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Phylogenetic analysis of *Jurinea* (Asteraceae) species from Turkey based on ISSR amplification

Bekir Dogan[1], Ahmet Duran[1] & Erdogan E. Hakki[2,*]

[1)] *Selcuk University, Faculty of Education, Biology Dept., Meram, 42090 Konya, Turkey*
[2)] *Selcuk University, Faculty of Agriculture, Field Crops Dept., 42079, Campus, Konya, Turkey (*corresponding author's e-mail: eehakki@selcuk.edu.tr)*

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Jurinea remains a taxonomically complex genus, many of the problems concentrating around *J. mollis*. In this study, *Jurinea* species native in Turkey were collected from different localities in Anatolia and DNA was isolated from the collected samples, using a commercial plant DNA extraction kit (Nucleon phytopure). Inter Simple Sequence Repeat (ISSR) was the fingerprinting method preferred due to its high level of reliability and consistency as compared with the Randomly Amplified Polymorphic DNA (RAPD). The data were analyzed with NTSYS-pc 2.1. The phytogeographical distribution of *Jurinea* was found to be closely related to the Anatolian Diagonal. Based on the Diagonal, two major taxonomic groups emerged, from which *J. kileae* was clearly separated both morphologically and phylogenetically.

Key words: Asteraceae, ISSR, *Jurinea*, molecular systematics, phylogeny

Introduction

The tribe Cardueae (Asteraceae) is generally classified into five subtribes named Echinopinae, Carlininae, Carduinae, Centaureinae and Cardopatiinae (Susanna *et al*. 2006). However, delimitation of these taxonomic entities is highly problematic. Also, some large genera in the tribes have generic delimitation problems, such as *Carduus* (90 species), *Cirsium* (250), *Centaurea* (400), *Cousinia* (800), *Jurinea* (100), and *Saussurea* (more than 300 species) (Garcia-Jacas *et al*. 2002). The extensive work conducted recently by Garcia-Jacas *et al*. (2000, 2001) and Font *et al*. (2002) have clarified the delimitation of *Centaurea*. Smaller studies also exist on *Cirsium* and *Carduus* (Haffner & Hellwig 1999), but most of the taxonomic problems still persist. The genus *Jurinea* constitutes a taxonomically complex group of plants with unsolved systematic problems, especially among the species near *J. mollis* (Danin & Davis 1975).

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.

Randomly Amplified Polymorphic DNA (RAPD) is a widely utilized fingerprinting method

with a wide range of applications (Williams *et al*. 1990). However, RAPD has a number of disadvantages that render it a sensitive method that should be utilized with special care. As compared with RAPDs, Inter Simple Sequence Repeat (ISSR) has much higher levels of repeatability, making it superior (Zietkiewicz *et al*. 1994, Prevost & Wilkinson 1999). ISSR has recently become one of the most widely utilized genetic diversity analysis methods.

Simple sequence repeats (SSRs), also known as microsatellites, are tandemly repeated di-, tri, tetra- or penta-nucleotide sequences (mainly within the range of 10–80 repeats of the core unit) that are abundant within eukaryotic genomes. High level of genomic variation is generated by the more or less evenly distributed microsatellite sequences present within the plant and animal genomes. The high levels of genomic variation are widely used both for genetic variation analysis of natural plant DNAs (Wolfe *et al*. 1998), and crop plant DNAs (Vosman & Arens 1997, Hakki *et al*. 2001). Microsatellites can be used in inter- as well as intra-species analyses (Soranzo *et al*. 1999). However, the technique requires prior sequence information for the locus-specific primers, a feature that renders it difficult to be applied to plants with no adequate genomic sequencing studies. Without considering their difficulty or cost (Hakki & Akkaya 2000), numerous microsatellite loci have been identified for economically important crops like wheat, rice and maize. In *Jurinea*, however, they have not been utilized.

In this study, *Jurinea* species, which are difficult to characterize with morphological traits, were collected from their natural habitats in Turkey. DNA was isolated and fingerprinting was performed using a highly reliable and reproducible technique that mimics the application ease of RAPDs. The method employed to assess the genetic diversity and to resolve the genetic relationships among the species is a derived technique of SSR characterization based on PCR amplification of ISSR regions primed by a single oligonucleotide corresponding to the targeted repeat motif. The SSR-containing primers are usually 16–25 base pair long oligonucleotides anchored at the 3´- or 5´-end by two to four arbitrary, and often degenerate, nucleotides (Fang *et al*. 1997). The primer can be based on any of the motifs found at SSR loci. In these conditions, only sequence regions flanked by the two adjacent identical and inversely oriented microsatellites will be amplified. Overall, the technique does not require prior sequence information (advantage against microsatellites) and its reliability is higher than the RAPDs.

The aim of this study was to determine the genetic relationships among the selected Anatolian-originated *Jurinea* species and to resolve their unclear and controversial status, based on conventional morphological characters, by using a DNA-based molecular marker system.

Material and methods

Specimen collection

Silica gel dried leaf samples belonging to 14 *Jurinea* species were collected from the natural flora of Turkey. The species and their localities are as follows: *J. consanguinea* (Konya-Aksehir), *J. cadmea* (Izmir-Bozdag), *J. mollis* (Izmir-Rasathane), *J. macrocalathia* (Tekirdag-Kumbag), *J. kileae* (Kirklareli-Kiyikoy), *J. pontica* (Izmit-Sapanca), *J. macrocephala* (Konya-Eregli), *J. ramulosa* (Kahramanmaras), *J. pulchella* (Van), *J. aucherana* (Erzincan), *J. brevicaulis* (Erzincan), *J. cataonica* (Erzincan), *J. alpigena* (Karabuk-Keltepe), and *J. ancyrensis* (Elazıg-Harput).

DNA extraction

Nuclear DNAs of the silica gel dried leaf samples were extracted according to the instructions of the 'Nucleon phytopure' plant DNA extraction kit (RPN 8510) from Amersham Life Science, England. For each sample, 100 mg of leaf was used and DNAs were isolated individually. After concentrations were determined (by an Eppendorf BioPhotometer), sample DNAs were diluted to the working concentration of 25 ng l^{-1}. To better quantify the DNAs and to assess the quality of the DNAs, samples were also ran in an agarose gel (0.9%), stained with ethidium bromide, against the standard of DNA with known concentrations. Stock DNAs were kept at –86 °C ultralow temperature freezer.

ISSR amplifications

Of 25 primers used during our initial screening, 8 primers that gave the most informative patterns (in terms of repeatability, scorability, and the ability to distinguish between varieties) were selected for fingerprinting. The characteristics of the primers used are given in Table 1.

Each reaction contained 2.5 mM $MgCl_2$; 10 mM Tris-HCl (pH 8.8); 50 mM KCl; 0.8% Nonidet P40; 200 mM of each of dNTPs; 0.5 μM primer; 25 ng DNA template and 0.4 units of *Taq* DNA Polymerase (Bioron) in a final reaction volume of 25 μl. 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 50 sec and 72 °C for 1 min in Eppendorf Mastercycler gradient thermocycler. A final extension was allowed for 10 min at 72 °C. Upon completion of the reaction, aliquots of PCR products (15 μl) were mixed with 3 μl of loading buffer (50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol) loaded onto a 2.0% agarose/1× Tris-Borate EDTA gel and electrophoresed at 4 V cm^{-1}.

Amplifications were repeated at least twice (in different time periods) for each primer using the same reagents and procedures.

Data collection and cluster analysis

Amplified fragments were visualized under a UV transiluminator 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 ver. 2.02 (Applied Biostatistic, Exeter Software, Setauket, New York, USA).

In cluster analysis of the samples the unweighted pair-group method with the arithmetic mean (UPGMA) procedure was followed (Rohlf 1992). In order to determine the ability of ISSR data to display the inter-relationships among the samples, principle co-ordinate analysis (PCA) of pairwise genetic distances (Nei 1972) was also conducted using NTSYS-pc package.

Results and discussion

Plants collected from 12 different natural habitats were taken to the laboratory silica-gel dried. The total number of species collected and used in the phylogenetic analysis was 14. DNA extractions were first tried with a standard 2X CTAB method. Due to the poor DNA quality produced by the CTAB procedure, a commercial kit (Nucleon phytopure) was used in all isolations and repeated extractions were conducted whenever necessary.

From an initial screening of 25 ISSR primers, eight primers generated 89 highly polymorphic fragments that were consistently amplified

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

Primer	Primer sequence	T_m (°C)	Size (bp)	GC (%)	T_{Ann} (°C)	Number of polymorphic bands
ISSR 1	$(ACC)^6$-G	63.1	19	68.4	61	15
ISSR 2	$(GA)^9$-C	56.7	19	56.7	56	12
ISSR 3	$(AG)^9$-C	56.7	19	52.6	56	14
ISSR 4	$(AC)^9$-G	56.7	19	52.6	56	7
ISSR 5	$(AC)^8$-CG	56	18	55.6	55.5	11
ISSR 6	$(AC)^8$-CC/T	54.8	18	52.8	55.5	11
ISSR 7	CAG-$(CA)^8$	56.7	19	52.6	56	9
ISSR 8	CGT-$(CA)^8$	56.7	19	52.6	56	10

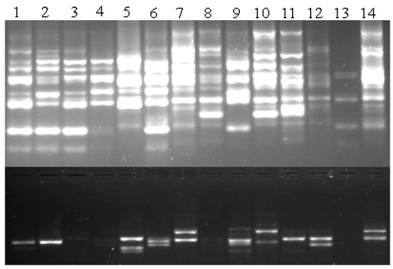

Fig. 1. Representative agarose gels where PCR products were amplified with the primers ISSR 1 (highest number of polymorphic bands, top) and ISSR 4 (lowest level of polymorphic bands, down). 1 = *Jurinea consanguinea*, 2 = *J. cadmea*, 3 = *J. mollis*, 4 = *J. macrocalathia*, 5 = *J. kileae*, 6 = *J. pontica*, 7 = *J. macrocephala*, 8 = *J. ramulosa*, 9 = *J. pulchella*, 10 = *J. aucherana*, 11 = *J. brevicaulis*, 12 = *J. cataonica*, 13 = *J. alpigena*, 14 = *J. ancyrensis.*

in repeated experiments conducted at separate dates. The GC percentages of the selected primers were within the range of 52.6%–68.4% (five of them being 52.6%). Six of the primers were 19-bp long and the two were 18-bp. Annealing temperatures of the oligos were about 1 °C lower than their melting temperatures. The characteristics as well as the sequences of the primers revealing a polymorphism are shown in Table 1. Primer ISSR 1 amplified the highest number (15 bands) and ISSR 4 the lowest number of polymorphic fragments (7 bands). In total, the average number of polymorphic fragments per primer used was roughly 11. A representative figure containing ISSR 1 and ISSR 4 banding patterns is in Fig. 1.

The analysis of the scored bands revealed that 13 *Jurinea* species were composed of two major groups that varied at a level of 55% similarity. The remaining species, identified as *J. kileae*, served as an outlier sample equally distinct from both groups (Fig. 2). *Jurinea kileae* was collected from Kirklareli-Kiyikoy in western Anatolia (European site) and is the only species collected from a coastal region of the Black Sea. The species mainly collected from coastal regions in western Anatolia (with the exception of *J. consanguinea* from central Anatolia) gathered in the first major group. Our studies clearly demonstrated that *J. mollis* is most closely related to *J. consanguinea*, followed by *J. cadmea*. The other major clade is composed of the species collected in eastern Anatolia, with the exception of *J. macrocephala* from Eregli in the province of Konya. *Jurinea aucherana* was closely related to *J. macrocephala* and less closely to *J. ramulosa*. Overall, the samples collected from Eastern Turkey grouped into three branches, the second branch formed by the species collected from Erzincan. From the dendrogram, it was impossible to distinguish *J. brevicaulis* from *J. cataonica*. However, the distinction was possible with the Principal Coordinate Analysis (PCA).

The related species were clearly separated by the second principal coordinate (Fig. 3). The last branch represented by *J. pulchella*, a species col-

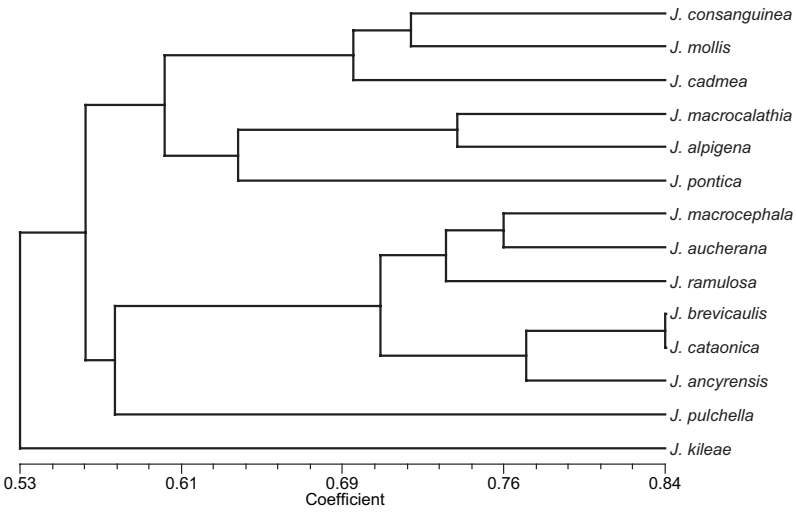

Fig. 2. Dendrogram showing the genetic relationship of *Jurinea* using inter simple sequence repeats.

lected from Van, was also found to be efficiently discriminated with PCA where it appeared between the two major groups. This separation was possible by the first principal coordinate (Fig. 3). Clear distinction of *J. kileae* was possible with both principal coordinates. All these findings were consistent with the morphological classifications made in the *Flora of Turkey* (Danin & Davis 1975).

The phytogeographical distribution of the genus *Jurinea* was found to be closely related to the Anatolian Diagonal. The Diagonal is rich in local endemic plants, of which there are more here than in any other Mediterranean region (Duran *et al*. 2005). The concept of the Diagonal was first proposed by Davis (1971) who defined it as an oblique belt running from the northeast south to the Anti-Taurus: it was then divided into two, with one branch to the Amanus (Amanos Dağları), the other to the Cilician Taurus. Thirty-three percent of the total number of species growing in Turkey are found along the Diagonal, while 5% are more or less restricted to it. One explanation for the present richness of the species is neo-endemism and distribution patterns of the plants related to the Diagonal (Ekim & Güner 1986).

Jurinea macrocephala, *J. ramulosa*, *J. aucherana*, *J. brevicaulis*, *J. cataonica*, and *J. ancyrensis* grow on and around the Anatolian Diagonal and they geographically differ from the remaining *Jurinea* species. Also molecular studies have proven the phylogenetically close relationship of the species that inhabit the Anatolian Diagonal. Although *J. pulchella* grows at the eastern parts of the Anatolian Diagonal and is both geographically and phylogenetically distinct from this group, it is still the most closely related species. *Jurinea pontica*, *J. alpigena*, *J. macrocalathia*, *J. cadmea*, *J. consanguinea* and *J. mollis* are geographically isolated from the remaining species. Molecular data supports a close relationship between these species. *Jurinea kileae*, which grows along the salty dune shores at the western part of the Anatolian Diagonal, constitutes a highly distinct group, both morphologically and phylogenetically different from the remaining *Jurinea* species.

Based on the physiographic disturbances that isolated the *Jurinea* species growing on the Anatolian Diagonal and its eastern parts from the

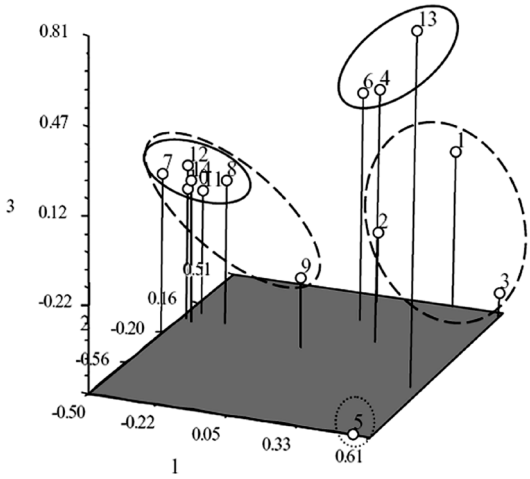

Fig. 3. Principal co-ordinate analysis of *Jurinea* species. There are two major groups separated by the first principal coordinate, while *J. kileae* (5) was efficiently distinguished by both coordinates. 1 = *Jurinea consanguinea*, 2 = *J. cadmea*, 3 = *J. mollis*, 4 = *J. macrocalathia*, 5 = *J. kileae*, 6 = *J. pontica*, 7 = *J. macrocephala*, 8 = *J. ramulosa*, 9 = *J. pulchella*, 10 = *J. aucherana*, 11 = *J. brevicaulis*, 12 = *J. cataonica*, 13 = *J. alpigena*, 14 = *J. ancyrensis*.

species growing along the western regions of the Diagonal, two major taxonomical groups were constituted. Our molecular data provided strong and reliable support to the biogeographic distribution patterns and the taxonomic relationships within *Jurinea*.

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References

Ash, G. J., Raman, R. & R. Crump, N. S. 2003: An investigation of genetic variation in *Carthamus lanatus* in New South Wales, Australia, using intersimple sequence repeats (ISSR) analysis. — *Weed Res.* 43: 208–213.

APG 2003: An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. — *Bot. J. Linn. Soc.* 141: 399–436.

Bremer, K., Backlund, A., Sennblad, B., Swenson, U.,

Andreasen, K., Hjertson, M., Lundberg, J., Backlund, M. & Bremer, B. 2001: A phylogenetic analysis of 100+ genera and 50+ families of euasterids based on morphological and molecular data with notes on possible higher level morphological synapomorphies. — *Pl. Syst. Evol.* 229: 137–169.

Danin, A. & Davis, P. H. 1975: *Jurinea* Cass. — In: Davis, P. H. (eds.), *Flora of Turkey and the East Aegean Islands* 5: 439–450. Edinburgh Univ. Press, Edinburgh.

Davis, P. H. 1971: Distribution patterns in Anatolia with particular reference to endemism. — In: Davis, P. H., Harper, P. C. & Hedge, I. C. (eds.), *Plant life of South-West Asia*: 15–28. Bot. Soc. Edinburgh.

Duran, A., Sağıroğlu, M. & Duman, H. 2005: *Prangos turcica* (Apiaceae), a new species from South Anatolia, Turkey. — *Ann. Bot. Fennici* 42: 67–72.

Ekim, T. & Güner, A. 1986: The Anatolian Diagonal: fact or fiction. — *Proc. Soc. Edinburgh* 89B: 69–77.

Fang, D. Q., Roose, M. L., Krueger, R. R. & Federici, C. T. 1997: Fingerprinting of trifoliate orange germplasm accessions with isozymes, RFLPs, and inter-simple sequence repeat markers. — *Theor. Appl. Genet.* 95: 211–219.

Font, M., Garnatje, T., Garcia-Jacas, N. & Susanna, A. 2002: Delineation and phylogeny of *Centaurea* sect. *Acrocentron* based on DNA sequences: a restoration of the genus *Crocodylium* and indirect evidence of introgression. — *Pl. Syst. Evol.* 243: 15–26.

Garcia-Jacas, N., Garnatje, T., Susanna, A. & Vilatersana, R. 2002: Tribal and subtribal delimitation and phylogeny of the Cardueae (Asteraceae): A combined nuclear and chloroplast DNA analysis. — *Mol. Phylogen. Evol.* 22: 51–64.

Garcia-Jacas, N., Susanna, A., Garnatje, T. & Vilatersana, R. 2001: Generic delimitation and phylogeny of the subtribe Centaureinae (Asteraceae): A combined nuclear and chloroplast DNA analysis. — *Ann. Bot.* 87: 503–515.

Garcia-Jacas, N., Susanna, A., Mozaffarian, V. & Ilarslan, R. 2000: The natural delimitation of *Centaurea* (Asteraceae: Cardueae): ITS sequence analysis of the *Centaurea jacea* group. — *Pl. Syst. Evol.* 223: 185–199.

Hakki, E. E., Savaskan, C. & Akkaya, M. S. 2001: Genotyping of Anatolian doubled-haploid durum lines with SSR markers. — *Euphytica* 122: 257–262.

Hakki, E. E. & Akkaya, M. S. 2000: Microsatellite isolation using amplified fragment length polymorphism markers: no cloning, no screening. — *Mol. Ecol.* 9: 2149–2154.

Joel, D., Portnoy, V., Tzuri, G., Greenberg, R. & Katzir, N. 1998: Molecular markers of the identification of *Orobanche* species. — In: Moreno, M. T., Cubero, J. T., Berner, D., Joel, D. M., Musselman, L. J. & Parker, C. (eds.), *Advances in parasitic plant research*, *Proc. 6th Int. Symposium on Parasitic Weeds*: 152–160. Consegeria des Agricultura Pesca, Cordoba.

Jump, A. S., Woodward, F. L. & Burke, T. 2003: *Cirsium* species show disparity in patterns of genetic variations at their range-edge, despite similar patterns of reproduction and isolation. — *New Phytol.* 160: 359–370.

Mengistu, L. W. & Messersmith, C. G. 2002: Genetic diversity of *Kochia*. — *Weed Sci.* 50: 498–503.

Nei, M. 1972: Genetic distance between populations. — *Am. Nat.* 106: 283–292.

Pharmawati, M., Yan, G. & McFarlane, I. J. 2004: Application of RAPD and ISSR markers to analyse molecular relationships in *Grevillea* (Proteaceae). — *Aust. Syst. Bot.* 17: 49–61.

Prevost, A. & Wilkinson, M. J. 1999: A new system of comparing PCR primers applied to ISSR fingerprinting of potato accessions. — *Theor. Appl. Genet.* 98: 107–112.

Rohlf, F. J. 1992: *NTSYS-pc: Numerical taxonomy and multivariate analysis system*, *version 2.0*. — State Univ. New York, Stony Brook, NY.

Soranzo, N., Provan, J. & Powel, W. 1999: An example of microsatellite length variation in the mitocondrial genome of conifers. — *Genome* 42: 158–161.

Susanna, A., Garcia-Jacas, N., Hidalgo, O., Vilatersana, R. & Garnatje, T. 2006: The Cardueae (Compositae) revisited: Insights from ITS, *trnl-trnf*, and *matk* nuclear and chloroplast DNA analysis. — *Ann. Missouri Bot. Garden* 93: 150–171.

Vosman, B. & Arens, P. 1997: Molecular characterization of GATA/GACA microsatellite repeat in tomato. — *Genome* 40: 25–33.

Williams, J. G. K., Kubelik, A. R. & Livak, K. J. 1990: DNA polymorphism amplified by arbitrary primers are useful as genetic markers. — *Nucleic Acids Res.* 18: 6531–6535.

Wolfe, A. D., Qiu-Yun, X. & Kepkart, S. R. 1998: Assessing hybridization in natural populations of *Penstemon* (Scrophulariaceae) using hypervariable intersimple sequence repeat (ISSR) bands. — *Mol. Ecol.* 7: 1107–1125.

Yang, W., De Oliveira, A. C., Godwin, I., Schertz, K. & Bennetzen, J. L. 1996: Comparison of DNA marker technologies in characterizing plant genome diversity: variability of Chinese sorghums. — *Crop Sci.* 36: 1669–1676.

Zietkiewicz, E., Rafalski, A. & Labuda, D. 1994: Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. — *Genomics* 20: 176–183.