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Phylogenetic relationships between *Malcolmia, Strigosella, Zuvanda,* and some closely related genera (Brassicaceae) from Turkey revealed by inter-simple sequence repeat amplification

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Abstract: The genus *Malcolmia* W.T.Aiton is taxonomically problematic, and some of its species have recently been transferred to the genera *Strigosella* Boiss. and *Zuvanda* Dvorák. Three species of this genus are native to Turkey. The revision studies based on molecular data, which were collected in the past few years, display the phylogenetic relations and the systematic positions of the taxa more reliably and apparently. Thus taxonomic problems of species are resolved through DNA-based molecular analyses, which are not affected by environmental factors compared to phenotypic studies. In the present study, the amplifications of the DNA fragments were carried out using ISSR primers, and the phylogenetic relationship among the taxa was revealed through a dendrogram produced as the outcome of the NTSYSpc 2.1 software. The infrageneric and intergeneric phylogenetic relationships among *Malcolmia* and other related genera are determined. A very close relationship was determined between *Malcolmia chia* and *M. flexuosa*. *Strigosella* and *Zuvanda* species were determined to be phylogenetically different from these 2 species. The phylogenetic relationships among the *Malcolmia, Strigosella*, *Zuvanda, Leptaleum* DC., *Neotorularia* Hedge & J.Léonard, and *Sisymbrium* L. taxa were investigated. The phylogenetic separation of *Malcolmia, Strigosella*, *Zuvanda Leptaleum*, *Neotorularia*, and *Sisymbrium* genera and their specimens constituted separate clades on the dendrogram.

Key words: Cruciferae, *Malcolmia*, ISSR, phylogeny, Turkey

Türkiye *Malcolmia, Strigosella, Zuvanda* ve diğer yakın cinsler (Brassicaceae) arasındaki filogenetik akrabalık ilişkilerinin inter-simple sequence repeat çoğaltımı ile analizi

Özet: *Malcolmia* W.T.Aiton cinsi taksonomik olarak problemli bir cinstir ve son yıllarda yapılan çalışmalarla bazı türleri *Strigosella* Boiss. ve *Zuvanda* Dvorák cinslerine aktarılmıştır. Bu cinsin Türkiye'de üç türü doğal olarak yetişmektedir. Son yıllarda moleküler verilere göre yapılmış olan revizyon çalışmaları taksonların filogenetik ilişkilerini ve sistematik pozisyonlarını daha güvenilir ve net bir biçimde ortaya koymaktadır. Böylece fenotipik analizlerin aksine çevre şartlarından etkilenmeyen DNA tabanlı moleküler genetik analizlerle taksonomik problemi olan türlerin sorunları

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çözülmeye başlanmıştır. Bu çalışmada ISSR primerleri kullanılarak DNA'ların çoğaltılması yapılmış ve taksonlar arasındaki filogenetik ilişkilerin dendrogramı NTSYSpc 2.1 programı kullanılarak elde edilmiştir. *Malcolmia* cinsi içi ve diğer yakın cinsler arasındaki filogenetik akrabalıklar tespit edilmeye çalışılmıştır. *Malcolmia chia* ve *M. flexuosa* arasında çok yakın bir akrabalık ilişkisi tespit edilmiştir. *Strigosella* ve *Zuvanda* türlerinin bu iki türden filogenetik olarak ayrı olduğu belirlenmiştir. *Malcolmia, Strigosella*, *Zuvanda*, *Leptaleum* DC., *Neotorularia* Hedge & J.Léonard ve *Sisymbrium* L. cinsleri arasındaki filogenetik ilişkiler incelenmiştir. *Malcolmia, Strigosella*, *Zuvanda*, *Leptaleum*, Neot*orularia* ve *Sisymbrium* cinsleri filogenetik olarak dendrogramda ayrı kladlarda yer almıştır.

Anahtar sözcükler: Cruciferae *Malcolmia*, ISSR, filogeni, Türkiye

Introduction

The vascular plant family *Brassicaceae* comprises about 338 genera and 3700 species (Al-Shehbaz et al., 2006). It is a cosmopolitan family, being particularly abundant in the northern hemisphere. Forty-four tribes in the *Brassicaceae* are now recognised and over 90% of the genera have been studied molecularly and assigned to tribes (Warwick et al., 2010).

A molecular approach has contributed much to the understanding of phylogenetic relationships within the *Brassicaceae*. Recent advances in the molecular phylogenetic studies on the *Brassicaceae* (Koch, 2003; Koch et al., 2003; Mitchell-Olds et al., 2005; Beilstein et al., 2006; Al-Shehbaz et al., 2006; Warwick et al., 2007; Beilstein et al., 2008; Khosravi et al., 2009; German et al., 2009; Couvreur et al., 2010; Warwick et al., 2010) have led to numerous changes in its generic delimitations. However, the division of the family into monophyletic tribes remained at its initial stages, although an attempt to address that has recently been made (Al-Shehbaz et al., 2006).

The genus *Malcomia* W.T.Aiton is represented by 10 taxa in the European flora (Ball, 1967), Palestine flora (Zohary, 1966), and Cyprus flora (Meikle, 1977). The genus *Malcomia* is represented by 3 species in Turkey: *M. chia* (L.) DC., *M. flexuosa* (Sibth. & Sm.) Sibth. & Sm., and *M. greca* Boiss. & Sprun.

M. exacoides (DC.) Spreng. (Özgökçe & Ünal, 2007) and *M. intermedia* C.A.Mey. (Ünal & Özgökçe, 2008) have been treated respectively as *Zuvanda exacoides* and *Strigosella intermedia* (Al-Shehbaz et al., 2007).

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

Randomly amplified polymorphic DNA (RAPD) is a widely utilised fingerprinting method with a wide range of applications (Williams et al., 1990). However, RAPD has a number of disadvantages that render it too sensitive a method that should be utilised with special care. When compared to RAPDs, inter-simple sequence repeat (ISSR) has much higher levels of reproducibility, hence making it superior to other options (Zietkiewicz et al., 1994, Prevost & Wilkinson, 1999; Dogan et al., 2007; Hakki et al., 2010). ISSR has recently become one of the most widely utilised methods for the analysis of genetic diversity.

Internal transcribed spacers (ITS) region of nuclear ribosomal DNA comparisons have also recently been widely utilised in vascular plant taxonomy. The fact that the use of ISSR covers the whole genome rather than a single sequence of around 700bp, ISSR analysis can be considered a good alternative for ITS phylogeny.

In recent studies of *Malcolmia*, all the Central Asian *Malcolmia* species (e.g., *M. africana*, *M. karilini*, and *M. brevipes*) were placed in the genus *Strigosella* Boiss. (Akhani, 2003). The genus *Malcolmia* R.Br. (ca. 10 species) was paraphyletic and was divided into 3 tribal clades, providing support for the separate genera *Malcolmia*, *Strigosella* Boiss. and *Zuvanda* (Warwick et al., 2007).

A molecular study involving the *Malcolmia* species that are present in Turkey has not been carried out so far. The aim of the present study was to determine the status of the taxonomically problematic species amongst the *Malcolmia* species based on the phylogenetic relations and to identify the phylogenetic relationships between the *Malcolmia* species and other closely related species.

Materials and methods

Plant materials: *Malcolmia* and the other genus specimens were collected by the authors from Antalya, Iğdır, İzmir, Şanlıurfa, Siirt, and Van provinces, and Syria between 2006 and 2009 (Figure 1). The collected specimens were dried according to the standard herbarium methods and marked with a collection number. *Flora of Turkey* (Cullen, 1965), *Flora Iranica* (Rechinger, 1968), *Flora Europaea* (Ball, 1967), *Flora of Iraq* (Townsend, 1980), *Flora of the USSR* (Komarov, 1970), *Flora Palestine* (Zohary, 1966), and *Flora of Cyprus* (Meikle, 1977) were used to identify the plant samples. The collected plant specimens are kept in Yüzüncü Yıl University Faculty of Science and Art, Department of Biology (VANF). The specimens' localities and the examined representative specimens are given in the appendix. The genera *Leptaleum*, *Neotorularia*, and *Sisymbrium* were selected as outgroups.

DNA isolation: Nuclear DNA samples were isolated (2X CTAB method) from leaves dried in silica gel, and leaves from the herbarium material belonging to the *Malcolmia* species along with the *Leptaleum*, *Neotorularia*, and *Sisymbrium* genera collected from Turkey (Sambrok et al., 1989). Total DNA was obtained from 50-75 mg of dried leaf tissue from 10 different individuals. The DNA samples were isolated with the Easy Nucleic Acid Isolation Kit (OMEGA) and the concentrations were determined by a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The sample DNAs were diluted to 25 ng/µL. Unused DNA samples were kept at -86 °C.

ISSR Amplifications: PCR amplifications using ISSR primers (Domenyuk et al., 2002; Galvan et al., 2003) were conducted in an Eppendorf Mastercycler Gradient Thermocycler (New York, USA). Each PCR reaction contained a 25 µL column consisting of 2.5 µL of PCR buffer (10 mM tris/50 mM KCl buffer, pH 8.0), 3 µL of 25 mM MgCl, 0.5 µL of each primer, 0.5 µL of dNTP mix, 0.4 µL of Taq DNA polymerase, 4 µL of each DNA, and 14.1 µL of 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) for 1 min and 72 °C for 1 min, annealing temperature, 1 min, and a final extension was allowed for 10 min at 72 °C. 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, 1× tris-borate-EDTA gel and electrophoresed at 4 V cm^{-1}. Amplifications were repeated at least twice (at independent times) for each primer using the same reagents and procedures. Reactions without DNA were used as negative controls. The optimum annealing temperature was determined for each primer. The characteristics of the primers used are given in the Table.

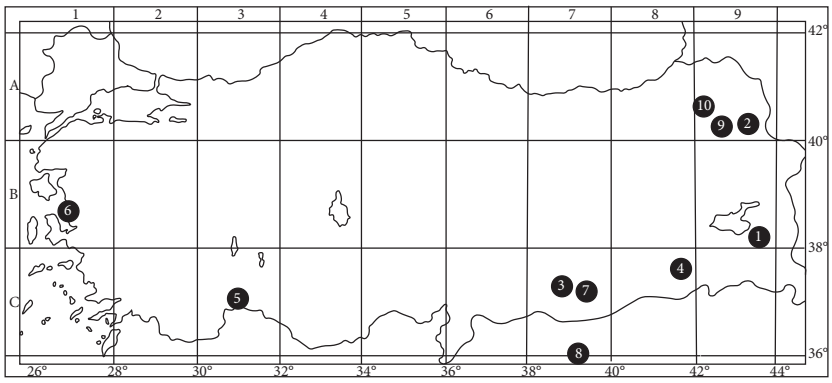

Figure 1. Distribution map of the examined specimens of the genera *Malcolmia, Strigosella, Zuvanda, Leptaleum*, *Neotorularia*, and *Sisymbrium*. 1-*Strigosella africana*, 2-*S. intermedia*, 3-*Zuvanda crenulata*, 4-*Z. exacoides*, 5-*Malcolmia chia*, 6-*M. flexuosa*, 7-*Sisymbrium orientale*, 8-*S. orientale* (Syrian specimen), 9-*Leptaleum filifolium*, 10-*Neotorularia torulosa*.

Table. ISSR primers used in this study and their specifications (Domenyuk et al., 2002; Galvan et al., 2003).

PRIMER	PRIMER SEQUENCE	T_m (°C)	SIZE (bp)	GC%	T_{an}
ISSR M1	$(AGC)^6$-G	63.1	19	68.4	63
ISSR M2	$(ACC)^6$-G	63.1	19	68.4	63
ISSR M3	$(AGC)^6$-C	63.1	19	68.4	63
ISSR M5	$(GA)^9$-C	56.7	19	56.7	56
ISSR F1	GAG-$(CAA)^5$	49.1	18	38.9	49
ISSR F2	CTC-$(GT)^8$	56.7	19	52.6	56
ISSR F3	$(AG)^8$-CG	56	18	55.6	56

Data collection and phylogenetic analysis: Amplified fragments were visualized under a UV transiluminator and photographed using a gel documentation system (Vilbert Lourmat, Eberhardzell, Germany). 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, New York, USA). Similarity coefficient method was used. In cluster analysis of the samples the unweighted pair-group method with the arithmetic mean (UPGMA) procedure was followed (Rohlf, 1992). Genetic distances calculated with the SM coefficient. In order to determine the ability of ISSR data to display the inter-relationships among the samples, analysis was conducted using the NTSYS-pc package.

Results and discussion

Through an initial screening of 23 ISSR primers, 7 primers revealed high levels of polymorphisms. These primers generated 78 highly polymorphic fragments that were consistently amplified in repeated experiments conducted on different dates. The GC percentages of the selected primers were within the range of 38.9%-68.4%. The characteristics and the sequences of the primers revealing polymorphism are shown in the Table. In the overall analysis, the average number of polymorphic fragments per primer used was roughly 11. A representative figure containing ISSR M1 and ISSR F1 banding patterns is shown in Figure 2. The genetic distances calculated with the SM coefficient ranged between 0.51 and 0.70.

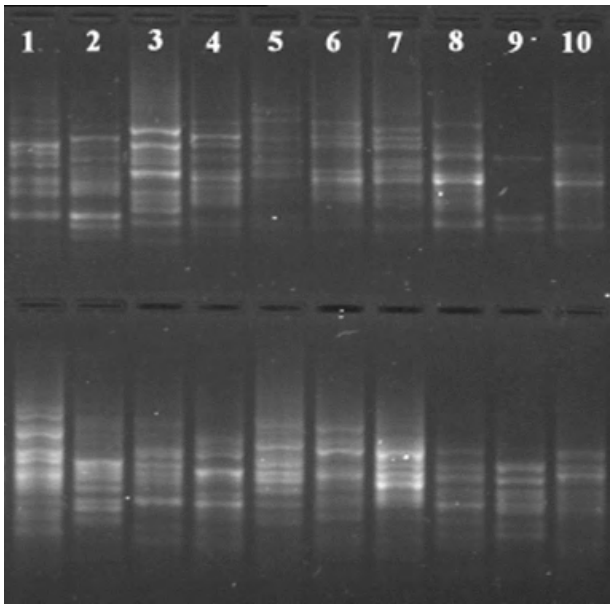

Figure 2. Representative agarose gels where PCR products were amplified with the primers ISSR M1 (top) and ISSR F1 (down). 1-*Strigosella intermedia*, 2-*S. africana*, 3-*Zuvanda crenulata*, 4-*Z. exacoides*, 5-*Malcolmia chia*, 6-*M. flexuosa*, 7-*Sisymbrium orientale*, 8-*S. orientale* (Syrian specimen), 9-*Neotorularia torulosa*, 10-*Leptaleum filifolium*.

The analyses of the scored ISSR bands revealed 4 genera and 10 species in our study. The dendrogram based on the ISSR bands revealed 4 clades, each of which matched 1 of the 4 genera, viz. *Malcolmia, Strigosella, Zuvanda, Sisymbrium*, *Neotorularia*, and *Leptaleum* (Figure 3). The genera *Sisymbrium*, *Neotorularia*, and *Leptaleum* displayed a much higher molecular phylogenetic similarity. There was a correlation between the morphologically diagnostic characters and the molecular taxonomic classification. The related species were clearly separated according to the results of the principal coordinate analysis (PCA) (Figure 4).

The *Malcolmia* species resided on the same clade while the other species were located in a separate clade in the phylogenetic dendrogram obtained. *M. chia* and *M. flexuosa* were positioned on the same clade and a close similarity was identified among them. *S. intermedia*, *S. africana*, *Z. crenulata*, and *Z. exacoides* were located on the same major clade but they were subdivided into 2 clades among themselves. According to these data, *S. intermedia* and *S. africana* as well as *Z. crenulata* and *Z. exacoides* were closely related. These obtained results are in conjunction with the results of the study by Warwick et al. (2007). Warwick et al. (2007) moved some species of the *Malcomia* genus into the genera *Strigosella* and

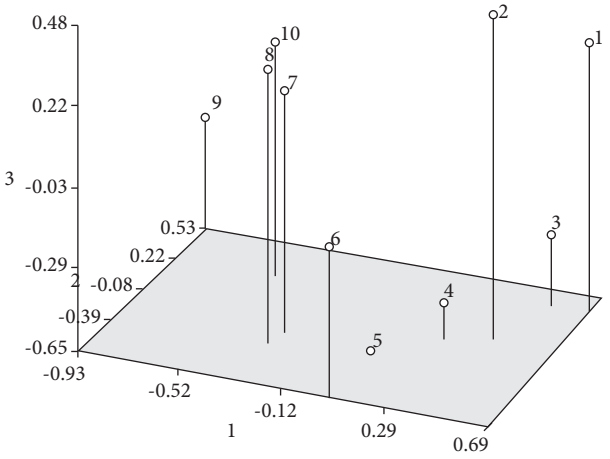

Figure 4. Principal coordinate analysis of *Malcolmia, Strigosella, Zuvanda, Leptaleum*, *Neotorularia*, and *Sisymbrium* species. 1-*Strigosella intermedia*, 2-*S. africana*, 3-*Zuvanda crenulata*, 4-*Z. exacoides*, 5-*Malcolmia chia*, 6-*M. flexuosa*, 7- *Sisymbrium orientale*, 8-*S. orientale* (Syrian specimen), 9-*Neotorularia torulosa*, 10-*Leptaleum filifolium*.

Zuvanda. *M. africana* was reported as *Strigosella africana* and *M. crenulata* was reported as *Zuvanda crenulata* in the study by Al-Shehbaz and Warwick (2007). Our results confirm these findings mentioned above. Moreover, Warwick et al. (2010) characterised a new tribe that was named Anastaticeae.

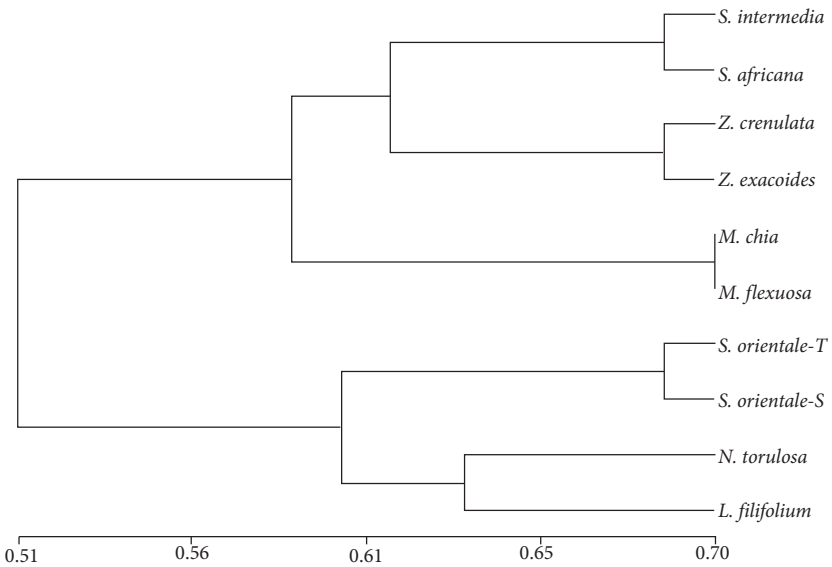

Figure 3. Dendrogram showing genetic relationships in *Malcolmia, Strigosella, Zuvanda, Leptaleum*, *Neotorularia*, and *Sisymbrium* species based on ISSR markers.

The other major clade in the obtained dendrogram hosted genera of *Sisymbrium*, *Neoorularia*, and *Leptaleum*. The sub-clade where the *Sisymbrium* samples reside indicates that these 2 samples are of a separate taxon. The *Neotorularia* and the *Leptaleum* taxa in the other clade were identified to be closer to each other than to *Sisymbrium* phylogenetically.

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Appendix

Examined representative specimens: – S*trigosella africana.* Turkey. B9 Van; Güzeldere (Hoşap), Üçgen Köyü çevresi, dere kenarı, 2017 m, 11.05.2009, 38°21′384″N, 43°49′548″E, *Murat* & *Fevzi* 10157. – *Strigosella intermedia*. A9 Iğdır; Tuzluca ve Alhanlı köy yolunun doğusu ve batısı, düzlük çayır, 950 m, 06.04.2009, 40^{0}06′085″N 43°39′154″E, *Murat* & *Fevzi* 10019. – *Zuvanda crenulata*. C7 Şanlıurfa; merkez, Osman Bey Kampüsü, İktisat Fakültesi yanı, bahçe içleri ve zayıf peyzaj alanları, 499 m, 31.03.2009, 37°10′071″N, 38°59′736″E, *Murat* & *Fevzi* 10043. – *Zuvanda exacoides*. C8 Siirt, Siirt′ten Güçlü Konak ilçesine doğru 30 km, Balıklı ile Yığınlı köyleri arası, tarla kenarı, 450 m, 28.03.2009, 37°44′066″N, 41°47′916″E, *Murat* & *Fevzi* 10011. – *Malcolmia chia*. C3 Antalya; Akdeniz Üniversitesi Kampüsü, Ziraat Fakültesi çevresi, derin vadi, 500 m, sık makili kayalık yerler. 39 m, 07.05.2009, 36°53′426″N, 30°39′466″E, *Murat* & *Fevzi* 10153. – *Malcolmia flexuosa*. B1 İzmir; Tuz işletme merkezleri civarı Homa Dalyanı, tuzlu sahil toprakları, 0-2 m, 05.05.2009, 38°32′066″N, 26°50′235″E, *Murat* & *Fevzi* 10145. – *Sisymbrium orientale*. C7 Şanlıurfa; merkez, Bağlarbaşı Mah, Açıksu mevkii, boş araziler, 581 m, 03.04.2009, 37°10′839″N, 38°46′104″E, *Murat* & *Fevzi* 10046. – *Sisymbrium orientale*. Suriye; Tedmur (Palmira′ya giderken), yol kenarı, 298 m, 25.04.2009, 34°33′021″N, 38°16′208″E, *Murat* & *Fevzi* 10136. – *Leptaleum filifolium*. A9 Iğdır; Tuzluca ve Alhanlı köy yolunun doğusu ve batısı, düzlük çayır, 950 m, 06.04.2009, 40°06′085″N, 43°39′154″E, *Murat* & *Fevzi* 10020. – *Neorularia torulosa*. A9 Iğdır; Tuzluca ve Alhanlı köy yolunun doğusu ve batısı, düzlük çayır, 950 m, 06.04.2009, 40°06′085″N, 43°39′154″E, *Murat* & *Fevzi* 10040.

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