairy
Flowering part branching: 0 = simple; 1 = branched	Vexillum margin: 0 = plane; 1 = wavy
Flowering part: 0 = leaf axils; 1 = bract axils; 2 = both	Vexillum apex: 0 = none; 1 = pitted; 2 = pitted-mucro
Peduncle length (mm)	Vexillum hair: 0 = absent, 1 = present
Peduncle indumentum: 0 = eglandular; 1 = glandular; 2 = mixed; 3 = glabrous	Vexillum shape: 0 = emarginate; 1 = rounded; 2 = retuse
Pedicel length (mm)	Wing width (mm)
Pedicel hair type: 0 = eglandular; 1 = glandular; 2 = mixed; 3 = glabrous	Wing claw length (mm)
Peduncle/pedicel ratio: 0 = longer; 1 = shorter; 2 = equal	Wing limb length (mm)
Pedicel/petiole ratio: 0 = longer; 1 = shorter; 2 = equal	Wing auricle length (mm)
Number of bracts	Wing auricle/claw ratio: 0 = longer; 1 = shorter; 2 = equal
Bract shape: 0 = entire; 1 = linear; 2 = triangular; 3 = dentate; 4 = subulate; 5 = ovate; 6 = absent	Carina limb length (mm)
Bract length (mm)	Carina length (mm)
Bract width (mm)	Carina width (mm)
Number of teeth on bracts	Carina shape: 0 = triangular; 1 = oblong; 2 = ovate; 3 = spatulate; 4 = rhombic; 5 = elliptic
Teeth length of bract (mm)	FRUIT
Bract hairiness: 0 = hairy; 1 = glabrous	Pod shape: 0 = oblong; 1 = ovate; 2 = elliptic
Bract surface: 0 = eglandular; 1 = glandular; 2 = both; 3 = glabrous	Legume shape: 0 = rhombic; 1 = plane; 2 = only ventral side curved; 3 = only dorsal side curved
Bracteoles: 0 = absent; 1 = present	Pod length (mm)
Calyx length (mm)	Pod width (mm)
Number of calyx teeth	Pod hair type: 0 = eglandular; 1 = glandular; 2 = mixed; 3 = glabrous
Calyx teeth shape: 0 = linear; 1 = lanceolate; 2 = cuspidate; 3 = subulate; 4 = linear-lanceolate	Pod hair shape: 0= pubescent; 1 = pilose; 2 = villous
Calyx teeth apex: 0 = acute; 1 = acuminate	Beak: 0 = absent; 1 = present
Calyx tooth length (mm)	Pod/beak length ratio: 0 = longer; 1 = shorter; 2 = equal
Calyx tube length (mm)	Beak indumentums: 0 = glabrous; 1 = hairy
Calyx tooth/tube ratio: 0 = longer; 1 = shorter; 2 = equal	Beak length (mm)
Calyx shape: 0 = strongly saccate; 1 = weakly saccate	
Calyx indumentums: 0 = hairy; 1 = glabrous	

Table 2. The characteristics of the ISSR primers.

Primer	Sequences	T_m (°C)	G/C ratio (%)	Length (bp)	Polymorphic bands
ISSR 5	5'-ACA CAC ACA CAC ACA CCG-3'	56.0	55.6	18	32
ISSR 8	5'-CGT CAC ACA CAC ACA CAC A-3'	56.7	52.6	19	42
F4	5'- AGA GAG AGA GAG AGA GTG- 3'	53.7	50.0	18	17
F9	5'-GAA GAA GAA GAA GAA-3'	39.6	33.3	15	29
UBC840	5'-GAG AGA GAG AGA GAG AYT-3'	56.5	47.2	18	36
M7	5´- AGA GAG AGA GAG AGA GAG C- 3´	56.7	52.6	19	19
M15	5'- CAC ACA CAC ACA CAC AAG -3'	53.7	50.0	18	57
M16	5'- CAC ACA CAC ACA CAC AGC -3'	56.0	55.6	18	21

absence). Every gel was scored in triplicate (independent scorings) and only the consistently scored fragments were used for analysis. The standardized ISSR data were initially analyzed using the NTSYSpc package program (Applied Biostatistics, Exeter Software) (Rohlf, 1992).

Many different types of characters were recorded. Continuous data, usually considered to be intrinsically ordered when coded into 'discrete' states, were scored as numbered states accounting for the natural ranges of variation. All characters were scored as 1–8 or multistate but were considered as unordered in the final analysis. The code of 9 was used to represent missing data. Where appropriate, nonvariable characters and characters for which there was a considerable amount of missing data were omitted during the analyses. The results of the incongruence length difference test showed that the 2 data sets, the numerical partial (1–8 characters) set and the present–absent (0,1) set, were congruent only at P = 0.01 (P = 1 – (99/100) = 0.010). This P-value is the threshold at which combining the 2 data sets would improve phylogenetic confidence. A binary data matrix was prepared and all data analysis was performed using the NTSYSpc package program. In both cluster analyses of the samples, the unweighted pair-group method with arithmetic mean (UPGMA) procedure was followed (Rohlf, 1992). The similarity coefficient method was used. Genetic distances were calculated with the simple matching coefficient. In order to determine the ability of ISSR data to display the interrelationships among the samples, analysis was conducted using the NTSYSpc package program. ISSR data obtained from 7 samples and a total of 253 bands were converted into a matrix of 253 × 7. Numerical data obtained from 7 taxa and a total of 125 traits were organized into a matrix of 125 × 7. The mean of 10 individual sample measurements related to the external morphologies was considered for every metric character of the taxa. Sneath's simple matching coefficient was used in the UPGMA clustering method. Cophenetic correlation can be calculated and used as an indication of degree of fit between the similarity matrix and the cophenetic value matrix based on the UPGMA cluster file. In order to determine the ability of numerical data to display the interrelationships among the samples, principal coordinate analysis (PCoA) of pairwise genetic distances (Nei, 1972) was also conducted using NTSYSpc and Minitab packages.

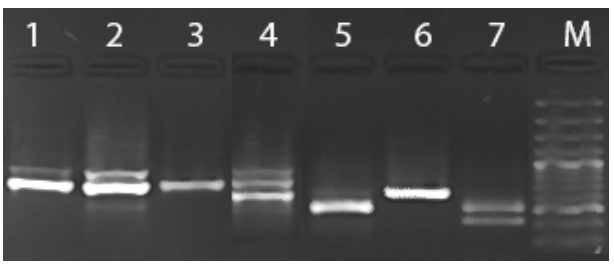

Figure 1. Representative agarose gels where PCR products were amplified with the primers ISSR F4. 1- *Bituminaria acaulis*, 2- *B. acaulis*, 3- *B. bituminosa*, 4- *Cullen jaubertianum*, 5- *Cicer anatolicum*, 6- *Vicia anatolica*, 7- *Astragalus emarginatus*.

3. Results

Classification of the taxa based on morphological characters is the gold-standard among the various well-established methods of taxonomy. However, problems in classification arise when the taxa display a large amount of variability due to phenotypic plasticity (Van den Berg and Groendijk, 1999), a situation where the most valuable contribution from the molecular biological tools may be obtained. In the present study, by using numerical classification methods, 7 taxa were evaluated morphologically on the basis of a data matrix generated from 125 characters (7 × 125). Cluster analysis was conducted based on both discrete and continuous morphological data that were previously standardized. Figure 2 shows the UPGMA phenogram comprising all operational taxonomic units (OTUs) in the present work.

Related to the molecular study, 5 primers from an initial screening with 8 ISSR primers revealed high

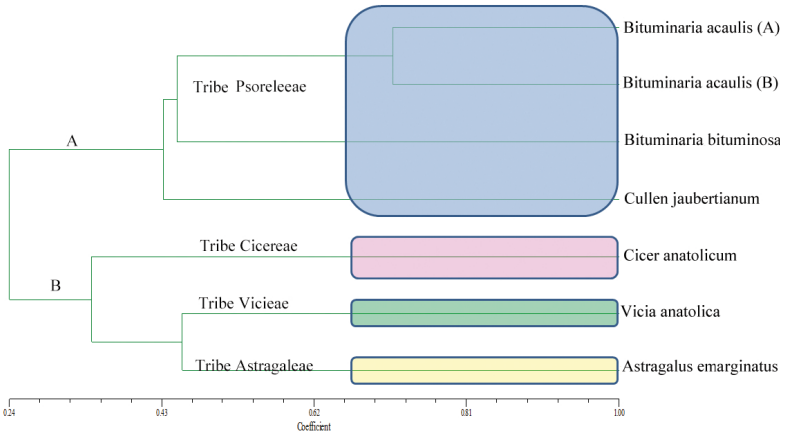

Figure 2. Phenogram showing the relationships of the genera *Bituminaria* and *Cullen* with outgroups.

levels of polymorphism. The ISSR primers generated 253 highly polymorphic fragments that were consistently amplified in repeated experiments. The molecular data set comprised these 253 × 7 characters. The GC percentages of the selected primers were 33.3%–55.6% (4 of them being 52.6%). Genetic distances were calculated with the simple matching coefficient. Figure 3 shows the UPGMA dendrogram comprising all OTUs in the present work.

Since the phenogram generated does not reflect the distinction of some taxa properly [e.g., the improper discrimination of *Bituminaria acaulis* (A) and *B. acaulis* (B)], we benefitted from the higher resolution offered by PCoA in determining these relationships more accurately (Figure 4), whereby the related subspecies were clearly separated by the first and the second principle coordinates (PCo1 and PCo2).

4. Discussion

Prior to 1977 (Stirton, 1981a), Psoraleeae was assumed to be closely related to Amorpheae, but recent studies (Lavin et al., 2001a; Wojciechowski et al., 2004) have shown that Psoraleeae is nested in Phaseoleae s.l. (Lewis et al., 2005).

Psoraleeae is a sister to the Phaseoleae subtribe Glycininae in a well-supported clade based on *rbcL* sequences (Doyle et al. 1997), encoding subunit 2 of the cytochrome oxidase gene (Adams et al., 1999; Doyle and Doyle, 2000). *Glycine* with the basally branching *Cullen* was sister to *Otholobium*, *Psoralidium*, and *Rupertia* in

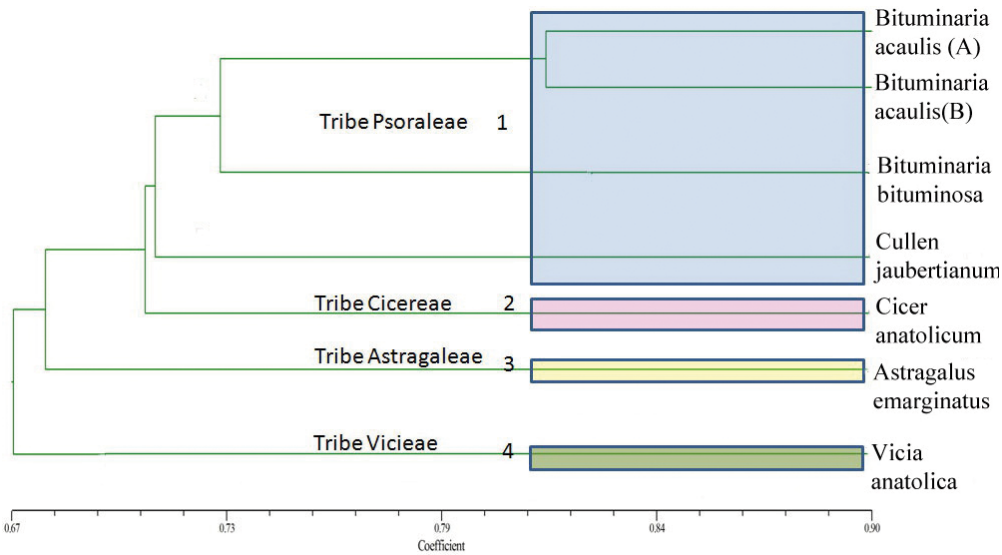

Figure 3. Dendrogram showing the relationships of the genera *Bituminaria* and *Cullen* with outgroups.

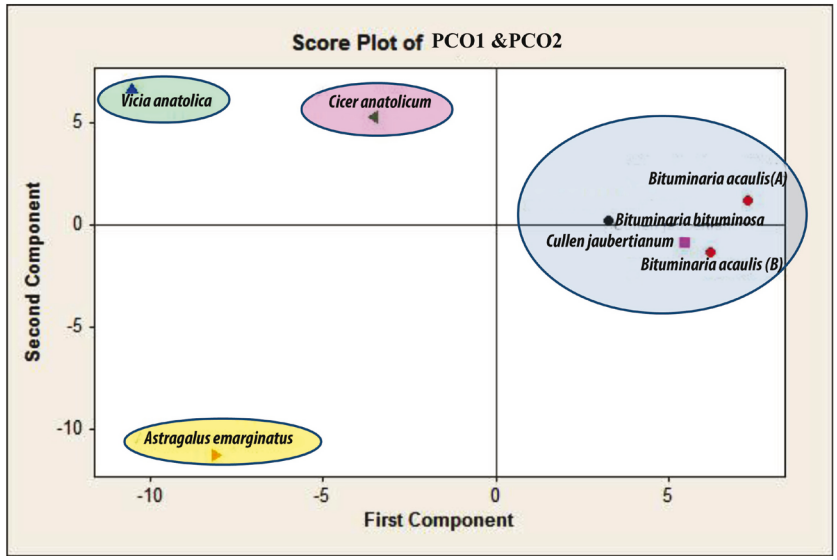

Figure 4. PCoA of the genera *Bituminaria* and *Cullen* with outgroups.

Figure 5. Herbarium samples of *Bituminaria acaulis*: A) collected in Artvin Province (*S.Toksoy* 1002), B) collected in Rize Province (*A.Duran* 9750 & *M.Öztürk*).

*trn*K-*mat*K analysis (Hu, 2000, Wojciechowski et al., 2004).

In this study the relationships of tribes Psoraleeae (*Bituminaria*, *Cullen*), Vicieae (*Vicia*), Astragalae (*Astragalus*), and Cicereae (*Cicer*) were determine by both taxonomic and molecular data for the first time in Turkey. According to the *Flora of Turkey*, the tribe Psoraleeae is after Astragalae and before tribes Cicereae and Vicieae.

According to the ISSR dendrogram, 2 major groups could be distinguished: (A) a major group with *Bituminaria acaulis*, *B. bituminosa*, and *Cullen jaubertianum*, or, in other words, the tribe Psoraleeae; (B) an outgroup with *Cicer anatolicum*, *Vicia anatolica*, and *Astragalus emarginatus* in tribes Vicieae, Astragalae, and Cicereae. The cophenetic correlation of the distance and the tree matrices obtained by the molecular method was 0.80, indicating a good fit of the dendrogram to the distance matrix (Rohlf, 1992).

The preliminary molecular results obtained in this study and the current phenotypic analyses were generally

in good agreement. Similarly, the phenogram generated by the numerical data presented a highly similar clustering profile on PCoA.

In order to clarify the interrelationships of some taxa at species level, we also conducted PCoA. The goal of PCoA is to permit the positioning of objects in a space of reduced dimensionality while preserving their distance relationships as much as possible. We performed PCoA using the product-moment correlation as a coefficient. The procedure calculates the distance matrix based on STAND data while the procedures EIGEN, PROJ, and MXPLOT were used to perform the PCoA. We preferred PCoA rather than principal components analysis because PCoA performs better on data sets with missing data (Rohlf, 1972). Using the first 2 dimensions (PCo1 and PCo2), it was observed that the improper discrimination of the target taxa, like that of *B. acaulis* (A) and *B. acaulis* (B), were resolved in a satisfying way due to the higher resolution of the PCoA method (Figure 4).

Among the field samples and herbarium materials, we recognized that 2 samples of *Bituminaria acaulis* (A) from Artvin (*S.Toksoy* 1002) and *Bituminaria acaulis* (B) from Rize (*A.Duran* 9750 & *M.Öztürk*) showed differences in the phenogram, dendrogram, and PCoA. They differed in features such as stem and petiole length; leaflet width × length, petiolulate, stipule length; inflorescence length, flowers on each peduncle, bracts and bracteoles width × length; and calyx, calyx teeth and tube length, standard, carina and wing length, claw and limb length (Figure 5).

According to morphological and molecular data, after revision of the genus *Psoralea* in Turkey, the genus *Psoralea* specimens were divided into the genera of *Bituminaria* and *Cullen* in accordance with Stirton's (1981b) classification. Although the genus *Psoralea* specimens' pedicel was subtended by a distinctive lobed cupulum, those of *Cullen* and *Bituminaria* were not. *Bituminaria* and *Cullen* are separated from each other by their fruit structures. The genus *Cullen* has fruit oval, conspicuously black glandular warty when mature, while the genus *Bituminaria* has fruit never black glandular warty. However *B. acaulis* has bracteoles and *B. bituminosa* does not.

The key to diagnosis of the genera *Psoralea*, *Cullen*, and *Bituminaria* is given below (Stirton 1981b).

1. Flower-pedicel subtended by a distinctive lobed cupulum ..... 1. Psoralea

1. Flower-pedicel never subtended by a cupulum

2. Fruit oval, conspicuously black glandular warty when mature ..... 2. Cullen

2. Fruit never black glandular warty .... 3. Bituminaria

Consequently, 2 of the 3 *Psoralea* taxa must be transferred to *Bituminaria bituminosa* and *B. acaulis*, and 1 must be transferred to *Cullen jaubertianum*. Thus, there is no occurrence of the genus *Psoralea* in Turkey.

Appendix.

Additional specimens examined (*: specimens used for DNA samples).

– **Bituminaria bituminosa**: Turkey. A2 Bursa: Uludağ, maquis, 460 m, 18.05.1975, *R.Çetik* 4405 (KNYA); Yalova: Arpalı surroundings, 10 km east of Termal, 150 m, 18.05.1975, *R.Çetik* 4406 (KNYA); A3 Sakarya: Sakarya University, behind Faculty of Science and Arts, roadside, 750–800 m, 15.06.2012, *S.Toksoy* 1012* (KNYA); A5 Amasya: Boğazköy, 18.06.1955, *R.Çetik* 394 (KNYA); B1 İzmir: Kuşadası Kalamaki National Park, maquis, 15 m, 27.05.1982, *R.Çetik* 7653 & *H.Ocakverdi*, *B.Eyce* (KNYA); C3 Antalya: Kumluca to Kemer, 15 km from Kumluca, 580 m, 14.05.1976, *R.Çetik* 5121 (KNYA); C4 Konya: Bucakkışla, Bıçakçı village, bridge surroundings, *Pinus brutia* forest clearing, 600 m, 30.05.1978, *M.Vural* 1835 (KNYA). – **Bituminaria acaulis**: Turkey. A8 Artvin (Çoruh): Maden, garden side, *R.Çetik* 2 (KNYA); Murgul, Petek village, Orta district, roadside slope, 835 m, 21.08.2012, *S.Toksoy* 1001 (KNYA); Rize: İkizdere, Ovit pass, Dereköy village, 1800 m, 30.8.2013, *A.Duran* 9533 & *M.Çelik* (KNYA); İkizdere, İkizdere-Cimil road, 22 km, 1700 m, 18.8.2013, *A.Duran* 9750 & *M.Öztürk** (KNYA). – **Cullen jaubertianum:** Turkey. C8 Kilis: Gaziantep-Kilis road, after Şahin Bey monument, 712 m, 15.05.2010, 36°52′51″N, 37°21′02″E, *M.Öztürk* 1531 & *A.Duran* (KNYA); Gaziantep-Kilis road, after Şahin Bey monument, 712 m, 2.06.2012, *A.Duran* 9363, *Ö.Çetin* & *M.Çelik** (KNYA). – **Cicer anatolicum:** Turkey. B7 Erzincan: Üzümlü, Keşişdağ road, above Üzümlü, 2120 m, 16.07.2009, roadside, 39°73′637″N, 39°69′417″E, *M.Öztürk* 1500 & *A.Duran** (KNYA); – **Vicia anatolica:** Turkey. C6 Mardin: Mazıdağ road, 4 km to Mazıdağ, 970 m, 12.05.2008, vineyards, 37°29′700″N, 40°31′350″E, *M.Öztürk* 1316 & *A.Duran** (KNYA). – **Astragalus emarginatus:** Turkey. C6 Kahramanmaraş: Ahır Mountain, above Kahramanmaraş, 1400 m, 20.09.2012, *M.Celik* 1059b* (KNYA).

References

Bandara NL, Papini A, Mosti S, Brown T, Smith LMJ (2013). A phylogenetic analysis of genus *Onobrychis* and its relationships within the tribe Hedysareae (Fabaceae). Turk J Bot 37: 981–992.

Barneby RC (1977). Daleae Imagines. Mem New York Bot Gard 27: 1–891.

Bello MA, Bruneau A, Forest F, Hawkins JA (2009). Elusive relationships within order Fabales: phylogenetic analyses using matK and rbcL sequence data. Syst Bot 34: 102–114.

Bornet B, Branchard M (2001). Nonanchored inter simple sequence repeats (ISSR) markers reproducible and specific tools for genome finger printing. Plant Mol Biol Rep 19: 209–215.

Davis PH, editor (1970). Flora of Turkey and the East Aegean Islands, Vol. 3. Edinburgh, UK: Edinburgh University Press.

Davis PH, Mill RR, Tan K (1988). Flora of Turkey and the East Aegean Islands (Suppl. 1), Vol. 10. Edinburgh, UK: Edinburgh University Press.

Doyle JJ, Doyle JL, Ballenger JA, Dickson EE, Kajita T and Ohashi H (1997). A phylogeny of the chloroplast gene rbcL in the Leguminosae: taxonomic correlations and insights into the evolution of nodulation. American Journal of Botany 84: 541-554.

Doyle JJ, Chappill JA, Bailey CD, Kajita T (2000). Towards a comprehensive phylogeny of legumes: evidence from rbcL sequences and non-molecular data. In: Herendeen PS, Bruneau A, editors. Advances in Legume Systematics, Part 9. Kew, UK: pp. 1–20.

Egan AN, Crandall KA (2008). Divergence and diversification in North American Psoraleeae (Fabaceae) due to climate change. BMC Biology 6: 55.

Erik S, Tarikahya B (2004). Türkiye florası üzerine. Kebikeç 17: 139–163 (in Turkish).

Güner A, Aslan S, Ekim T, Vural M, Babaç MT, editors (2012). Türkiye Bitkileri Listesi (Damarlı Bitkiler). İstanbul, Turkey: Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayını (in Turkish).

Hutchinson J (1964). The genera of flowering plants, Vol 1. Oxford University Press.

Lavin M, Herendeen P, Wojciechowski MF (2005). Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the Tertiary. Systematic Biology 54: 530–549.

Hu J-M (2000). The phylogenetic relationships of the tribe Millettieae and allies – the current status. In: Herendeen PS & Bruneau A (eds.). Advances in Legume Systematics, part 9, pp. 299-320, Royal Botanic Gardens, Kew.

Lavin M, Pennington RT, Klitgaard BB, Sprent JI, Lima HC, de & Gasson PE (2001a). The Dalbergioid legumes (Fabaceae): delimitation of a pantropical monophyletic clade. American Journal of Botany 88: 503-533.

Lewis G, Schrire B, Mackinder B, Lock M (2005). Legumes of the World. Kew, UK: Royal Botanical Gardens.

Maddison WP, Donoghue MJ, Maddison DR (1984). Outgroup analysis and parsimony. Syst Zool 33: 83–103.

Meher RS, Maassoumi AA, Saidi A, Osaloo ShK, Nohooji MG (2012). Morphological cladistic analysis of some bifurcate hairy sections of *Astragalus* (Fabaceae) in Iran. Turk J Bot 36: 434–442.

Nei M (1972) Genetic distance between populations. The American Naturalist 106: 283–292.

Özhatay N, Kültür Ş, Aslan S (2009). Check-list of additional taxa to the supplement Flora of Turkey IV. Turk J Bot 33: 191–226.

Persson C (2001). Phylogenetic relationships in Polygalaceae based on plastid DNA sequences from the trnL-F region. Taxon 50: 763–779.

Polhill RM (1994). Classification of the Leguminosae. In: Bisby FA, Buckingham J, Harborne JB, editors. Phytochemical Dictionary of the Leguminosae. Plants and Their Constituents, Vol. 1. London, UK: Chapman and Hall, pp. 16–37.

Prenner G, Klitgaard BB (2008). Towards unlocking the deep nodes of Leguminosae: floral development and morphology of the enigmatic *Duparquetia orchidacea* (Leguminosae, Caesalpinioideae). Am J Bot 95: 1349–1365.

Rafalski JA, Vogel JM, Morgante M, Powell W, Andre C, Tingey SV (1996). Generating and using DNA markers in plants. In: Birren B, Lai E, editors. Nonmammalian Genomic Analysis. San Diego, CA, USA: Academic Press, pp. 75–134.

Rohlf FJ (1992). NTSYSpc: Numerical Taxonomy and Multivariate Analysis System, Version 2.0. Stony Brook, NY, USA: State University of New York at Stony Brook.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory Manual, Vol. 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Stirton CH (1981a). Psoraleeae. In: Polhill RM, Raven PH, editors. Advances in Legume Systematics. Kew, UK: Royal Botanic Gardens, pp. 337–343.

Stirton CH (1981b). Studies in the Leguminosae-Papilionoideae of southern Africa. Bothalia 13: 317–325.

Tucker SC (1987). Floral initiation and development in legumes. In: Stirton CH, editors. Advances in Legume Systematics, Part 3. Kew, UK: Royal Botanic Gardens, pp. 183–279.

Tucker SC (1997). Floral evolution, development, and convergence: the hierarchical-significance hypothesis. Int J Plant Sci 158: 143–161.

Tucker SC (2003). Floral development in legumes. Plant Physiol 131: 911–926.

Tucker SC, Douglas AW (1994). Ontogenetic evidence and phylogenetic relationships among basal taxa of Legumes. In: Ferguson IK, Tucker S, editors. Advances in Legume Systematics, Part 6: Structural Botany. Kew, UK: Royal Botanic Gardens, pp. 11–32.

Tucker SC, Stirton CH (1991). Development of the cymose inflorescence, cupulum and flower of *Psoralea pinnata* (Leguminosae: Papilionoideae: Psoraleeae). Bot J Linn Soc 106: 209–227.

Wojciechowski MF, Lavin M, Sanderson MJ (2004). A phylogeny of Legumes (Leguminosae) based on analysis of the plastid matK gene resolves many well-supported subclades within the family. American Journal of Botany 91 (11): 1845-1861.

Van den Berg RG, Groendijk-Wilders N (1999). Numerical analysis of the taxa of series *Circaefolia* (*Solanum* sect. *Petota*). In: Nee M, Symon D, Lester R, Jessop J, editors. Solanaceae IV. Kew, UK: Royal Botanic Garden, pp. 213–226.