African Journal of Biotechnology Vol. 9(12), pp. 1741-1745, 22 March, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

A phylogenetic analysis of *Jurinea* (Compositae) species from Turkey based on ITS sequence data

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Accepted 10 March, 2010

In this study, the taxa which belong to the *Jurinea* Cass. genus, grown naturally in Turkey and collected from different localities, are compared in terms of their internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (nrDNA). The DNAs of the taxa were isolated by using a commercial kit (Nucleon Phytopure). Sequences were aligned with Clustal X. Phylogenetic relationships are assessed by sequence analysis, conducted with the DNASTAR software after the amplification of ITS regions with the universally recognized primers, named as 17SE and 26SE. In conclusion, morphologically similar taxa are also clustered together when a phylogenetic analysis is conducted based on molecular data. *Jurinea* species of the Anatolian peninsula are closely related with the topography of the region and certain level of molecular isolation of the species is evidenced.

Key words: *Jurinea*, Asteraceae, molecular systematics, phylogeny, internal transcribed spacer, Turkey.

INTRODUCTION

Cardueae (Asteraceae) is the tribe that is generally accepted to be classified into five subtribes named Echinopinae, Carlininae, Carduinae Centaureinae and Cardopatiinae (Susanna et al., 2006). However, delimitation of these taxonomic entities is highly problematic, hence, there are some conspicuous incongruences. Not only the limits of the tribes, but also the boundaries between these units are very difficult to be established. Again, some large genera of the tribes have generic delimitation problems: *Carduus* L. (90 species), *Cirsium* Mill. (250 species), *Centaurea* L. (400 species), *Cousinia* Cass. (800 species), *Jurinea* Cass. (100 species) and *Saussurea* DC. (more than 300 species) (Garcia-Jacas et al., 2002). Extensive work conducted recently by Garcia-Jacas et al. (2000, 2001) and Font et al. (2002) have clarified the delimitation of *Centaurea*. Limited studies also exist on *Cirsium* and *Carduus* (Haffner and Hellwig, 1999), but most of the taxonomic problems persist. The genus *Jurinea* Cass. (100 species) constitutes a taxonomically complex group of plants with systematically unsolved problems, especially within the species surrounding *Jurinea mollis* (L.) Reichb. (Danin and Davis, 1975).

In recent years, whenever classification is problematic, revision studies of the families, employed by morphological means, are supported by the molecular phylogenetics (APG, 2003). In contrast with the cladistic morphological analysis, DNA-based molecular genetic methods are not influenced by the environmental conditions; hence they serve as powerful tools in resolving the taxonomical problems of the species.

Nuclear genomic analyses are applied for all the taxonomical categories and populations for the reconstruction of phylogenetic relationships among Angiosperms. nrDNA is organized as individual chromosomal units that are repeated thousands of times in the genomes of most

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Abbreviations: ITS, Internal transcribed spacer; **nrDNA,** nuclear ribosomal DNA; **RNA,** ribonucleic acid; **rRNA,** ribosomal RNA; **dNTPs,** deoxynucleoside 5'-triphosphate; **EDTA,** ethylene diamine tetraacetic acid; **PCR,** polymerase chain reaction; **CTAB,** cetyl trimethyl ammonium bromide; **ISSRs,** inter simple sequence repeats.

of the higher plants. Every unit contains the genes coding for 5.8S, 18S and 26S ribosomal RNA (rRNA) subunits, but it also contains several different spacer DNA regions. Highly variable ITS regions (ITS-1 for the region in between the coding regions 18S and 5.8S; ITS-2 for the spacer DNA in between the coding regions 5.8S and 26S) are extensively used for the systematic analysis of closely related taxa (closely related genera as well as species level) in order to resolve their taxonomical problem (Jorgensen and Cluster, 1988; Swofford and Olsen, 1990; Soltis et al., 1999; Plovanich and Panero, 2004; Wang et al., 2005; Haffner and Helwig 1999). In this study, *Jurinea* species, which are difficult to characterize with the use of morphological traits, were collected from the natural flora of Turkey. DNAs were isolated and partial ITS sequences were used in order to reveal the phylogenetic relationships of the Turkish *Jurinea* species.

MATERIAS AND METHODS

Specimen collection

Silica gel dried plant leaf samples belonging to 18 *Jurinea* species were collected from the natural flora of Turkey. The *Jurinea* species collected and their localities are as follows: *Jurinea consanguinea* (Konya-Aksehir), *Jurinea cadmea* (Izmir-Bozdag), *J. mollis* (Izmir-Rasathane), *Jurinea macrocalathia* (Tekirdag-Kumbag), *Jurinea kileae* (Kirklareli-Kiyikoy), *Jurinea pontica* (Izmit-Sapanca), *Jurinea macrocephala* (Konya-Eregli), *Jurinea ramulosa* (Kahramanmaras), *Jurinea pulchella* (Van), *Jurinea aucherana* (Erzincan), *Jurinea brevicaulis* (Erzincan), *Jurinea cataonica* (Erzincan), *Jurinea alpigena* (Karabuk-Keltepe), *Jurinea ancyrensis* (Elazig-Harput), *Jurinea cypria* (Mersin-Mut), *Jurinea stoechadifolia* (Çankiri), *Jurinea turcica* sp. nov. (Kirklaerli-Kiyiköy), and *Jurinea tortumensis* sp. nov. (Erzurum-Tortum).

DNA extraction

Nuclear DNAs of silica gel dried leaf samples collected in the field were extracted first according to the usual CTAB procedure (Sambrook et al., 1989). A total of 30 mg dried leaf material was used and DNAs were isolated individually. Due to the low DNA quality, DNA extractions were repeated with a commercial kit (Nucleon Phytopure, Amersham-Pharmacia) according to the manufacturer`s suggestions. After concentrations were determined (by an Eppendorf BioPhotometer), sample DNAs were diluted to the working concentration of 25 ng/µl. 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 λ DNA with known concentrations. Stock DNAs were kept at -86°C ultralow temperature freezer.

ITS amplifications and sequencing

Double-stranded DNA of the ITS region was mostly amplified using 17SE as the forward primer and 26SE as the reverse primer (Sun et al., 1994). In our study, reaction mix 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, 1 µM primer, 25 ng DNA template and 0.4 units of *Taq DNA Polymerase* (Bioron) in a final reaction volume of 25 µl. The profile used for amplification included a hot start at 94°C for 5 min. Then, 40 cycles of amplification were carried out under the following conditions: 94°C for 1 min, 54°C for 1 min and 72°C for 1.5 min. With a final extension step of 10 min at 72°C the reactions were completed. Aliquots of PCR products (15 µl) were mixed with 3 µl of loading buffer, loaded onto a 2.0% agarose/1x Tris-Borate EDTA gel and electrophoresed at 4 V/cm. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA). Direct sequencing of the amplified DNA segments was performed using a BigDye Terminator Cycle Sequencing v3.1, following the manufacturer's protocol and analysed on an ABI PRISM 3730 DNA analyser (PE Biosystems). The sequences were edited using Chromas 1.45.

Phylogenetic analysis

DNA sequences were aligned via Clustal X software (Thompson et al., 1997). Phylogenetic relationships were determined *via* DNASTAR Lasergene sequence analysis software (DNASTAR, Madison, WI, USA), based on partial ITS-1 sequences (Table 1).

RESULTS AND DISCUSSION

DNA extractions were first tried with a standard 2X CTAB method. Due to the poor DNA quality with the CTAB procedure, a commercial kit (Nucleon Phytopure) was used in all isolations and repeated extractions were conducted whenever necessary.

ITS sequences of *Jurinea* taxa were aligned with the computer program Clustal X (data not given) and phylogenetic tree, based on the partial ITS-1 sequences were generated by use of a DNA sequence analysis software, named as DNASTAR (Figure 1).

The maximum size of the amplification product from the *Jurinea* taxa was 660 bp long. Sequence analysis was conducted from forward direction of ITS-1 region. When all the sequences were aligned with the multiple sequence alignment freeware, Clustal X, spacer DNAs were found to be highly variable. Based on these sequences, the dendrogram was constructed with DNASTAR software and the taxa were found to be basically clustered in accordance with their individual morphological structures (Figure 1).

J. cataonica, J. cypria, J. brevicaulis, J. macrocephala, J. ramulosa, J. aucherana, J. ancyrensis, J. macrocalathia and *J. stoechadifolia* were the Jurinea taxa that constituted one of the major groups. Common characteristics of all taxa (but *J. macrocalathia*) within this group were: leaves whole, linear or linear-lanseolate, branched flowering sites and usually in corymbus style. Distibution of the species of this group, with the exception of *J. macrocalathia*, were found to be closely related to the topography of the Anatolian peninsula. That is, because these species (but *J. stoechadifolia*) are naturally grown at the Eastern part of the Anatolian Diagonal. *J. stoechadifolia* is distributed in Bulgaria and former Soviet

Table 1. Origin of the materials and herbaria where the vouchers are deposited and Genbank accession numbers.

Species	Origin, voucher	Genbank accession numbers
J. consanguinea	Turkey, Konya: Sultan Mountains, 1130 m, 37°14.913'N, 038° 49.427'E, 18.07.2006, B.Dogan 1501 (KNYA).	EU760464
J. tortumensis	Turkey, Erzurum: From Erzurum to Tortum, 1880 m, 40°.15.08'N, 041°.31.59'E, 31.05.2003, A.Duran 6170 (KNYA), Endemic.	EU760475
J. alpigena	Turkey, Karabuk, Keltepe, 1630 - 1680 m, 41°03.796'N, 032°27.781'E, 21.07.2006, B.Dogan 1510 (KNYA), Endemic.	EU760458
J. cadmea	Turkey, Izmir, Odemis, Bozdag, 1650 - 1750 m, 38°20.671' N, 028°06.403' E, 19.07.2006, B.Dogan 1503 (KNYA), Endemic.	EU760462
J. mollis	Turkey, Kirklareli, from Vize to Sergen, *Quercus* sp. forest, 310 - 320 m, 41° 37.312' N, 027° 40.950' E, 07.07.2005, B.Dogan 1504 (KNYA).	EU760470
J. macrocalathia	Turkey, Tekirdag, Malkara, Kumbag, 5 - 25 m, 40° 51.852' N, 027°27.604' E, B.Dogan 1507 (KNYA).	EU760468
J. turcica	Turkey, Kirklareli, Kiyikoy, 5 - 10 m, 41° 38.283' N, 028° 05.661' E, 08.07.2005, B.Dogan 1008 (KNYA), Endemic.	EU760467
J. pontica	Turkey, Kocaeli, Sapanca lake slopes, 40-50 m, 40°44.118' N, 033°36.871' N, 09.07.2005, B.Dogan 1011 (KNYA), Endemic.	EU760471
J. pulchella	Turkey, Van, Campus of Yüzüncü Yil University, 1650 m, 05.08.2005, B.Dogan 1025 (KNYA).	EU760472
J. kilaea	Turkey, Kirklareli, Kasatura (Kastros), beach dunes, 1 - 5 m, 41°35.273' N, 028°08.566' E, 08.07.2005, B.Dogan 1010 (KNYA).	EU760466
J. stoechadifolia	Turkey, Çankiri, from Çankiri to Ilgaz, 830 m, 39°54.362' N, 040°41.903' E, 23.09.2006, B. Dogan 1522, A. Duran and H. Duman (KNYA).	EU760474
J. cypria	Turkey, Mersin, Mut, Mountain pasture of Kozlar, 1320 m, 37°14.913' N, 038°49.427' E, 28.07.2006, B.Dogan 1500 (KNYA)	EU760465
J. macrocephala	Turkey, Konya, Aydos Mountain, Kayasaray village, 1700 m, 37°22.249' N, 034°17.308' E, 31.07.2005, B.Dogan 1017 (KNYA).	EU760469
J. aucherana	Turkey, Erzincan, Spikor mountain, 760-20 95 m, 39°53.429' N, 039°45.778' E, 22.08.2006, B.Dogan 1519 (KNYA)	EU760460
J. ramulosa	Turkey, Kahramanmaras, Ahir Mountain, 1390 m, 37°37.057' N, 036°52.074' E, B.Dogan 1513 (KNYA).	EU760473
J. brevicaulis	Turkey, Erzincan, Spikor Mountain, 1420 m, 39°47.363' N, 039°29.940' E, 07.08.2005, B.Dogan 1028 (KNYA), Endemic.	EU760461
J. cataonica	Turkey, Erzincan, Spikor Mountain, 10 km, 1750 m, 39°47.954' N, 039°30.343' E, 07.08.2005, B.Dogan 1029 (KNYA), Endemic.	EU760463
J. ancyrensis	Turkey, Elazig, from Keban to Agli, 800 - 850 m, 38°48.692' N, 038°43.929' E, 21.08.2006, B.Dogan 1516 (KNYA).	EU760459

Union (Iljin, 1962; Kozuharov, 1964). This species is the only taxon within *Jurinea* that is localized to gypsy soil and its distribution is free from the restrictions of the Anatolian Diagonal. Results of the present study overlap that of the ISSR study, conducted recently by Dogan et al. (2007).

The remaining taxa of the phylogenetic tree, with the only exception of *J. pulchella*, are distributed at the Western part of the Anatolian Diagonal. The common characteristics of these species include the presence of single capitula (except *J. pontica* and *J. pulchella*) and the fragmented leaf shapes (from pinnatilobed to pinnatisect). These taxa are: *J. mollis, Jurinea cadmea, Jurinea kilaea, J. consanguinea, J. alpigena, J. turcica, J. pontica* and *J. pulchella*. The species *J. mollis, J. cadmea* and *J. pulchella* constituted a separate clade within the phylogenetic tree. *J. mollis* and *J. cadmea* are the two species that are also morphologically very similar. *J. pulchella* that is distributed at the Eastern parts of the Diagonal presents both pinnatisect and whole leaves. This suggests that it is a transition taxon and it can be found within both groups. That is why it is meaningful that in this study it was placed within the Western group with fragmented leaves. That also is an indication that leaf-shape is a more descriptive characteristic for the genus *Jurinea*. This was also evidenced by the close relationships of the taxa *J.*

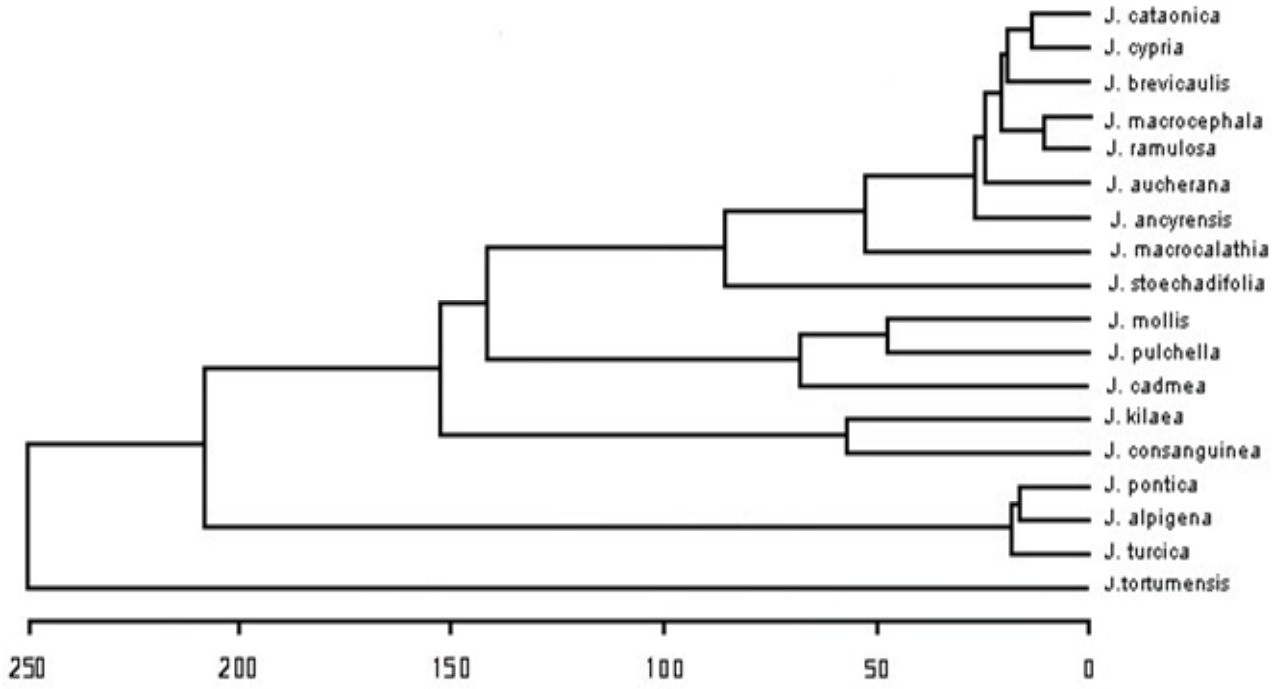

Figure. 1. Phylogenetic tree showing the genetic relationship of *Jurinea* using ITS region.

kilaea and *J. consanguinea* that share similar leaf characters (that is, they have in both fragmented and whole leaves). However, *J. kilaea* within the genus has unique physiognomy with its distribution at the salty dune coastal regions.

J. pontica, *J. alpigena* and *J. turcica* are the species that were placed together as a sub-clade with considerably low level of variation. *J. turcica* and *J. alpigena* share an unbranched shoot and single capitula. Beyond the fragmented leaves (a distinguishing character in *Jurinea*) that *J. pontica* shared with the other two taxa, its shoot morphology and capitual structure are completely different. That is, *J. pontica* presents a branched shoot and the number of its capitula is more than two, hence its place in this phylogenetic tree was not found very meaningful.

In a recent study that used ISSRs as a fingerprinting method, 14 *Jurinea* taxa that occur naturally in Turkey were used and a high level of coherence was found with the classification on the basic morphological characteristics and also with the topographical state of the Anatolian Diagonal. The taxa used were clearly separated into two groups. The first group was comprised of the taxa *J. consanguinea, J. mollis, J. cadmea, J. macrocalathia, J. alpigena,* and *J. pontica*. These species are naturally grown at the West parts of the Diagonal. The other group was constituted of the species *J. macrocephala, J. aucherana, J. ramulosa, J. brevicaulis, J. cataonica, J. ancyrensis* and *J. pulchella*. These are all species that occur at the Eastern part of the Diagonal (Dogan et al., 2007).

In conclusion, morphologically similar taxa are also clustered together when a phylogenetic analysis is conducted based on molecular data. *Jurinea* species of the Anatolian peninsula are closely related with the topography of the region and certain level of molecular isolation of the species is evidenced.

Our investigation reported here has demonstrated that resolution of genetic relationships within controversial taxonomic entities may be enhanced by ITS sequence analysis. When compared with the phenotypical characters, molecular data generally provide more reliable results (Meerow et al., 2003; Garnatje et al., 2005). However, recent studies suggest that ITS studies alone are usually inadequate for phylogenetic reconstruction (Maurin et al., 2007). For a holistic understanding, phylogenetic studies using systematic data from various sources, including sequences from plastid DNAs, ITS sequences of nrDNAs, other genomic sequences as well as morphologies of the taxa should be considered. According to our best knowledge, this is the first report on the use of ITS sequences of *Jurinea* from Turkey.

ACKNOWLEDGEMENTS

We express our thanks to TUBITAK (Project no. TBAG-105T355) and The Scientific Investigation Projects Co-

ordinate Office of The Selcuk University (Project no: 05401046) for financial support.

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