Genetic characterization of Allium tuncelianum: An endemic edible Allium species with garlic odor

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Abstract

Allium tuncelianum (Kollman) Özhatay, Matthew & Şiraneci is a native species to the Eastern Anatolia. Its plant architecture resembles garlic (Allium sativum L.) and it has mild garlic odor and flavor. Because of these similarities between two species, A. tuncelianum has been locally called “garlic”. In addition, both A. tuncelianum and garlic has 16 chromosomes in their diploid genomes. Recently, A. tuncelianum has been suggested as the wild progenitor species of garlic. In this study, amplified fragment length polymorphisms (AFLP) markers and nucleotide sequence analysis of the internal transcribed spacer region (ITS) were used to assess genetic and phylogenetic relationships among A. tuncelianum, garlic and some other Allium species. AFLP analysis demonstrated that A. tuncelianum and garlic are genetically distinct and they are likely different species. Phylogenetic analyses based on the nucleotide sequence of ITS suggested that A. tuncelianum and garlic are distinct species and placed A. tuncelianum, garlic, Allium ampeloprasum and Allium scorodoprasum into the same clade in the neighbor joining dendrogram and in the consensus tree of parsimony analysis. However, A. tuncelianum was phylogenetically less related to garlic than either A. ampeloprasum or A. scorodoprasum, suggesting that A. tuncelianum may not be the immediate wild ancestor species of garlic. Further studies to generate hybrid progeny between A. tuncelianum and garlic (if possible) could provide more information on the homology between the chromosomes of A. tuncelianum and garlic and genetic relationships between these two species.

Keywords: AFLP; ITS; Phylogeny; Allium sativum; Ancestor species

1. Introduction

Garlic (Allium sativum L.) has been cultivated since the ancient times and its progenitor species has been suggested but not yet identified. Fritsch and Friesen (2002) have suggested that if there is a wild ancestor species of garlic, it should grow in the region from Mediterranean to south Central Asia, based on their taxonomic studies. A wild garlic relative from central Asia, Allium longicuspis, was proposed as a progenitor species of garlic (Vvedensky, 1944). However, recent studies based on the DNA markers demonstrated that A. longicuspis is not a distinct species from common garlic (Maass and Klaas, 1995; Al-Zahim et al., 1997; Ipek et al., 2003). Mathew (1996) suggested that Allium tuncelianum which is utilized as garlic in Eastern region of Turkey might be the wild ancestor of garlic. The plants of three species, A. sativum, A. longicuspis, and A. tuncelianum share some common characteristics such as odor, coiling of the flower stem before anthesis, pale colored, small, glabrous, rather narrow perianth segments, and glabrous filaments with very long lateral cusps (Mathew, 1996; Etoh and Simon, 2002).

A. tuncelianum is originally named as Allium macrochaetum Boiss and Haussk subsp. tuncelianum Kollmann (Etoh and Simon, 2002). Although, it is native to ‘Tunceli’ province (especially at Platos of Munzur Mountains in Ovacik district) of Turkey, it naturally grows in the limited region located between Sivas and Erzurum provinces (Baktir, 2005). Due to its resemblance to common garlic, it is locally called as ‘Tunceli garlic’ or ‘Ovacik garlic’ in the region.
A. tuncelianum usually forms single cloved white bulb, unlike garlic which has a multiple cloved bulb. The flower scape of A. tuncelianum coils early in its elongation, which is a typical characteristic of some garlic types. While A. tuncelianum forms non-bulbiferous inflorescences with fertile flowers, all flowering garlic genotypes have bulbils formation in their inflorescences along with the flowers. Bulbil formation in the garlic inflorescence has been suggested as a cause of garlic sterility (Koul and Gohil, 1970), but this has been refuted by studies generating true seed (reviewed by Simon and Jenderek, 2003). Cytological studies on A. tuncelianum has demonstrated that its genome is diploid with 2n = 16 chromosomes, which is the same number of chromosomes with most of the widely cultivated edible Allium species, except with leek that has predominantly tetraploid genome (Ozhatay, 2002; Mathew, 1996). Although A. tuncelianum has been considered as very close relative to garlic, the exact genetic or phylogenetic relationship of this species with garlic and other similar Allium species is not known to our knowledge.

The internal transcribed spacer region (ITS) consisting of 5.8S ribosomal RNA gene (rDNA) and flanking internal transcribed spacer 1 and 2 (ITS-1 and ITS-2) has been successfully used to characterize the phylogenetic relationships among the species of the genus Allium (Dubouzet and Shinoda, 1998, 1999; Mes et al., 1999; Friesen et al., 2000; Fritsch and Friesen, 2002). The genus Allium consists of about 750 species and most of them naturally grow in northern hemisphere (Stearn, 1992). Controversial taxonomy of this genus has been revised and will probably continue to be revised. The genus, Allium has been classified under Liliaceae, Amaryllidaceae, and recently Alliaceae family (Mes et al., 1999). Based on nucleotide sequence of ITS of nuclear ribosomal DNA (rDNA), Fritsch and Friesen (2002) recognized 14 monophyletic subgenera in this genus. According to phylogenetic analyses, A. sativum L., A. porrum L., and Allium ameloprasum L. that are morphologically similar species to A. tuncelianum have been classified under subgenus Allium section Allium (Hanelt, 1990; Mes et al., 1999; Ricroch et al., 2005). In this respect, the purpose of this study was to investigate phylogenetic and genetic relationships of A. tuncelianum with garlic and other related Allium species using amplified fragment length polymorphisms (AFLP) and the sequence of ITS region.

2. Materials and methods

2.1. Plant materials

Two different accessions of A. tuncelianum, four accessions of garlic, one accession of A. longiscapus and 17 other Allium species were included to this study (Table 1). A. tuncelianum accessions were obtained from Tunceli province of Turkey. Other Allium species except Allium cepa were obtained from US Department of Agriculture, Western Region Plant Introduction Station, Pullman, WA, USA. Seeds of Allium species were planted to pots in the Walnut Street, Greenhouse at University of Wisconsin, Madison, WI, USA, and young leaves were sampled from single plant of each accession. Accessions of A. tuncelianum, A. longiscapus and garlic were also planted in a field in Bursa province of Turkey to observe the morphology of these species. ITS sequence of A. cepa (gi:216278871) were obtained from the National Center for Biotechnology Information (NCBI) GenBank databases (http://www.ncbi.nlm.nih.gov) and included to phylogenetic analysis.

2.2. DNA extraction and PCR procedures

Lyophilized leaf samples were powdered using a paint shaker and approximately 150 mg of powder obtained were transferred to micro centrifuge tubes for DNA extraction. DNA samples were extracted according a modified CTAB method described by Fütteterer et al. (1995). ITS regions (ITS-1, 5.8S rDNA subunit, ITS-2) were amplified using primer combination of ITS A and ITS B (Blattner, 1999). Each 25 μL polymerase chain reaction (PCR) consisted of 0.75 Units of DNA polymerase (PanVera, Madison, WI, USA) with the reaction buffer supplied at 1× concentration, 0.8 μM of each primer, dNTPs at 200 μM each, and 40–60 ng template DNA. The reactions were heated to 94 °C for 2 min followed by 35 cycles of 95 °C for 20 s, 55 °C for 45 s, and 68 °C for 1 min and final extension of 72 °C for 5 min. For these reactions, a Perkin Elmer model 9600 Thermal Cycler was used. PCR products were size fractionated by electrophoresis through 1.5% (w/v) agarose in 1× TAE buffer (40 mM Tris–acetate, pH 8.0 and 1 mM EDTA) with a GeneRuler™ 100 bp DNA ladder (Fermentas, Hanover, MD, USA) as DNA molecular weight marker. Gels were stained with ethidium bromide (0.5 μg mL⁻¹) (Sigma, St Louis, MO, USA) and photographed.

Table 1 Accession numbers and the length of ITS regions in the Allium species analyzed in this study

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Species</th>
<th>Length of ITS (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI592999</td>
<td>A. roylei</td>
<td>641</td>
</tr>
<tr>
<td>W612754</td>
<td>A. oschaninii</td>
<td>642</td>
</tr>
<tr>
<td>W612755</td>
<td>A. pokemense</td>
<td>627</td>
</tr>
<tr>
<td>PE280549</td>
<td>A. altaicum</td>
<td>643</td>
</tr>
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<td>PE219754</td>
<td>A. fistulosum</td>
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</tr>
<tr>
<td>PI576875</td>
<td>A. altyunicolium</td>
<td>632</td>
</tr>
<tr>
<td>PI371880</td>
<td>A. schoenoprasum</td>
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</tr>
<tr>
<td>W621059</td>
<td>A. lineare</td>
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<td>PI576906</td>
<td>A. hymenorrhizum</td>
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</tr>
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<td>W618966</td>
<td>A. polyrhizum</td>
<td>633</td>
</tr>
<tr>
<td>PI369526</td>
<td>A. senescens</td>
<td>643</td>
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<td>A. scorodoprasum</td>
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<td>PI576926</td>
<td>A. ramosum</td>
<td>646</td>
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<td>PI576957</td>
<td>A. tuberosum</td>
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<td>W620304</td>
<td>A. cernuum</td>
<td>655</td>
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<tr>
<td>gi:216278871</td>
<td>A. cepa</td>
<td>639</td>
</tr>
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<td>Turkey</td>
<td>A. tuncelianum 1</td>
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</tr>
<tr>
<td>Turkey</td>
<td>A. tuncelianum 2</td>
<td>643</td>
</tr>
<tr>
<td>U094-4</td>
<td>A. longiscapus</td>
<td>642</td>
</tr>
<tr>
<td>U074</td>
<td>A. sativum</td>
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<td>PI515971</td>
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<td>PI383817</td>
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<tr>
<td>PI497949</td>
<td>A. sativum</td>
<td>642</td>
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</tbody>
</table>
2.3. Sequencing of ITS region

Purification of PCR products from the agarose gel, cloning and sequencing of them were carried out according to procedures previously described by Ipek et al. (2005) except for plasmid DNA extraction procedures. Three to four bacterial colonies from each purified and cloned PCR product were picked up and subjected to PCR amplification using T7 and SP6 universal primers of cloning vector. Each PCR reaction contained the same reaction mixture described above and a single bacterial colony as a DNA template. PCR reaction conditions were 94 °C for 2 min, then 35 cycles of 95 °C for 20 s, 50 °C for 1 min, 68 °C for 2 min and a final extension of 72 °C for 5 min. To remove single stranded DNA fragment such as primers, PCR products were treated with ExoSap (USB, Cleveland, OH, USA) by following the manufacturer’s protocol and 2 μl of each ExoSap treated PCR products was used for sequencing reaction.

2.4. Data analyses

Sequences obtained were manually edited with CHROMAS v.2.31 (Technelysium Pty. Ltd.). All sequences were aligned using CLUSTAL W option in BioEdit sequence alignment editor (Hall, 1999). No manual adjustment of alignment was made to avoid introducing subjective bias.

Aligned ITS sequences were evaluated with bootstrap analysis 2000× and Kimura (1980) distances were calculated to construct a neighbor joining (NJ) dendogram using TREECON v. 1.3b program (van de Peer and de Wachter, 1994) with the program defaults. The NJ dendogram was visualized by using TREECON program. The first 100 most parsimonious trees were generated by DNAPENNY in PHYLIP v. 3.6 (Felsenstein, 2002) utilizing sophisticated “branch and bound” algorithm and using the program defaults. A consensus tree of the first 100 most parsimonious trees was calculated by CONSENSE in PHYLIP v. 3.6 using extended majority rule option. During construction of the NJ tree and calculation of most parsimonious trees, ITS nucleotide sequences of Nothoscordum bivalve (gi:11595756), which was obtained from NCBI GenBank was included into analysis for rooting trees as it was suggested by Dubozet and Shinoda (1999).

2.5. AFLP analysis

AFLP analysis was performed according to procedures described by Vos et al. (1995) with the modification of Ipek et al. (2003). EACGG/MCTC primer combination was used to compare the AFLP band profiles of A. tuncelianum and garlic.

3. Results

3.1. Plant morphology

Plant architecture and leaf structure of A. tuncelianum resembled the garlic in the field in 2006 (Fig. 1A). The accessions of A. tuncelianum had coiling of their scapes before anthesis, like garlic (Fig. 1A). Although, the accessions of both garlic and A. tuncelianum were planted to field at the same time (November, 2005 in Bursa, Turkey), A. tuncelianum started bolting much earlier than garlic (Fig. 1A,C). Flowering time of both species should be synchronized or pollen should be stored in future studies if an attempt to obtain hybrid progeny between A. tuncelianum and garlic is desired. During anthesis, A. tuncelianum had pinkish-purple fertile flowers with well-exserted filaments and styles, but the umbels did not contain any bulbls which are present in the umbels of all flowering garlic accessions, along with flowers (Fig. 1B; Etoh and Simon, 2002; Simon and Jenderek, 2003). The flowers in the umbels of A. tuncelianum set fertile black seeds, like other Allium species. A. tuncelianum had single bulb with a single large round clove in contrast to garlic clones which has bulbs composed of 10–20
cloves of relatively similar size. These observations were in agreement with the earlier description of *A. tuncelianum* (Mathew, 1996). In addition to the similarity of plant architectures and morphologies of *A. tuncelianum* and garlic, *A. tuncelianum* has mild garlic odor and flavor. Therefore, it has been called “garlic” in the region where it grows in the wild, is collected and consumed as garlic. The species is not cultivated, but it is only collected from the wild and consumed locally with increasing popularity. Because of this unconscious collection from the wild, the species faces a serious threat of extinction. In an attempt to counteract this, studies and local conservation efforts with international collaboration (a project supported by Small Grants Programme (SGP) of Global Environment Facility (GEF) of the United Nations Development Programme (UNDP)) have been initiated to take *A. tuncelianum* under cultivation and adapt it in different regions of Turkey (Yanmaz et al., 2006; http://sgp.undp.org/web/projects/8149/promotion_of_cultivation_and_conservation_of_tunceli_garlic_allium_tuncelianum_project_phase_ii.html).

### 3.2. AFLP analysis

DNA markers have been extensively used to analyze genetic diversity in many plant species, including garlic (Al-Zahim et al., 1997; Ipek et al., 2003; Volk et al., 2004). Studies based on isozyme, RAPD and AFLP markers did not suggest that *A. longicuspis* is a distinct species from garlic (Pooler and Simon, 1993; Maass and Klaas, 1995; Al-Zahim et al., 1997; Ipek et al., 2003). Therefore, we attempted to compare AFLP banding profile of *A. tuncelianum* with garlic accessions and found that the banding profiles of these two species were very different (Fig. 2). A limited number of AFLP markers (approx. 15–20%) shared by garlic and *A. tuncelianum* suggested that *A. tuncelianum* and garlic might be distinct species. We have demonstrated in our previous study that AFLP markers of the same size in different garlic clones do not always have same nucleotide sequence identity and homology among the AFLP bands sharing the same position on a polyacrylamide gel depends on the phylogenetic relationship in garlic (Ipek et al., 2006). Therefore, the AFLP markers shared by both *A. tuncelianum* and garlic may not have the homologous nucleotide sequences and sequence identity of these AFLP markers should be characterized to make any phylogenetic conclusion.

### 3.3. ITS sequences and phylogenetic analysis

The internal transcribed spacer region (ITS) has been used for phylogenetic analyses in the genus *Allium* (Dubouzet and Shinoda, 1998, 1999; Mes et al., 1999; Friesen et al., 2000; Fritsch and Friesen, 2002). Dubouzet and Shinoda (1999) suggested DNA sequence analysis of ITS as a useful tool for intragenic classification of *Alliums*. The ITS region in *A. tuncelianum*, garlic and some other *Allium* species were sequence-characterized in this study to analyze the phylogenetic relationship among these *Allium* species. The lengths of ITS region in the *Allium* species evaluated in this study ranged

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Fig. 2. Part of autoradiogram demonstrating the differences of AFLP banding profile in *A. sativum* (1, 2), *A. schoenoprasum* (3, 4), *A. ampeloprasum* (5, 6) and *A. tuncelianum* (7, 8). The AFLP banding profile is generated using the primer combination EACGG/MCTC.
from 627 bp in *Allium pskemense* to 655 bp in *Allium cernuum* (Table 1). *A. tuncelianum* had an ITS region of 643 bp while ITS regions of garlic, *A. longicuspis*, *A. ampeloprasum* and *Allium scorodoprasum* accessions were consist of 642 bp (Fig. 3). The difference in length between the *A. tuncelianum* and garlic was due to the insertion of thymine residue at 130 bp position. In addition, *A. longicuspis* (U094-4) and a garlic accession (U074) in this study had identical ITS sequences (Fig. 3).

The ITS sequences of various *Allium* species were compared to determine the phylogenetic relationship among *A. tuncelianum* and other *Allium* species. Both the neighbor joining dendrogram and the consensus tree of parsimony analysis has the same tree topology (Fig. 4A,B). Phylogenetic relationships among *Allium* species were in agreement with previous studies (Dubouzet and Shinoda, 1999; Mes et al., 1999; Friesen et al., 2000; Fritsch and Friesen, 2002; Ricroch et al., 2005). All *Allium* species formed a monophyletic group (Fig. 4A).

The neighbor joining dendrogram and the consensus tree of parsimony analysis placed *A. tuncelianum* into the clade of subgenus *Allium* along with garlic, *A. longicuspis*, *A. ampeloprasum* and *A. scorodoprasum* (Fig. 4A,B). According to these phylogenetic dendograms, *A. tuncelianum* separated as species before the speciation of *A. ampeloprasum*, *A. scorodoprasum* and garlic. Therefore, our phylogenetic analysis based on the nucleotide sequences of ITS did not support the hypothesis that *A. tuncelianum* is the immediate progenitor species of garlic.

### 4. Discussion

*A. longicuspis* has been proposed to be the ancestor species of garlic (Vvedensky, 1944). Several studies based on DNA markers demonstrated that *A. longicuspis* is not genetically distinct from garlic. Our results based on nucleotide sequence of ITS in this study also suggested that *A. longicuspis* and *A. sativum* are the same species since a garlic accession (U074) in this study had identical ITS sequences. Recently, *A. tuncelianum* has been proposed as progenitor species of garlic due to the common morphological features of both species (Mathew, 1996; Etoh and Simon, 2002; Fritsch and Friesen, 2002). *A. tuncelianum* also has mild garlic odor and flavor, and it has been locally consumed as garlic in the region where it grows in the wild. Our study indicates that *A. tuncelianum* is not a likely progenitor of garlic, based upon inflorescence structure, flowering time and bulb morphology. Furthermore, our AFLP analysis revealed that *A. tuncelianum*
and garlic are genetically different and they are likely distinct species. Phylogenetic analysis of ITS nucleotide sequences further supported the idea that *A. tuncelianum* is a different species from both garlic and leek. Although, *A. tuncelianum* was placed in the clade of subgenus *Allium*, *A. tuncelianum* separated before the separation of garlic, *A. ampeloprasum* and *A. scorodoprasum* as a species.

Garlic-like but odorless *Allium* species, ‘Mushuu-ninniku’, from Japan was characterized to determine its relationship with garlic and other related *Allium* species (Ariga et al., 2002). According to morphological and biochemical characteristics, karyotype, restriction fragment length polymorphism, and isozyme markers analyses, ‘Mushuu-ninniku’ was found to be more similar to leek than to garlic, although bulb structure with multiple cloves of this plant is similar to garlic. In another study, genetic characterization of bulbous leek-like *Allium* species consumed as garlic in the Chinese diet has revealed that it was also a leek variant (Bohanec et al., 2005). Both of these recently characterized *Allium* accessions seem to be a member of subgenus *Allium*. *A. tuncelianum* could be another leek-like *Allium* species with mild garlic odor and flavor according to our results. Therefore, comparing genetic relationship of *A. tuncelianum* with these leek-like accessions could also be helpful to identify the origin of *A. tuncelianum* and to obtain a more conclusive result about the phylogeny of subgenus, *Allium*.

In conclusion, our results did not suggest *A. tuncelianum* as the immediate wild progenitor species of garlic. Further
between garlic chromosomes and studies to generate hybrid progeny to understand the level of homology between *A. tuncelianum* in situ. Evidence testing the suggestion of Mathew (1996) can be investigated through phylogenetic analyses using a broader diversity of *Allium* species that are native to Flora of Turkey and other molecular marker types such as plastid cpDNA can make possible to identify the origin of the *A. tuncelianum* and garlic.

References


