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Morphometric and Molecular Diversity in Some Species of the Genus *Alopecurus* L. in Iran

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Abstract
Morphometric and molecular studies were performed on the three species and two varieties of *Alopecurus*. Numerical taxonomic studies were performed on 14 populations using 37 morphological characters. Clustering and PCA ordination separated the species studied in different clusters. Factor analysis identified the most variable morphological characteristics to be used in *Alopecurus* species delimitation. Out of ten RAPD primers used, five primers produce 115 bands. Six bands were common to all three species while the other bands were polymorph. Some bands were specific to *A. myosuroides*, while some other bands were specific for *A. textilis* and may be considered as loci for discriminating between these two species. UPGMA clustering and PCO ordination of the *Alopecurus* species based on RAPD data showed the species distinctiveness supporting the morphological results. *A. arundinaceus* also showed greater similarity to *A. myosuroides* in its molecular characteristics indicating the use of RAPD data in *Alopecurus* species delimitation.

Keywords: Alopecurus, Numerical taxonomy, RAPD.

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تنوع مورفولوژیکی و مولکولی چند گونه *Alopecurus* L. در ایران

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چکیده
مورفومتری و مطالعه مولکولی تعداد سه گونه و دو واریته *Alopecurus* L. ایران انجام شد. تاکسونومی عددی ۱۴ جمعیت با استفاده از تعداد ۳۷ صفت‌ریختی انجام گرفت. دندروگرام ها و رسته بندی گونه‌ها جدایی جمعیت‌های مختلف یک گونه را از دیگر گونه‌ها مشخص نمود. آنالیز فاکتور متغیرترین صفات ریختی متمایز کننده گونه‌ها را به منظور استفاده در تاکسونومی *Alopecurus* شناسایی نمود. از میان تعداد ۱۰ پرایمر RAPD استفاده شده، ۵ پرایمر تولید باند کردند که از این میان ۶ باند در تمامی گونه‌ها و جمعیت‌های مطالعه شده مشترک بودند. برخی از باندها منحصر بفرد یک گونه بودند و تعدادی دیگر برای یک جمعیت خاص انحصاری بودند. دندروگرام‌ها و رسته‌بندی گونه‌ها بر اساس داده‌های مولکولی جدایی جمعیت‌های مختلف یک گونه را از دیگر گونه‌ها و نیز کاربرد مارکرهای مولکولی RAPD را در تفکیک گونه‌های *Alopecurus* نشان داد.

واژه‌های کلیدی: تاکسونومی عددی، RAPD، *Alopecurus*.

Introduction

The genus *Alopecurus* L. (foxtail), a member of the tribe Aveneae, contains 29 species and is distributed throughout almost all non-tropical regions of both hemispheres and alpine tropics, with its main area of distribution in southwest Asia. The genus contains important fodder plants, such as *A. pratensis*, *A. arundinaceus*, *A. aequalis*, *A. geniculatus* L., *A. myosuroides* and *A. bulbosus*. Many arctic, alpine or high mountain species of the genus including *A. mucronatus*, *A. textilis*, *A. glacialis*, *A. vaginatus*, *A. borealis*, *A. aucheri*, *A. laguroides* and *A. lanatus* are valuable pasture grasses.

Alopecurus species are hermaphroditic out-breeding plants, caespitose, rhizomatous or stoloniferous and annuals, biennials or perennials. Their inflorescence is a spike-like panicle, oblong to cylindrical or ovate. The spikelets are laterally compressed, with 1 floret. The base chromosome number of the genus *Alopecurus* is x = 7 and somatic chromosome numbers of 2n = 14, 28, 42, 56 have been reported (Watson and Dallwitz, 1992).

Bor (1970) in *Flora Iranica* reported eleven *Alopecurus* species growing in Iran which are annuals, biennials or perennials, with membranous ligules. The leaf blades are linear, acuminate and flat to convolute. Its leaf sheaths are glabrous or hairy, the inflorescence is panicle and the spikelets are laterally compressed, with a single floret. Rhachilla disarticulating below the glumes, which are equal to subequal, acute to obtuse or terminating in an aristate point, are 3-veined and are always connate below. Lemma is 3-, 4- or 5-veined with the margins connate below, dorsally awned (rarely awn ±absent). Palea is 1- veined, keeled, sometimes absent, lodicules absent, stamens are three, styles connate below, caryopsis laterally compressed, obliquely obovate from the side view, glabrous and the embryo is 1/3 x caryopsis.

Various studies on *Alopecurus* species from different parts of the world include chromosome number reports (Devesa *et al.*, 1990; Goukasian & Nazarova 1998; Montgomery *et al.*, 1997), a morphological and cytogenetical study of the inter-specific hybrids (Sieber and Muray, 1981a, b), anatomical studies (Metcalfe, 1960), floral morphology (Dogan 1985, 1991a) and numerical taxonomy (Dogan, 1991b, 1997). However such studies are totally absent from Iran, therefore the present study comprises a numerical taxonomy and molecular (RAPD) analysis of three species and two varieties of *Alopecurus* in Iran for the first time.

RAPD markers have been used for studying the genetic diversity and species relationships in different plant species. These molecular markers provide the opportunity for direct genetic study and comparison of the plant materials without any other influences (Weising *et al.*, 2005).

Material and Methods

Morphometric studies

Numerical taxonomic studies were performed on 14 populations of three species and two varieties of *Alopecurus* namely: 1- *Alepecurus myosuroides* Huds. var. *breviaristatus*, 2- *A. myosuroides* var. *myosuroides*, 3- *A. arundinaceus* Poir., and 4- *A. textilis* Boiss..

The plant specimens studied were collected from their natural habitats and also studied in the Noshahr station herbarium of the Research Institute of Forests and Rangelands. At least 3 plants from each population were used in numerical analysis. The voucher specimens have been deposited in the herbarium of Shahid Beheshti University (HSBU) and in the Noshahr station herbarium of the Research Institute of Forests and Rangelands.

Thirty-seven morphological characters (14 quantitative and 23 qualitative characters, Table 1) were studied which were taken from flora and published materials related to *Alopecurus* (Bor, 1968, 1970; Tzvelev, 1984; Dogan, 1985). For numerical analyses, the means of quantitative characteristics were used while qualitative characteristics were coded as binary or multistate characteristics. In order to determine the species inter-relationships, cluster analysis using UPGMA (Unweighted Paired Group

with Arithmetic Mean) and WARD (Minimum variance spherical clusters) as well as ordination based on principal component analysis (PCA) and principal coordinate analysis (PCO) were performed. The Euclidean, squared Euclidean and taxonomic distances were used as dissimilarity coefficients in cluster analysis of the morphological data (Ingrouille, 1986).

The analysis of variance (ANOVA) followed by the least significant difference test (LSD) was used to show significant difference between quantitative characterstics, while factor analysis was performed to identify the most variable morphological characteristics among the species and populations studied (Chatfield and Collins, 1995).

Random Amplified Polymorphic DNA (RAPD) Analysis

DNA was extracted from fresh leaves collected from germinating seeds. The PCR reaction mixture consisted of 1 ng template DNA, 1 x PCR buffer

Table 1- Morphological Characters and their Coding.

Row	Character	Character States
1	Habit	1-annual 2-perenial
2	Stem length	cm
3	Number of stem nodes	1-one 2-two
4	Rhizome	1-present 2-absent
5	Covering of basal sheath	1-hairy 2-glabrous
6	Blade length	cm
7	Blade width	cm
8	Ligule shape	1-obtuse 2-acute 3-serrate 4-truncate
9	Ligule size	cm
10	Panicle shape	1-narrow-cylindrical 2-broadly cylindrical 3-ovate
11	Panicle length	cm
12	Panicle width	cm
13	Spikelet shape	1-urceolate 2-elliptic
14	Spikelet length	cm
15	Spikelet width	cm
16	Shape of glume apex	1-aristate 2-acute
17	Glume length	cm
18	Glume width	cm
19	Color of glume apex	1-purple 2-not purple
20	Glumes at apex	1-parallel or convergent 2-divergent
21	Glumes awn	1-present 2-absent
22	Inner glumes margins	1-ciliate 2-not ciliate
23	Hair-covering of glumes	1-all over 2-only on keels
24	Shape of lemma apex	1-obliquely truncate 2-acute
25	Lemma length	cm
26	Lemma width	cm
27	Lemma-Glume ratio	1-equal 2unequal
28	Lemma covering	1-ciliate 2-notciliate
29	Color of lemma awn	1- purple 2-not purple
30	Structure of lemma awn	1-strong 2-slender
31	Lemma awn length	cm
32	Lemma awn exerted from spikelet	1-yes 2-no
33	Awn-Lemma ratio	1-longer 2-shorter
34	Awn position	1- lower 1⁄3 of lemma 2-lower 1⁄2 of lemma
35	Awn form	1-geniculate 2-straight
36	Palea	1-present 2-absent
37	Anther length	cm

(10 mM Tris-HCJ pH 8.8, 250 mM KC12), 200 µM dNTPs, 0.80 µm 10-base random primers and 1 unit of Taq polymerase, in a total volume of 25 µl. DNA amplification was performed on a palm cycler GP-00 1 (Corbet, Australia). Template DNA was initially denatured at 94^0C for 3 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 1 min at 92^oC, primer annealing for 1 min at 36^oC and primer extension for 2 min at 72^0C. A final incubation for 10 min at 72^0C was performed to ensure that the primer extension reaction proceeded to completion.

The PCR amplified products were separated by electrophoresis on 1.5% agarose gel using a 0.5 X TBE buffer (44.5 mM Tris/Borate, 0.5 mM EDTA, pH 8) or 12% polyacrylamide gel. The gels were stained with ethidium bromide and visualized under UV light or silver stained for added sensitivity. RAPD markers were named by primer origin, followed with the primer number and the size of amplified products in base pairs. Thirty random primers of Operon technology (Alameda, Canada) were used.

The experiment was repeated three times and reproducible RAPD bands were used for further analysis. The bands obtained were treated as binary characters and coded accordingly (presence =1, absence = 0). Jaccard similarity was determined among the genotypes studied for use in clustering. The genotypes showing similarity in their RAPD characteristics were grouped by using UPGMA and PCO ordination (Sheidai *et al.*, 2008). In order to test the fitness of different clustering trees to the original similarity of the species studied, cophenetic correlation was determined. SPSS ver. 9 (1998) and NTSYS ver. 2.02 (1998) and DARwin5 (2008) were used for statistical analyses.

Results

Morphometry

The ANOVA and LSD tests showed significant difference among the species and varieties studied. The preliminary analysis revealed that the first four factors comprise about 81% of total variance. In the first factor, with about 43% of total variance, quantitative characteristics of the width of the lemma and the length of the floret along with most of the qualitative characteristics possess the highest positive or negative characters (>0.6 or <-0.6). In the second factor, with about 22% of total variance, the other quantitative characteristics (except length of lemma) showed the highest correlation. Qualitative morphological characteristics showed the highest correlation in the third and forth factors.

Different clustering methods as well as PCA ordination based on morphological characters produced similar results, placing the populations of each *Alopecurus* species together and in a separate cluster or group (Figs. 1 and 2). UPGMA clustering showed the highest cophenetic correlation (>0.80) and is discussed here.

In general, three major clusters are formed. The first major cluster comprises two sub-clusters, formed by the populations of *A. myosuroides* var. *breviaristatus* and *A. myosuroides* var. *myosuroides*, supporting their taxonomic status. Three populations of *A. arundinaceus* formed the second major cluster showing more similarity to *A. myosuroides* var. *breviaristatus* and *A. myosuroides* var. *myosuroides*. Three populations of *A. textilis* comprised the third major cluster standing at some distance from the other two species. PCA ordination supported the clustering results (Fig. 2). The first PCA axis separates the three *Alopecurus* species while the second PCA axis separates *A. arundinaceus* from the other two species.

Species abbreviations: mb 1 & 2 = Noshahr and Kajoor populations of *Alopecurus myosuroides* var. *breviaristatus* respectively, mm1-6 = Noshahr, Chaloos, Ghareghoshoon, Azarbayejan, Ghezelozan river, Arak and Shahriar populations of *A. myosuroides* var. *myosuroides* respectively, ar 1-3 = Firoozkooh, Touchal and Charmahlobakhteyari populations of *A. arundinaceus* respectively and tx 1-3 = Touchal, Noshahr and Kandovan populations of *A. textilis*.

RAPD analysis

Out of ten RAPD primers used, five primers were able to produce 115 bands in the *Alopecurus* species studied (Fig. 3). Six bands of OPI05-15, OPI05-17,

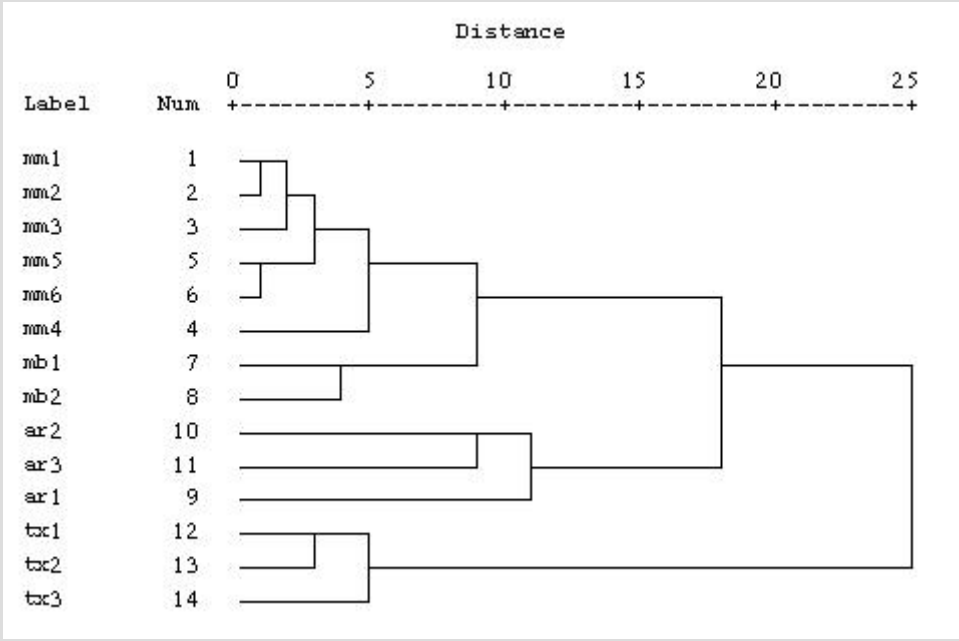

Figure 1. UPGMA clustering of morphological characteristics.

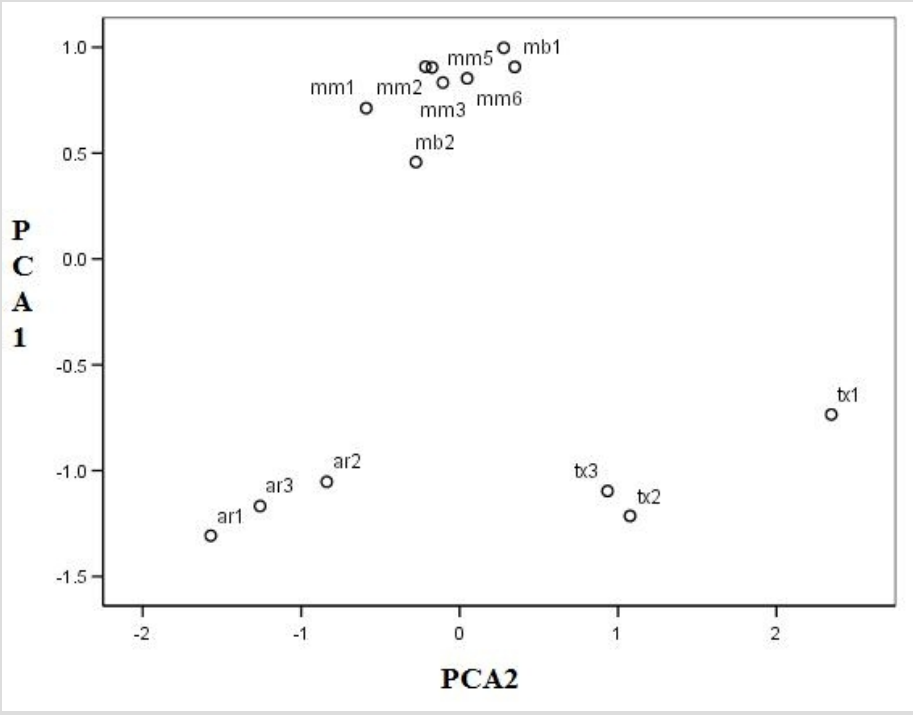

Figure 2. PCA ordination of *Alopecurus* species based on morphological characters. Species abbreviations as in Fig. 1.

OPI05-20, OPI05-21 and OPI05-22 were common to all three species while the other bands were polymorph. The bands (loci) OPI05-11, OPI05-14, OPI05-25, OPI03-9, OPI07-15 and OPI07-23 were specific to *A. myosuroides*, while the bands OPI05-2, OPI05-6 and OPI05-10 were specific for *A. textiles*; therefore, these bands/loci may be considered as discriminating loci for the species studied.

The bands OPI05-1, OPI05-19, OPI03-3 and OPI03-5 were specific to the Chaloos population of *A. myosuroides var. myosuroides* while bands OPI03-1 and OPI03-2 were specific to the Noshahr population of *A. myosuroides var. myosuroides*. The band OPI05-18 was present in all populations except in the Chaloos population of *A. myosuroides var. myosuroides*, indicating the loss of band/loci possibly due to population genomic changes.

The band OPI05-5 was specific to the Namrood population of *A. arundinaceus*, while bands OPI05-31 and OPI03-4 were specific to the Shahriar population of *A. arundinaceus*. Similarly bands OPI05-6, 7, 8 and 9 were specific to the Shahkooh population of *A. textilis* and bands OPI05-30, OPI05-32 were specific to the Touchal population of *A. textilis*.

Discussion

The ANOVA test indicates that most of quantitative and qualitative characteristics may be of use in the delimitation of *Alopecurus* species. This is further supported by factor analysis of morphological characteristics. Therefore all these characteristics are among the most variable morphological characteristics which may be used in species delimitation within the genus *Alopecurus*.

Both cluster analysis and PCA ordination based on morphological characteristics place the populations of each *Alopecurus* species together as well as in a separate cluster or group. In this study, three populations of *A. arundinaceus* show greater similarity to *A. myosuroides* var. *breviaristatus* and *A. myosuroides* var. *myosuroides*. Dogan (1999) in his taxonomic treatment of the genus in Turkey also considered *A. arundinaceus* closer to *A. myosuroides* than to *A. textilis*, although in *Flora Iranica* (Bor, 1970) *A. arundinaceus* is close to *A. textilis*.

In molecular analysis, specific bands were observed in populations of each species. This may indicate the genomic distinctness of the populations studied. UPGMA clustering, PCO and PCA ordination of the *Alopecurus* species based on RAPD data produced similar results supporting the morphological analyses (Figs. 4 and 5). The three species studied formed separate clusters indicating their genetic distinctness. *A. arundinaceus* showed more similarity to *A. myosuroides* in its molecular characteristics similar to morphological analyses. Therefore it seems that RAPD data may be of use in *Alopecurus* species delimitation which is reported for the first time.

Figure 3. RAPD profile of *Alepecurus* species by primer OPI05.

Columns 1-7 = 1- Molecular ladder, 2- Touchal population of *A. textilis*, 3- Shahkooh population of *A. textilis*, 4- Shahriar population of *A. arundinaceus*, 5- Namrood population of *A. arundinaceus*, 6- Chaloos population of *A. myosuroides* var. *myosuroides* and 7- Noshahr population of *A. myosuroides* var. *myosuroides*.

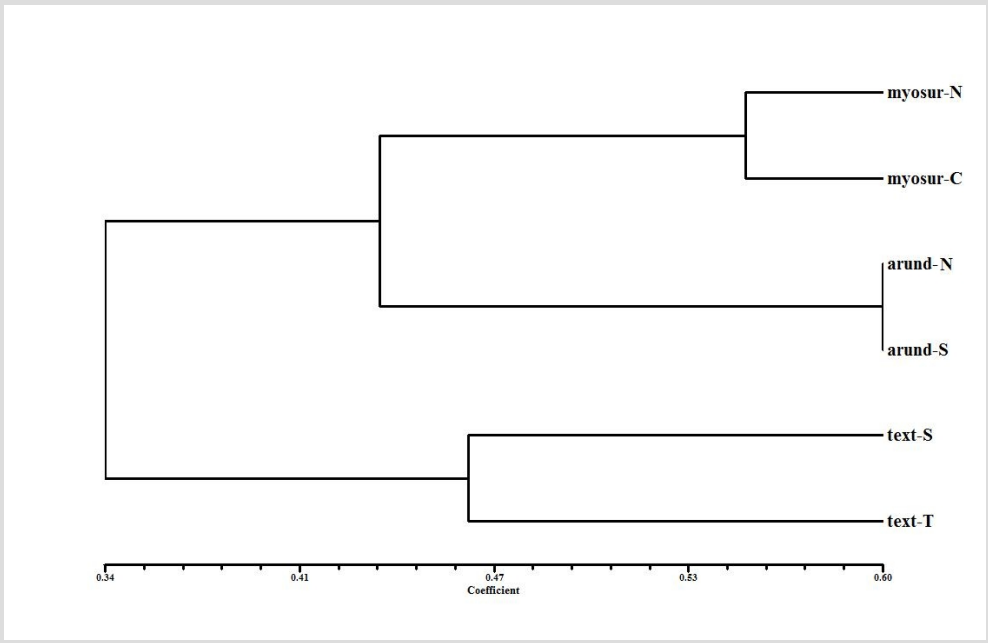

Figure 4. UPGMA clustering of *Alopecurus* species based on RAPD data.

Species abbreviations: mayosur-N = Noshahr population of *A. myosuroides* var. *myosuroides*, mayosur-C = Chaloos population of *A. myosuroides* var. *myosuroides*, arund-N = Namrood population of *A. arundinaceus*, arund-S = Shahriar population of *A. arundinaceus*, text-S = Shahkooh population of *A. textilis* and text-T = Touchal population of *A. textilis*.

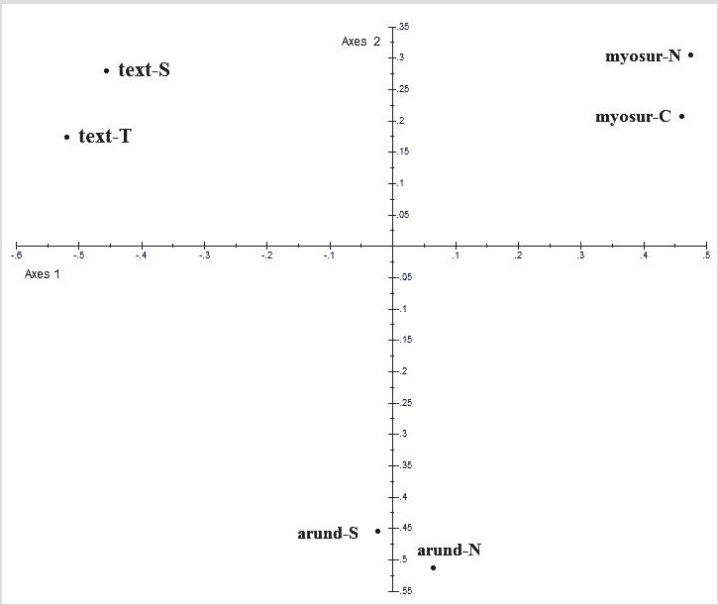

Figure 5. PCA ordination of *Alopecurus* species based on RAPD data.
(Species abbreviations as in Fig. 3).

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