

**PHYLOGENETIC RELATIONSHIPS AMONG THE TAXA OF THE GENUS
JOHRENIA DC. (APIACEAE) FROM TURKEY
BASED ON MOLECULAR METHOD**

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Keywords: Johrenia; Dichoropetalum; Apiaceae; Taxonomy; ISSR; Turkey.

Abstract

In the present study, ISSR markers were employed to determine the phylogenetic relationships among the taxa of *Johrenia*. The genera *Angelica* and *Xanthogalum* were selected as outgroups. Unweighted pair group method with arithmetic mean (UPGMA) and Principal Coordinate Analyses were conducted to view the molecular relationships. *Johrenia alpina*, *J. depauperatum* and *J. aurea* are transferred to the genus *Dichoropetalum*. The infrageneric and intergeneric phylogenetic relationship among the *Johrenia* and *Dichoropetalum* genera are determined.

Introduction

The Apiaceae family is represented by approximately 400 genera and 3500 species worldwide (Constance, 1971; Pimenov and Leonov, 1993). The family consists of 102 genera and 434 species in Turkey (Erik and Tarıkahya, 2004). The Apiaceae includes many commonly grown vegetables (carrot, parsnip) and condiments (chervil, cumin, parsley, dill). They owe their distinctive flavour largely to diverse essential oil compounds in the fruits, seeds and leaves. The family also encompasses widespread weeds and toxic plants (Downie *et al.*, 2000).

A molecular approach has contributed much to understanding the evolutionary relationships of Apiaceae. Phylogenetic analyses of the family using chloroplast DNA (cpDNA) sequences (Downie *et al.*, 1996), cpDNA restriction sites (Plunkett and Downie, 1999), and nuclear ribosomal DNA internal transcribed spacer (ITS) sequences (Downie and Katz-Downie, 1996) provided an alternative classification of Drude (1898). Revision based on molecular data provide better resolution of the systematic positions. The taxonomic problems at the species level have begun started to be solved with DNA-based molecular analyses which are not affected by environmental conditions, in contrast to the phenotypical analyses.

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Probably, the centre of the genetic diversity of the genera *Johrenia* and *Dichoropetalum* are in Anatolia. *Johrenia* is represented by nine taxa in Turkey, namely, *Johrenia selinoides*, *J. porteri*, *J. dichotoma* subsp. *dichotoma*, *J. dichotoma* subsp. *sintenisii*, *J. tortuosa*, *J. polyscias*, *J. alpina*, *J. berytea*, and *J. aurea* (Chonberlair, 1972). Five of these species are endemic to Turkey, viz *J. selinoides*, *J. dichotoma* subsp. *sintenisii*, *J. alpina*, *J. berytea*, and *J. polyscias*. The sample of *J. dichotoma* subsp. *sintenisii* was collected by G. Post 120 years ago and was only known from the type locality in Mardin province. *Johrenia aurea* species was collected 150 years ago which was only known from the type locality on Aslandağ mountain in Kayseri province (Chamberlain, 1972).

Johrenia was described first by Candolle (1829) based on *J. dichotoma* DC. Later, *Dichoropetalum* Fenzl, based on *D. alpinum* Fenzl, was described (Fenzl, 1842) but soon after sunk by its author into synonymy with *Johrenia* (Fenzl, 1843). The basis for the modern *Johrenia* taxonomy was laid by Boissier (1844), who placed 10 species in *Johrenia*, and divided them into two informal groups. The first contains six species (*J. selinoides*, *J. dichotoma*, *J. fungosa*, *J. graeca*, *J. alpina* and *J. berytea*). The second group was again subdivided into two subgroups. The first subgroup comprises *J. candollei*, *J. platycarpa*, and the other subgroup contains *J. aurea* and *J. juncea*. Drude (1898) maintained Boissier's classification of the genus. Bornmüller (1930) compiled a synopsis of the *Johrenia* species and included descriptions of some new species, among which *J. polyscias* is adopted in Turkish flora (Chamberlain, 1972).

Pimenov *et al.* (2007) made a comprehensive taxonomic analysis of *Dichoropetalum*, *Johrenia*, *Zeravschania* and other related genera of Apiaceae based on 32 morphological diagnostic characters. According to results from their comparative multivariate analysis, 28 new nomenclatural combinations are validated and lectotypes are designated for several names. From the genus *Johrenia*, Pimenov *et al.* (2007) transferred *J. alpina*, *J. aromatica*, *J. berytea*, *J. aurea*, *J. golestanica*, *J. paucijuga*, *J. platycarpa* and *J. ramosissima* into the genus *Dichoropetalum*. In addition, *Johrenia westii* was transferred to the genus *Ferulago*. Transferred species *J. alpina*, *J. berytea* and *J. aurea* are endemic to Turkey. According to the latest taxonomic analysis on some of the genera in Apiaceae family, the *Johrenia* and *Dichoropetalum* genera are represented by six taxa and three species respectively in Turkey. These taxa are as follows: *Johrenia selinoides*, *J. porteri*, *J. dichotoma* subsp. *dichotoma*, *J. dichotoma* subsp. *sintenisii*, *J. tortuosa*, *J. polyscias* in *Johrenia* genus; *Dichoropetalum alpinum*, *D. aureum* and *D. depauperatum* (Syn. *J. berytea*) in *Dichoropetalum* genus (Pimenov *et al.*, 2007).

Currently morphological revisions of various plant taxa are often supported by molecular data (APG, 2003). As compared with morphological data, DNA data are not influenced by the environmental conditions in which the plants have grown; hence they serve as a powerful tool in resolving taxonomical and systematical problems.

The aim of our study was to determine the infrageneric and intergeneric phylogenetic relationship among the *Johrenia* and *Dichoropetalum* genera employing ISSR method. Also, we selected *Angelica sylvestris* (M. Bieb.) Sprengel and *Xanthogalum purpurascens* Lallemand to resolve their controversial status by using a DNA based molecular marker system.

Materials and Methods

Plant materials: *Johrenia* and *Dichoropetalum* specimens were collected by the authors from Amasya, Mersin, Adana, Niğde, Kahramanmaraş, Bursa, Konya, Osmaniye and Kayseri provinces between of 2003-2008 (Fig. 1). The Flora of Turkey (Chamberlain, 1972), Flora Iranica (Rechinger, 1987), and Flora Europaea (Tutin, 1968), were used to identify the collected plant samples. Specimens are kept in Selçuk University Education Faculty Herbarium. The specimens' localities (Fig. 1) and examined representative specimens are in the appendix. The genera *Angelica* and *Xanthogalum* were selected as outgroups. These genera are closest to *Johrenia* in respect to phylogeny in Turkish flora.

DNA isolation: Nuclear DNA was isolated from leaves both from herbarium and fresh materials using CTAB method (Sambrook *et al.*, 1989). Total DNA was obtained from 50-75 mg dried leaf tissue from 10 different individuals. DNAs were isolated with the Easy Nucleic Acid Isolation Kit (OMEGA) and concentrations were determined by Nanodrop. Sample DNAs were diluted to 25 ng/μl. Stock DNAs were kept at -86°C.

ISSR amplifications: ISSR primers (Galvan *et al.*, 2003) were amplified in a PCR thermal cycler. The characteristics of the primers used are given in Table 1. Each PCR reaction contained 25 μl containing 2.5 μl PCR buffer (10 mM TRIS/50 mM KCl buffer, pH 8.0), 3 μl 25 mM MgCl₂, 0.5 μl of each primer, 0.5 μl of dNTP mix, 0.4 μl Taq DNA polymerase, 4 μl of each DNA and 14.1 μl distilled water. After a pre-denaturation step of 3 min at 94 °C, amplification reactions were cycled 40 times at 94°C for 1 min, at annealing temperature (Table 1) for 1 min and 72°C for 1 min and a final extension was allowed for 10 min at 72°C in an Eppendorf Mastercycler Gradient Thermocycler. 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 electrophoresed at 4V cm⁻¹. Amplified fragments were visualized under a UV transilluminator and photographed using a gel documentation system (Vilbert Lourmat, Infinity model).

Data analysis: 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 2.02 (Applied Biostatistic, Exeter Software, Setauket, New York, USA). Similarity coefficient method was used. In cluster analysis of the samples the unweighted pair-group method with arithmetic mean (UPGMA) procedure was followed (Rohlf, 1992). Genetic distances calculated with the Simple Matching coefficient. In order to determine the ability of ISSR data to display the inter-relationships among the samples analysis was conducted using NTSYS-pc package.

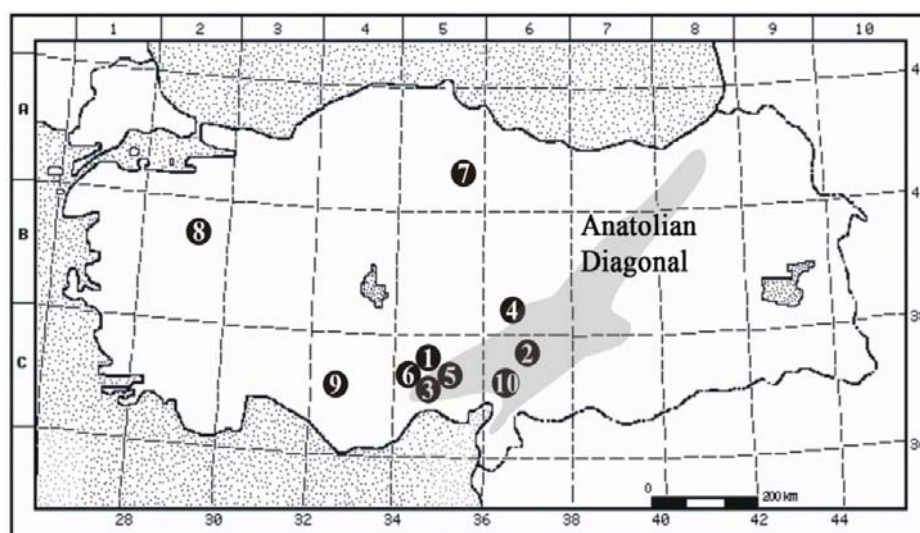


Fig. 1. Distribution map of the examined specimens; 1. *Dichoropetalum aytachii*, 2. *D. depauperatum*, 3. *D. alpinum*, 4. *Johrenia porteri*, 5. *J. selinoides*, 6. *J. dichotoma*, 7. *J. polyscias*, 8. *J. tortuosa*, 9. *Xanthogalum purpurascens*, 10. *Angelica sylvestris*.

Results and Discussion

From an initial screening of 25 ISSR primers, nine primers revealed high levels of polymorphisms. These primers generated 90 highly polymorphic fragments that were consistently amplified in repeated experiments. The GC percentages of the selected primers were within the range of 38.9-66.7% (five of them being 52.6%). In total, the average number of polymorphic fragments per primer used was roughly 11. Genetic distances calculated with the SM coefficient ranged from 0.46 to 0.99.

As a result of the evaluation of the ISSR data, *Dichoropetalum depauperatum* showed 95% similarity with and *D. aytachii*.

The clade composed of *J. dichotoma* and *J. porteri* are very similar according to the morphological characters. These similarities are supported with the ISSR data and these species show much more similarity in the dendrogram than any other species (Fig. 2).

Pimenov *et al.* (2007) selected 33 morphological characters for phenetic analyses of *Johrenia*, *Dichoropetalum*, *Zeravschania* and related genera. Selected characters were used to perform UPGMA analysis, and the resulting phenogram showed the relationships among *Johrenia*, *Dichoropetalum*, *Zeravschania*, and related genera. In the phenogram, three major groups matched with one of the three genera. The molecular dendrogram of the genera *Dichoropetalum* and *Johrenia* showed parallelism among the major groups like the phenogram of *Johrenia*, *Dichoropetalum*, *Zeravschania* genera of Pimenov *et al.* (2007).

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

PRIMER	PRIMER SEQUENCE	T _{melting} (⁰ C)	SIZE (bp)	GC%	T _{annealing}
ISSR M8	ACACACACACACACACACG	56.7	19	52.6	56
ISSR M12	GACACGACACGACACGACAC	61.4	20	60	60
ISSR M15	CACACACACACACACAAG	53.7	18	50	53
ISSR N2	GTGGTGGTGGTGGTG	53.3	15	66.7	52
ISSR F1	GAGCAACAACAACAACAA	49.1	18	38.9	49
ISSR F2	CTCGTGTGTGTGTGTGTGT	56.7	19	52.6	56
ISSR F5	AGAGAGAGAGAGAGAG	49.2	16	50	49
ISSR F6	CCACCACCACCACCA	53.3	15	66.7	53
ISSR F7	ACACACACACACACAC	49.2	16	50	48

The present study based on ISSR data revealed four clades, each clade matching with one of the four genera, viz. *Johrenia*, *Dichoropetalum*, *Angelica* and *Xanthogalum*. The genus *Angelica* with *Xanthogalum*, and *Dichoropetalum* with *Johrenia* shows much more molecular phylogenetic similarity. There is a correlation between the morphologic diagnostic characters and molecular taxonomic classification.

The concept of the “Diagonal” was first proposed by Davis (1971), who defined it as an oblique belt running from the north east, south to the Anti-Taurus; it then divides into two, with one branch to the Amanus (Amanos Mountains), and the other to the Cilician Taurus (Fig. 1). Thirty three percent of the total species growing in Turkey are found along the diagonal, while 5% are more or less restricted to it (Ekim and Güner, 1986).

The spread of genus *Dichoropetalum* is on the Anatolian diagonal (Fig. 1). The genus occurs in the Anti-Taurus region and branch (Aladağ, Amanos and Bolkar mountains) of the Anatolian diagonal. In Anatolia, almost all mountain peaks so far examined abound in endemics (Zohary, 1973). All species of *Dichoropetalum* are grown on mountain peaks and endemics in Anatolia. The Anatolian Diagonal and its adjacent areas are one of the most important centers of genetic diversity in Turkey. The Amanos mountain range is an interesting area, occupying an intersection of the Mediterranean phytogeographical

region and the Anatolian Diagonal, with many Euro-Siberian phytogeographical region enclaves (Ekim and Güner, 1986). The area is very rich in paleo and neo endemic plants.

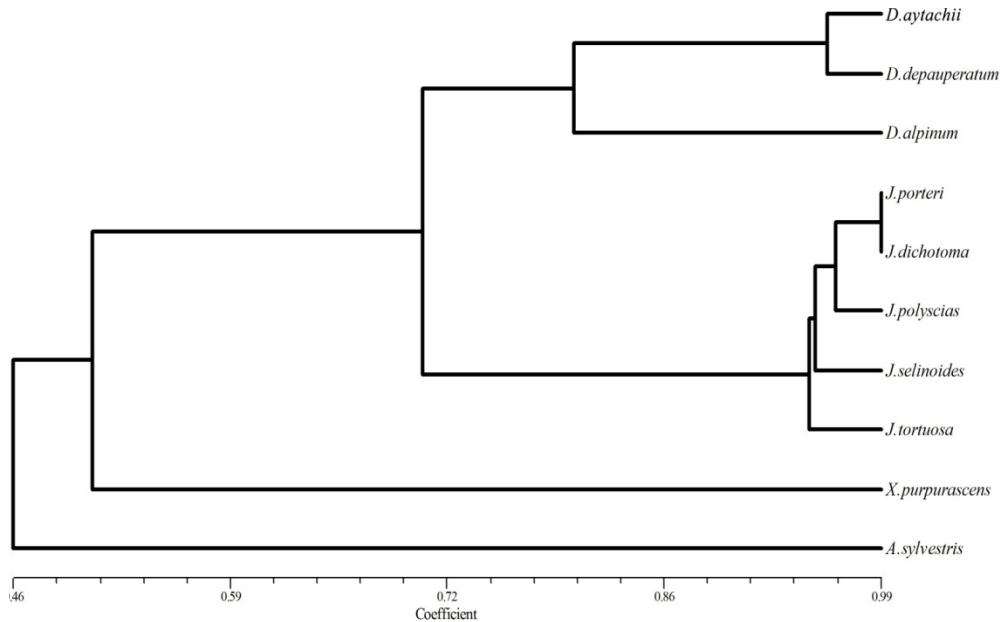


Fig. 2. Dendrogram showing genetic relationship of *Johrenia*, *Dichoropetalum*, *Angelica* and *Xanthogalum* species based on ISSR markers.

An interesting *Dichoropetalum* specimen, *D. aytachii*, (A. Duran 7699, Bağcı & Dinc) was collected from Aladağlar Mountain (Niğde) in 2007. It is rather different specimens in *Dichoropetalum* genus. On the specimen is required more comprehensive morphological, anatomical, palynological and cytotaxonomical studies. The specimen of the genus *Dichoropetalum* examined with other samples, and its species placed in different clade in dendrogram (Fig. 2). The related species were clearly separated by the principal coordinate analysis (Fig. 3).

Although the genus *Johrenia* is distributed primarily in the East Mediterranean region, some species maintain an interrupted spread in the ecotone zone in Central Anatolia. They grow on the Anatolian diagonal or western side of the diagonal except for *J. dichotoma* ssp. *sintensisii*. This taxon is only known from the type locality and is a very local endemic. This genus represents six species in the world and five species in Turkey (*J. distans* spread in Greece and F.Y.R. Macedonia). Four species of *Johrenia* endemic to Turkey are found on the Anatolian Diagonal. Taxa of *Johrenia* are poorly occurring and have local population in Anatolia. Opposite to the genus *Dichoropetalum*, the genus *Johrenia* is distributed in the lowest altitude of the mountains in Turkey.

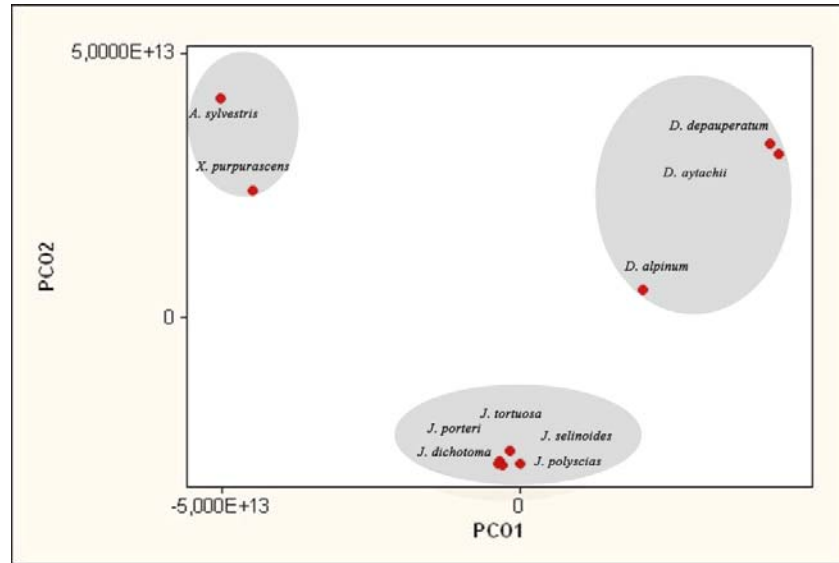


Fig. 3. Principal coordinate analysis of *Johrenia*, *Dichoropetalum*, *Angelica* and *Xanthagalum* species.

As a result the *Johrenia* and *Dichoropetalum* taxa in Turkey are classified according to the molecular data. *J. alpina*, *J. depauperatum* and *J. aurea* species have been transferred to the genus *Dichoropetalum*. Therefore, *Johrenia* and *Dichoropetalum* contain five and three species, respectively as revealed from molecular phylogenetic studies.

Acknowledgements

We would like to thank to Selcuk University (BAP Project no: 05401075) for financial support during this study.

Appendix

EXAMINED REPRESENTATIVE SPECIMENS: – *Dichoropetalum alpinum*. C5 Mersin: Gülek, Maden road, Karlıboğaz, 2400 m, 10.08.2007, steppe, Duran *et al.*, 7711, (Duran, Bağcı & Dinc) – *D. depauperatum*. C6 Kahramanmaraş: Göksun, Çardak, from Ericek village to Berit Mountain 2100-2400 m, 08.08.2007, steppe, Duran *et al.*, 7687, – *D. aytachii*. C5 Niğde: Çamardı, Aladağlar, Emli mountain pass, 2100 m, 09.08.2007, calcareous slopes, 38°00.844'N, 36°49.579'E Duran *et al.*, 7699, – *Johrenia porteri*. B6 Kayseri: Sarız, between Yalak (Yeşilkent)-Körkuyu, 1450 m, 7.8.2007, Duran *et al.*, 7686, – *Johrenia selinoides*. C5 Adana: Gülek mountain pass, between Akçatekir-Adana, 850 m, 10.08.2007, open forest, calcareous stony slopes, 37°13.271'N, 34°48.621'E, Duran *et al.*, 7708, – *Johrenia dichotoma*. C5 Mersin: Karakütük village, 700 m, 05.06.2007, roadsides, open machia, Duran *et al.*, 7381, – *Johrenia polyscias*. A5 Amasya: Hartna Castle, 450-630 m, 26.08.2007, rocky slopes, 40°39.314'N, 35°49.568'E, Bağcı *et al.*, 3688, – *Johrenia tortuosa*. B2 Bursa: Harmançık- Dursunbey road, 450 m, 21.07.2006, open *Pinus brutia*, Duran 7292. – *Xanthagalum purpurascens*. C4 Konya: Hadim, Gevne valley, Beyreli village, 1300, 25.7.2003, streamside, Duran 6332. – *Angelica sylvestris*. C6 Osmaniye: Yarpuz, 850 m, 12.09.2003, wet places, Duran 6354.

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