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Phylogenetic Relationships of Some *Ajuga* L. Taxa Based on the Ribosomal DNA Internal Transcribed Spacer Region

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Abstract

Ajuga L. is represented by 14 species and 27 taxa in the flora of Turkey. Eleven species of Ajuga have been studied for phylogeny based on sequence data of the nuclear ribosomal DNA region (ITS). According to the findings, morphologically similar species collected in the same group. The polymorphism in sequences of internal transcribed spacers ITS1 and ITS2 and 5.8 S rDNA coding region was used to determine relationships among different species of genus Ajuga. The taxonomy of several Ajuga species is need of revision based on the ITS phylogeny. Understanding the phylogenetic relationships among Ajuga species would contribute to the systematics of the genus, comprehension of the origins and evolution of species and sections and the use of species of genus Ajuga.

Keywords: *Ajuga*, ITS, phylogenetic, sequence

1. Introduction

The genus *Ajuga* L. consists of approximately 90 species, mostly distributed in the north temperate zone of the old world. *Ajuga* is also known as bugleweed, ground pine, carpet bugle, or just bugle, and is a genus of annual and perennial herbaceous flowering plants in the mint family Lamiaceae, with most species native to Europe, Asia, and Africa, but also two species in southeastern Australia (D.J. MABBERLEY [1]). *Ajuga* is represented by 14 species and 27 taxa in Turkey (P.H. DAVIS [2], H. DUMAN [3]).

Most genera in the Lamiaceae including the genus *Ajuga* have insect pollination systems. Their special floral structure suggests that intricate pollination mechanisms reflect a long history of adaptive coevolution between plants and pollinators (R.B. HUCK, [4]).

The ribosomal internal transcribed spacer (ITS) sequences are frequently used in researching phylogenetic relationships of plants at the lower taxonomic levels (B.G. BALDWIN & al. [5]). The ITS region consists of three parts: the ITS1, ITS2 and the highly conserved 5.8S rDNA exon. The total length of this region varies between 500 and 750 bp in angiosperms (P. POCZAI & J. HYVÖNEN [6]).

Asteraceae, Fabaceae, Orchideaceae, Poaceae, Brassicaceae, and Apiaceae are the plant families that have been studied most intensively by using molecular approaches. The information which is provided by the rDNA locus in the phylogenetic research can be used at different taxonomic levels since the specific regions of the rDNA loci are conserved differentially. Information from the spacer regions of the rDNA locus could be very useful to assign the samples within different levels of taxa in plant systematics. ITS has proven especially useful for elucidating relationships among congeneric species and closely related genera in Asteraceae (5-6).

It was reported that the ITS region could provide a valuable set of characters for addressing lower level phylogenetic relations between flowering plants. Despite a limited number of identified nuclear DNA regions, the ITS region is particularly effective for angiosperm systematists, especially for determining morphological and molecular similarities.
The purpose of this study is to determine the phylogeny ofsome *Ajuga* taxapresent in Turkey and to put them in the correct taxa based on their similarities and differences using sequence data of nuclear ribosomal DNA region.

2. Materials and Methods

2. 1. Plant Material

Plant samples were collected from various parts of Turkey (Table 1).

Table 1. Localities of plant samples

***Ajuga* taxa**	**Genbank Accession No.**	**Origin**
A. bombycina	KJ578645	C4 Antalya: Alanya, around castle, slopes, 102 m, 36^0 32'N 31^0 59'E, 24/03/2010.
A. chamaepitys subsp. *chia* var. *chia*	KJ578653	B3 Kütahya: 10 km to Domaniç, 39^o 44' 844'' N 29^o 32' 440''E, 31 v 2010
A. chamaepitys subsp. *cuneatifolia*	KJ578644	C2 Burdur, Bucak, Çobanbeli, stony places, 847 m, 37° 10'N 30° 29'E, 08/07/2008.
A. chamaepitys subsp. *cypria*	KJ578643	C2 Muğla: Fethiye, Babadağ, summit, stony, 1935 m, 36° 31'N 29° 10'E, 27 v 2010
A. chamaepitys subsp. *glaerosa*	KJ578647	C3 Antalya: Akseki, Güzelsu, Morca plateu, stony, 2090 m, 07/07/2008
A. chamaepitys subsp. *mesoginata*	KJ578649	C3 Antalya: Akseki, Güzelsu road, 1017 m, stony, 36° 54'N 31° 49'E, 07/07/2008
A. chamaepitys subsp. *palaestina*	KJ578652	B2 Denizli: Between Tavas-Denizli, 10 km to Denizli, slopes, 37° 38'N 29° 13'E, 30/05/2010
A. genevensis	KJ578651	A1 (E) Kırklareli: Dereköy road, 10 km to Dereköy, Quercus forest, 449 m, 41° 50'N 27° 18'E, 22/04/2009.
A. laxmannii	KJ578650	A1 (E) Kırklareli: Between Kıyıköy-Vize, *Quercus* forest, 298 m, 41° 39'N 27° 53'E, 16/06/2008.
A. orientalis	KJ578646	A2 (A) Bursa: Uludağ, open area, 1773 m., 40° 06'N 29° 08'E, 19/06/2008.
A. reptans	KJ578648	A2 (A) İstanbul: Sarıyer, İstanbul Ü. Orman Fak. Research forest, Fındık suyu, Carpinus forest, 41° 09'N 29° 00'E, 20/04/2009.

2. 2. DNA Extraction, PCR Amplifications, and Sequencing

Total genomic DNA was isolated from leaf tissue using the GE Nucleon PhytoPure Genomic DNA Extraction Kit (GE Healthcare UK Limited, Buckinghamshire, UK) according to the manufacturer's instructions. The quality of the total DNA was verified on 1% agarose gels.
PCR products for the internal transcribed spacers (ITS-1 and ITS-4) were obtained using RedTaqTM ReadyMixTM PCR Mix (Sigma–Aldrich) in a total volume of 25 ul. The

addition of BSA (5%) was necessary to improve the amplification. For double stranded PCR amplification and sequencing, the primers described by White et al. (T.J. WHITE & al. [7]) were used.

To amplify the ITS genes, specific primer sets (ITS-1 and ITS-4) were used. The sequence of the forward primer was ITS-1 (5'-TTC GTA GGT GAA CCT GCG G-3'). The reverse primer was ITS-4 5'- TCC TCC GCT TAT TGA TAT GC -3' (T.J. WHITE & al. [7]). The following conditions were used for amplification: a cycle of 95^{o}C for 2 min, 35 cycles of 95^{o}C for 60 s, 55^{o}C for 45 s, and 72^{o}C for 1.5 min; plus an extension step of 5 min at 72^{o}C. Negative controls were included with no addition of template DNA. Five microliters of PCR products were loaded onto 1% agarose gels in 1X Tris Acetic acid-EDTA (TAE) buffer. The gels were stained using ethidium bromide (0.2 μg/mL) and visualized and photographed under a UV transilluminator. PCR products were purified using the Wizard PCR and Gel Purification Kit (Promega) and stored at -20°C until required.

DNA sequencing was performed by Beckman CEQ 8000 genetic analyser. The DNA sequences were analysed using the BLAST homology search program, which is available at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) to identify close matches using BLAST (S.F. ALTSCHUL & al. [8]).

2. 3. Nucleotide Accession Numbers

The data of ITS sequence of nuclear ribosomal DNA have been deposited in the GenBank database under accession numbers; KJ578643, KJ578644, KJ578645, KJ578646, KJ578647, KJ578648, KJ578649, KJ578650, KJ578651, KJ578652 and KJ578653 (Table 1).

2. 4. Data Analysis

DNA sequence analyses were carried out through the BLAST, FASTA, and Chromas Lite programs (S.F. ALTSCHUL & al. [8], F. BANEYX [9], J.D. THOMPSON & al. [10]). Multiple gene alignments were carried out using MUSCLE 3.7 and Gblocks 0.91 b modules of "Phylogeny.fr." Phylogenetic analysis was done using PhyML 3.0 aLRT, and cladograms were generated using TreeDyn 198.3 module of Phylogeny.fr. All the above software and modules are freely available at http://www.phylogeny.fr/ (A. DEREEPER & al. [11]).

3. Results and discussion

We have analysed 13 accessions encompassing eleven *Ajuga* taxa. Sequencing data of the amplification products are obtained from ITS genes. The number of reads per accession varied because their quality also varied. Phylogenetic similarities are revealed by comparing with BLAST program in NCBI (National Center for Biotechnology Information).The nearest representatives of gene sequences in the gene bank are shown Table 2.

Table 2. Close relatives and similarity ratio in Genbank

Specimen	Closest relative in Genbank	Affinity ratio	Similar loculi	GenBank No.
A. chamaepitys subsp. *cuneatifolia* (ITS4)	*A. taiwanensis* voucher NSC-702 18S ribosomal RNA gene, partial	%87	435/497	HQ688669.1
A. chamaepitys subsp. *cypria* (ITS4)	*A. taiwanensis* voucher NSC-702 18S ribosomal RNA gene, partial	%89	475/529	HQ688669.1
A. bombycina (ITS4)	*A. taiwanensis* voucher NSC-702 18S ribosomal RNA gene, partial sequence;	%85	459/539	HQ688669.1
A. orientalis (ITS4)	*A. reptans* internal transcribed spacer 1, partial sequence; 5.8S	%93	579/616	EF508061.1
A. chamaepitys subsp. *glareosa* (ITS4)	*A. reptans* internal transcribed spacer 1, partial sequence; 5.8S	%89	462/517	EF508061.1
A. reptans (ITS4)	*A. reptans* internal transcribed spacer 1, partial sequence; 5.8S	%96	598/618	EF508061.1
A. chamaepitys subsp. *mesoginata* (ITS4)	*A. reptans* internal transcribed spacer 1, partial sequence; 5.8S	%93	556/619	EF508061.1
A. laxmannii (ITS4)	*A. reptans* internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	%83	436/524	EF508061.1
A. genevensis (ITS1)	*A. reptans* internal transcribed spacer 1, partial sequence; 5.8S	%81	562/614	EF508061.1
A. chamaepitys subsp. *palaestina* (ITS1)	*A. nipponensis* voucher NSC-704 18S ribosomal RNA gene, partial	%88	413/469	HQ840773.1
A. chamaepitys subsp. *palaestina* (ITS4)	*A nipponensis* voucher NSC-704 18S ribosomal RNA gene, partial	%87	468/533	HQ840773.1
A. chamaepitys ssp. *chia* var. *chia* (ITS1)	*A. nipponensis* voucher NSC-704 18S ribosomal RNA gene, partial	%87	599/681	HQ840773.1|
A. chamaepitys ssp. *chia* var. *chia* (ITS4)	*A. nipponensis* voucher NSC-704 18S ribosomal RNA gene, partial	%87	470/537	HQ840773.1|

The sequences ranged from 469 to 619 bp. Non-aligned ITS sequence lengths ranged to 469 bp in *A. chamaepitys* subsp. *palaestina* (KJ578652 in Table 2), and to 619 bp in *A. chamaepitys* subsp. *mesoginata* (KJ578649 in Table 2).The phylogenetic tree shows that well supported clades (Figure 1).

DNA sequence of *A. reptans* has been found similar to (96%) previously reported data in the database. Additionally, the base sequence of *A. orientalis* and *A. chamaepitys* subsp. *mesoginata* have also been found similar to *A. reptans* at a ratio of 93% (M. HUANG & al. [12]) (Table 2).

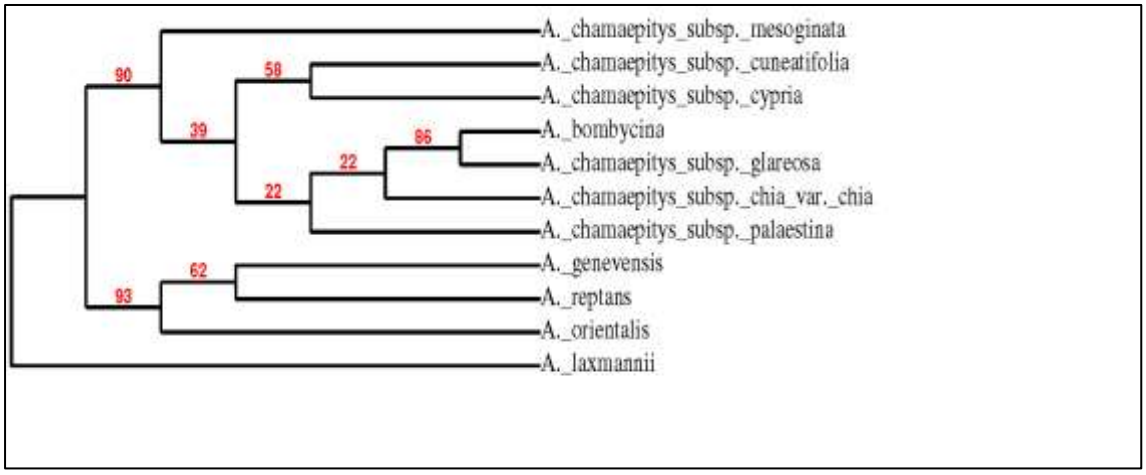

Figure 1. The cladogram shows the genetic relationships in *Ajuga* species based on ITS1-5.8S-ITS4 (ITS) sequences, evaluated by the Neighbour Joining method.

According to nucleotide sequence analysis of ITS regions, subspecies of *A. chamaepitys* formed a single group. *A. bombycina* was also included in the group of *A. chamaepitys*, suggesting that *A. bombycina* might be a subspecies of *A. chamaepitys.*

The phenotype of the blue-purple flowered species formed a separate group including *A. reptans, A. genevensis*, and *A. orientalis*. Species with purple-veined cream flowers formed a second group based on *A. laxmannii*.

When working on molecular phylogenies, choosing the genetic markers is an important process. These markers need to be chosen based on their ability to differentiate between taxa at the level that the research requires. Currently, the most common genetic marker used for phylogenetic studies in plants is the Internal Transcribed Spacer (ITS) region found in the nuclear genome of plants. This refers to the region of 18S, 5.8S and 26S ribosomal genes, along with two internal transcribed spacers. It is widely applied in phylogenetic studies, from fungi to higher terrestrial plants (T.J. WHITE & al. [7], I. ÁLVAREZ & J.F. WENDEL [13])

Although the genome diversity of plant species is studied with increasing intensity, the vast majority of plant species remains unknown. This study shows that ITS has high resolving power for discriminating closely related species with a high PCR and sequencing success rate across a broad range of plant species. Especially the PCR success rate with the ITS primer set was very high in our study. ITS PCR amplification success ranged from 100% to 80%. There were no problems with PCR amplification of ITS and also to obtain a high-quality PCR product in our study. The sequence data from type specimens will increasingly be essential to the stability of plant nomenclature. Continuing discovery of novel biodiversity and sequencing of the ITS region for systematic studies, as we performed in this study, supplies

useful data to solve the taxonomic problems among closely related plant species (B.G. BALDWIN & al. [5], B.G. BALDWIN [14], L. GIELLY & al. [15]). ITS1 and ITS2 are currently the most useful and practical nuclear sequences for addressing lower-level relationships in plants because of their relatively small size, high mutation rate, and easy amplification (B.G. BALDWIN & al. [5]).

Molecular phylogenetic has been increasingly used as a means of elucidating evolutionary histories and refining taxonomy where other means fail (e.g. the study group has ambiguous morphological data). Methods of obtaining molecular data have consequently improved to the point that these data are now as easy to obtain for phylogenetic purposes as any other type of data.

This important study provides new molecular data about genus *Ajuga* from Turkey to science. Phenotypic and genotyping characteristics of the Turkish *Ajuga* could be a very useful contribution to understanding their evolutionary differentiation mechanism geographically.

4. Conclusion

Ajuga species that were collected from different parts of Turkey have not previously been addressed by molecular methods. Some of these *Ajuga* species are closely related, and it has not been possible to identify these taxa based on simple morphological characters, especially subspecies of *A. chamaepitys*. Consequently, the relationships among these samples were evaluated by ITS sequences.

A. chamaepitys is a perennial, biennial or annual herb and very variable morphologically. Stems may be prostrate or ascending, variously hairy or glabrous. There is no absolutely sharp line of morphological differentiation between subspecies of *A. chamaepitys*. For instance, leaf and indumentum which are affected by environmental conditions are often used in the diagnosis of subspecies. *A. bombycina* is an endemic species from southwest Anatolia. Morphologically, it is very similar to *A. chamaepitys*, with only the densely tomentose leaves are different.

Out of *A. chamaepiys*, other species has blue-purple and cream flowers and erect stem. *A. laxmannii* is very different morpholigically from other species. According to our molecular results, this species is monophyletic.

The present study is a preliminary study in order to determine whether the internal transcribed spacer can provide additional useful information for the relationships of the subgeneric level of the genus or not; however, additional molecular data and a broader taxon sampling are needed for better conclusions.

Understanding the phylogenetic relationships among *Ajuga* species would contribute to the systematics of the genus, comprehension of the origins and evolution of species, and the use of species belonging to the genus *Ajuga*. A molecular phylogeny including more Turkish species (especially endemic species) should be used in the future. A global molecular phylogeny can also be used to look at species relationships within the group, many of which are still largely unclear.

We have shown here that ITS sequences could be suitable for revealing the taxonomic position of *Ajuga* samples. However it may be necessary to employ more than one locus to attain species-level discrimination across all *Ajuga* species.

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