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Phenetic relationships among *Lolium* s.l. (Poaceae) in Iran based on flavonoids spot profiles and quantitative morphology

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Abstract
Relationships between species of *Lolium* and *Festuca* have long been an interesting subject in taxonomy of the subtribe Loliineae. This study was concerned with the phenetic relationships of *Lolium* s.l. (including *Festuca* subgen. *Schedonorus*) using flavonoids spot profiles and quantitative morphological characters. Measurement of morphological characters and densitometry of flavonoids spots and their profile plots were performed by using calibrated digital images and ImageJ software package. Multivariate analyses (clustering and ordination) performed by using NTSYS-pc software package. Each species was described based on its flavonoid spot profile, and R*f* values and percentage of each spot in the corresponding profile were reported. Variation in flavonoid spot profiles of *Lolium rigidum*, *L. perenne* and *Festuca pratensis* revealed that flavonoids spot profiles revealed that they may be useful characters for further studying the variations within the species level. Cluster analysis of quantitative morphological characters separated the species in well defined groups and further separated *L. persicum* population Ardabil from other *L. persicum* populations. Separation of *F. arundinacea* populations into two distinct groups was also interesting which suggested that the existence of two forms of this species in Iran is probable.
Key words: *Festuca*, Flavonoids, *Lolium*, Morphology, Phenetics, Quantitative

Introduction

Relationships between species of *Lolium* L. and *Festuca* L. have long been remained as a controversy in taxonomy of the subtribe Loliineae. *Lolium* which was first classified in the tribe Triticeae Dumortier based on the morphology of inflorescence, was later transferred to the tribe Poeae (= Festuceae) by Nevski (1934). Many non-morphological evidences from different sources of data supported this transfer (see Darbyshire, 1993). Inter-generic hybridization between *Festuca* subgen. *Schedonorus* (P. Beauvois) Petermann and outbreed species of *Lolium* (specially, *L. perenne* L.) along with other evidences from cytology, anatomy, molecular markers and genomic and plastid DNA, supported the union of *F.* subgen. *Schedonorus* and *Lolium*. Darbyshire (1993) suggested the realignment of *F.* subgen. *Schedonorus* with the genus *Lolium*, and introduced new combinations: *Lolium* subgen. *Schedonorus* Darb., *L. arundinaceum* Darb., *L. giganteum* Darb., *L. mazzettianum*

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Darb., and *L. pratense* Darb. *Festuca* subgen. *Festuca* sect. *Ovinae* Fries (syn.: sect. *Festuca*), encompasses two controversial aggregates namely *F. ovina* L. and *F. rubra* L., according to the classification proposed by Hackel (1882), and with hundreds of species, subspecies, varieties, subvarieties, and formes, published so far. Stace *et al.* (1992) reduced all named morpho-anatomical variations into those two aggregates. He stated that only two characters (Sheaths of young tiller-leaves: fused/free, and tillers: extravaginal/ intravaginal) could definitely be used to distinguish between *Festuca ovina* and *F. rubra* aggregates; supporting the notion proposed by Hackel (1882) who divided the section into two main groups; *Intravaginales* and *Extravaginales vel Mixtae*. Phylogenetic studies based on cpDNA-RFLP (Darbyshire and Warwick, 1992) and ribosomal ITS (Charmet *et al.*, 1997; Gaut *et al.*, 2000; Torrecilla and Catalan, 2002) have demonstrated the paraphyly of *Festuca* and that, *Festuca* may include *Lolium* and *Vulpia* C.C.Gmel., therefore, choosing between the transfer of *F*. subgen. *Schedonorus* as a new genus (Soreng and Terrell, 1998), or realignment of it with genus *Lolium* (Darbyshire, 1993) remained to be studied. Torrecilla and Catalan (2002) worked on two main lineages in festucoids, namely "fine-leaved" and "broad-leaved fescues", and demonstrated that *Lolium* species were close relatives of broad-leaved fescues, and that the polyphyletic *Vulpia* was a close relative of fine-leaved fescues lineage. They confirmed that a monophyletic *Festuca* might encompass species from *Vulpia*, *Leucopoa* Griseb., *Schedonorus* and *Lolium*, while realignment of *Schedonorus* with *Lolium* would remain the rest of the clade as a large polyphyletic assemblage. Phylogeny of the festucoids based on nucleotide sequences of ITS and *trn*L-F regions (Catalan *et al.* 2004) showed that *Lolium*, *Mycropyropsis* and *Festuca* subgen. *Schedonorus* were close relatives, and they fell into a single monophyletic clade. Although several appreciated studies have been already performed in this complex group (Catalan *et al.*, 1997; Catalan, 2002; Torrecilla *et al.*, 2003; Catalan *et al.*, 2004; Torrecilla and Torrecilla *et al.*, 2004; Catalan, 2006; Muller and Catalan, 2006), the festucoids, *Festuca* s. str. and *Lolium* s.l. are still open and interesting subjects to be studied for more details.

Bor (1970) described six species of genus *Lolium* s. str. for Iran: *L. perenne* L., *L. multiflorum* Lam. (syn: *L. italicum* A.Br.), *L. rigidum* Gaud. (syn: *L. strictum* Presl.), *L. persicum* Boiss. and Hohen. ex Boiss., *L. temulentum* L. and *L. loliaceum* (Bory and Chaud.) Hand.-Mazz which was later considered as a synonym for *L. rigidum* subsp. *lepturoides* Sennen and Mauricio (legitimate name). They coincided with members of *Festuca* subgen. *Shedonorus* in high mountain elevations and mesic habitats along Alborz and Zagros chains. Flavonoids have long been proved as important characters in plant systematics and biosystematics researches, and they were still continue to take part specially in biosystematics researches (Sharifi-Tehrani and Ghassemi Dehkordi, 2011; Ghassemi Dehkordi *et al.*, 2012; Sharifi-Tehrani *et al.*, 2012).

This study was aimed to evaluate the relationships in *Lolium sensu* Darbyshire in Iran, using flavonoids spot profiles and quantitative morphological characters. *Lolium* specimens were studied here along with specimens from *Festuca* subgen. *Schedonorus* and from more distantly sister group, *Festuca* subgen. *Festuca*. This was the first report on the numerical analysis of quantitative morphological characters of *Lolium* s.l. in Iran. This study was also the first one to report application of digitally measured flavonoids spot profiles in the chemotaxonomy of the group in Iran. Relevance of this study was due to the importance of members of *Lolium* s.l. as economic hay and forage plants in Iran and the neighboring countries in the west of Mediterranean region, and also the relative absence of *F. sclerophylla* Boiss. ex Bisch. and *L. persicum* in previous studies.

Materials and Methods

Plant material

Plant materials were collected from wild populations throughout their distribution ranges in Iran (Table 1). Specimens were paper-dried, and determined using identification keys available in Flora Iranica and Flora of Turkey (Bor, 1970; Davis *et al.*, 1988). Seventy eight samples were chosen for flavonoid extractions or morphological studies (Tables 1, 2). Total flavonoids were extracted from 0.6 to 1.0 gram of dried leaves of 59 selected specimens belonging to ten species from three closely related genera.

Table 1. Plant material collected from wild populations in Iran. Acc: accession numbers of each specimen; TLC lane: corresponding flavonoid profile in Figure 1; S: corresponding plot in Figure 2

Alt. (m)	Loc	Acc	TLC Lane	S	Alt. (m)	Loc	Acc	TLC Lane	S
Festuca arundinacea					***Lolium rigidum***				
2095	Ardabil, Sabalan	643	1		2030	Sarab, Sabalan	724	28	
1880	Fars, Arzhan	633	2		2014	Ardabil, Sabalan	728	29	
2448	Hamadan, Tuyserkan	606	3		2014	Ardabil, Sabalan	730	30	
2448	Hamadan, Tuyserkan	607	4	A	1534	Urmia, Shahrchay	709	31	
2450	Kashan, Ghohroud	637	5		1534	Urmia, Shahrchay	727	32	I
2560	Kerman, Sardouyeh	624	6		1534	Urmia, Shahrchay	758	33	
2000	Yasouj, Sisakht	632	7		-	Fars, Sarvestan	747	34	
1790	Yasouj, Sisakht	669	8		-	Kerman, Estahban	752	35	
2150	Yasouj, Sisakht	671	9		-	Kerman, Estahban	759	36	
2450	Lurestan, Gahar	675	10		1790	Yasouj, Sisakht	712	37	
2898	Hamadan, Alvand	605	-		1326	Kud, Zaribar Lake	704	38	
2000	Yasouj, Sisakht	625	-		500	Ramsar	735	39	
2560	Kerman, Sardouyeh	623	-		40	Ramsar to Chaboksar	737	40	J
2095	Ardabil, Sabalan	641	-		2050	Semnan, Nekarman	740	41	
2104	Ardabil, Sabalan	640	-		2050	Semnan, Nekarman	767	42	
1700	Kashan, Ghamsar	638	-		1960	Tehran, Firouzkouh	718	43	
2450	Kashan, Ghohroud	637	-		1960	Tehran, Firouzkouh	738	44	E
1880	Fars, Arzhan	633	-		1960	Tehran, Firouzkouh	769	45	
1880	Fars, Arzhan	634	-		1960	Tehran, Firouzkouh	768	-	
festuca pratensis					***Lolium persicum***				
2000	Sarab, Sabalan	831	11		1618	Ardabil, Khalkhal	799	46	
2187	Chalaous, Moroud	841	12	B	35	Rasht, Parrehsar	821	47	
2196	Hamadan, Alvand	843	13		160	Tonekabon, Road 2000	804	48	K
2606	Isfahan, Semirom	835	14		500	Kheyroudkenar Jungle	819	49	
2000	Ramsar, Javaherdeh	826	15		900	Ramsar to Javaherdeh	802	-	
2000	Ramsar, Javaherdeh	834	16		1790	Yasouj, Sisakht	805	-	
2050	Semnan, Nekarman	824	17		1880	Fars, Arzhan	798	-	
1960	Tehran, Firouzkouh	830	18		***Vulpia myuros***				
2050	Semnan, Nekarman	878	20		1686	Asalem to Khalkhal	862	50	L
2050	Semnan, Nekarman	881	21	D	250	Asalem to Khalkhal	233	51	
2050	Semnan, Nekarman	885	22		460	Azadshahr	231	52	
Festuca sclerophylla					-	Herbarium Loan	1365	53	
2100	Karaj, Gachsar	852	19	C	460	Azadshahr	868	54	
Lolium perenne					1850	Yasouj, Sisakht	857	55	
1850	Chalous, Moroud	782	25	G	500	Kheyroudkenar Jungle	854	56	M
2050	Semnan, Nekarman	693	26	H	***Festuca gigantea***				
-	Herbarium loan	1369	27		-	Galougah to Timaj	1366	57	N
2847	Hamadan, Alvand	844	-		-	Asalem to Khalkhal	1367	58	
2187	Ardabil, Sabalan	837	-		***Festuca alaica****				
2000	Ramsar, Javaherdeh	834	-		-	Herbarium Loan	1368	59	O
2150	Yasouj. Mt. Dena	833	-		***Lolium multiflorum***				
2187	35 Km Chalous Road, Moroud village	782			-	Karaj	787	23	
1880	Fars, Arzhan	784	-		-	Karaj	786	-	
					1880	Fars, Arzhan	795	24	F

Table 2. List of morphological characters

Organ	No.	Character name	Organ	No.	Character name
Stem	1	Stems; node length	Floret	35	Floret; width of callus
	2	Nodes; width		36	Floret; length
	3	Stem; width adjacent to node		37	Floret; width
Leaf	4	Leaf; length	Lemma	38	Lemma; length
	5	Leaf; width		39	Lemma; width
	6	Leaf; thickness		40	Lemma; length complete - in CS
	7	Leaf; number of leaf veins		41	Lemma; thickness
Flag leaf	8	Flag leaf; length		42	Lemma; number of veins
	9	Flag leaf; width	Palea	43	Palea; length
	10	Flag leaf; thickness		44	Palea; width
	11	Flag leaf; number of veins		45	Palea; length complete - in CS
Sheath	12	Sheath; width		46	Palea; thickness
	13	Sheath; width-complete		47	Palea; number of veins
	14	Sheath; thickness	Awn	48	Awn; length
	15	Ligule; length		49	Awn; lemma tip to awn base, distance
	16	Auricle; length		50	Awn; width
	17	Auricle; cilia length		51	Awn; length of pubescent
Rachilla	18	Rachilla; inter-node length	Stamen	52	Stamen; number
	19	Rachilla; inter-node width		53	Anther; length
Glume	20	Base of glume; width		54	Anther; width
	21	Base of glume; cilia length		55	Filament; length
	22	Lower glume; length	Gynoecium	56	Ovary+Stigma; length
	23	Lower glume; width		57	Ovary; width
	24	Lower glume; width-complete		58	Stigma; length
	25	Lower glume; thickness		59	Style; width
	26	Lower glume; number of veins		60	Lodicule; number
	27	Upper glume; length		61	Lodicule; length
	28	Upper glume; width		62	Lodicule; width
	29	Upper glume; width-complete		63	Caryopsis; length
	30	Lower glume; thickness		64	Caryopsis; width
	31	Lower glume; number of veins	Terminal spikelet	65	Terminal spikelet; lower glume length
Spikelet	32	Spikelet; length		66	Terminal spikelet; lower glume width
	33	Spikelet; number of florets		67	Terminal spikelet; upper glume length
	34	Spikelet; axis inter-node length		68	Terminal spikelet; upper glume length

Extraction of flavonoids and TLC

Plant materials (leaves) were ground to a fine powder using mortar and pestle. Extraction was performed using 80% MeOH for 36 h according to Markham (1982) with modifications. Solvent of the filtrate was evaporated using rotary evaporator in 50-60° C under relative vacuum. Dried extracts were dissolved in distilled water and filtered to discard fatty substances. Aqueous extract was dried again and dissolved in 5 ml MeOH. One dimensional thin layer chromatography was performed using 20 cm × 10 cm Aluminum sheets covered with silica gel 60F254 (Merck). Solvent system consisted of

water: 20, acetic acid: 20, iso-propanol: 10, butanol: 50. Separated flavonoid spots on TLCs were visualized under UV 254nm and 366nm and digitally photographed using a Canon EOS 500D digital camera. Skewness of images was corrected in Adobe Photoshop software ver. 13.0 CS6 x64 extended. Then, images were calibrated in ImageJ software ver. 1.47s (Rasband, 2008) and for each specimen, the flavonoid spots profile were plotted. Migration distance for each spot was measured in ImageJ software, and data transferred to Microsoft Excel 2013 to calculate the R*f* values (Table 3). The area under each flavonoid spot in each profile was measured in ImageJ software and data were transferred to Microsoft Excel 2013 to calculate the percentage of each spot in its corresponding profile, where the bar graphs for each profile were drawn.

Table 3. R*f* values and percentage of each identified flavonoid spot in its corresponding profile. Letters in first column are same as letters in Figure 2. Numbers in first row are numbers of spots on the chromatogram from small R*f*s to large R*f*s (right to left on chromatogram)

	Spots		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
A	*F. arundinacea*	R*f*	0.127	0.349	0.419	0.521	0.584	0.627	0.662	0.703	0.745	0.801	
		%	3.33%	4.43%	8.53%	8.36%	17.11%	26.59%	12.28%	8.08%	5.56%	5.73%	
B	*F. pratensis*	R*f*	0.07	0.176	0.286	0.394	0.451	0.511	0.614	0.667	0.698	0.836	
		%	3.42%	5.97%	12.63%	16.53%	10.92%	10.37%	19.86%	12.00%	6.74%	1.55%	
C	*F. sclerophylla*	R*f*	0.115	0.239	0.309	0.374	0.42	0.473	0.589	0.65	0.717	0.93	
		%	4.33%	2.90%	4.11%	3.23%	6.42%	4.04%	33.32%	26.34%	8.30%	7.01%	
D	*F. pratensis*	R*f*	0.063	0.145	0.22	0.281	0.376	0.439	0.515	0.603	0.657	0.823	
		%	2.43%	4.40%	2.99%	11.66%	19.80%	8.14%	10.72%	16.19%	21.71%	1.97%	
E	*L. rigidum*	R*f*	0.096	0.195	0.272	0.318	0.382	0.5	0.625	0.732	0.807		
		%	4.30%	4.29%	6.88%	4.03%	8.84%	13.85%	43.48%	8.08%	6.25%		
F	*L. multiflorum*	R*f*	0.119	0.239	0.297	0.369	0.463	0.604	0.641	0.695	0.784		
		%	1.44%	4.25%	3.40%	4.66%	10.18%	33.21%	16.93%	23.07%	2.86%		
G	*L. perenne*	R*f*	0.095	0.18	0.299	0.361	0.585	0.639	0.708				
		%	7.94%	9.63%	8.94%	9.34%	38.44%	17.20%	8.51%				
H	*L. perenne*	R*f*	0.095	0.217	0.272	0.328	0.381	0.434	0.478	0.593	0.7	0.772	0.803
		%	6.30%	5.32%	8.32%	3.36%	4.30%	7.90%	4.32%	35.60%	10.83%	7.30%	6.46%
I	*L. rigidum*	R*f*	0.086	0.201	0.322	0.396	0.599	0.729	0.798	0.833			
		%	3.96%	8.03%	3.98%	9.48%	59.64%	5.37%	3.99%	5.55%			
J	*L. rigidum*	R*f*	0.082	0.179	0.388	0.607	0.676	0.732					
		%	15.36%	17.31%	13.77%	33.19%	14.76%	5.61%					
K	*L. persicum*	R*f*	0.136	0.189	0.366	0.461							
		%	18.15%	27.71%	27.56%	26.59%							
L	*V. myuros*	R*f*	0.117	0.18	0.368	0.468	0.613	0.678					
		%	18.07%	24.41%	22.96%	16.82%	12.23%	5.51%					
M	*V. myuros*	R*f*	0.045	0.123	0.37	0.448							
		%	27.80%	22.40%	24.80%	24.99%							
N	*F. gigantea*	R*f*	0.113	0.409	0.495	0.592							
		%	18.45%	49.60%	18.56%	13.39%							
O	*F. alaica*	R*f*	0.062	0.179	0.304	0.448	0.611						
		%	16.05%	9.39%	13.12%	44.60%	16.84%						

Morphology

Sixty eight quantitative morphological characters from both vegetative (17) and reproductive (51) characters were used for morphological study (Table 2). Each character was measured 3 to 6 times (independent measures on same specimen) to calculate averages and standard deviations (Table 4). Measurements were performed on several digital images taken with 15 megapixels Cannon EOS 500D camera capable to connect to stereomicroscope, and using millimeter-papers to calibrate the images. Calibration of images was performed using ImageJ software, and measurements were transferred to Excel datasheets to calculate the basic statistics (min, max, average, and SD). Inapplicable characters such as 'upper glume length' for *Lolium* spp. specimens were considered as missing values. Data were converted to NTS format of NTSYS-pc software and analyzed using Simint, Njoin, SAHN, Eigen and Mod3D modules. Cosine distance (dissimilarity) coefficient ($C_{ij} = \sum_k (x_{ki} - x_{kj}) / \sum_k x_{ki}^2 \sum_k x_{kj}^2$) was used for calculating dissimilarity matrix, and UPGMA was used as the sorting method in cluster analysis. Same coefficient was used for PCO analysis.

Results and Discussion

Flavonoids spot profiles

Solvent system was optimized to achieve best separation of flavonoid spots in one dimensional TLCs (Figure 1). Two dimensional test TLCs moved spots on diameter of TLC and confirmed efficient separation of spots using one dimensional TLCs. To document the process of optimization of solvent system, a database system (unpublished) was designed to help storage and retrieval of our TLCs data containing components of each tested solvent, list of samples on each chromatogram, TLC images, and to making specialized reports.

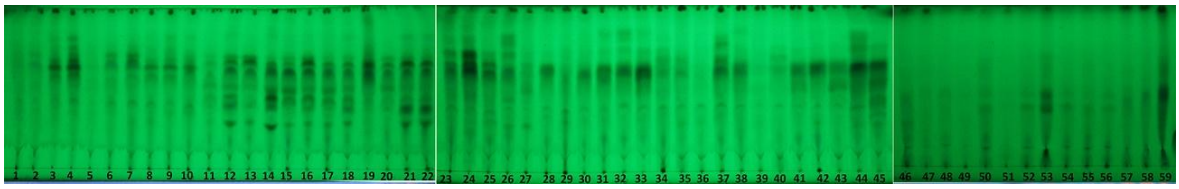

Figure 1. TLC chromatograms of selected specimens. Number beneath each lane refers to numbers in 'TLC lane' and corresponding specimen in Table 1

Flavonoids were separated in range R*f* = 0.045 to R*f* = 0.93. Number of identified flavonoid spots (separate R*f* values) in each extract ranged from 4 spots in *L. persicum* and *V. myuros* (K, M in Figure 2), to 11 spots in *L. perenne* (H in Figure 2). Percentage of spots in their corresponding extracts ranged from 1.44% (spot 1 in *L. multiflorum*; F) to 59.64% (spot 5 in *L. rigidum*; I).

Plots of *F. arundinacea*, *F. pratensis*, *F. sclerophylla* and *L. multiflorum* (A; B, D; C; F in Figure 2) were topologically similar, showing large spots in R*f* range 0.5-0.7. Plots of *L. perenne* samples (G, H in Figure 2) were close to this group, but with less strong spots (area under curve, or percentage of picks). Four plots belonging to *V. myuros*, *L. persicum* and *F. gigantea* were also similar, although the members of this group were distantly connected together. Plot of *F. alaica* was distinct, containing a large spot in R*f* 0.45. Flavonoid spot profile of *L. persicum* was most similar to that of *F. gigantea* (syn. *L. giganteum sensu* Darbyshire). Percentage of each spot and its corresponding R*f* value for all specimens are presented in Table 3.

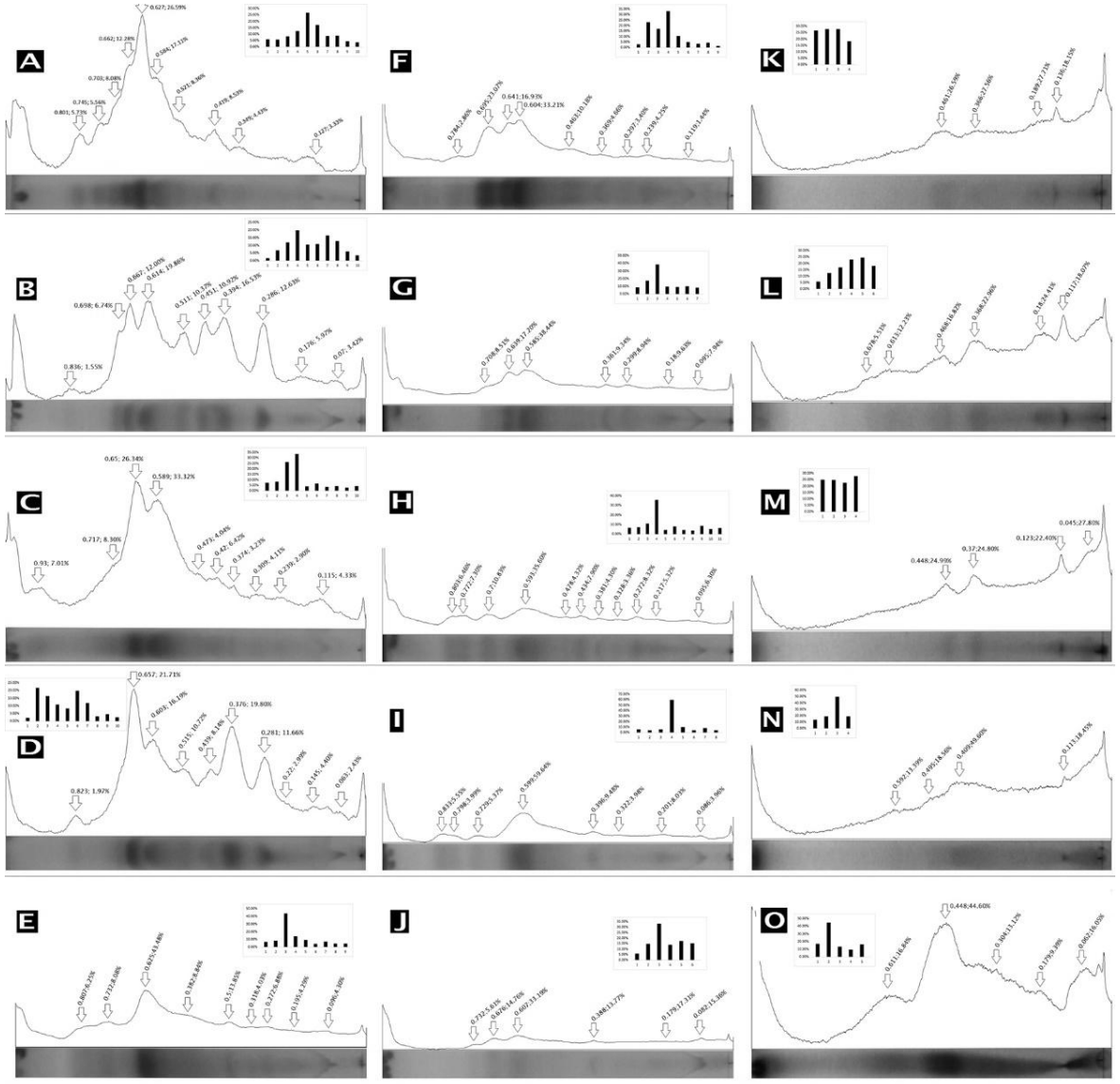

Figure 2. Plots showing location (R*f*), intensities and percentage of each flavonoid spot in selected specimens. Arrows point to pick of each spot, numbers above each arrow: first number corresponds to R*f* values, and second numbers are percentage of each spot in its profile. Bar charts are drawn according to percentage values. A. *Festuca arundinacea*; B. *F. pratensis*; C. *F. sclerophylla*; D. *F. pratensis*; E. *L. rigidum*; F. *L. multiflorum*; G. *L. perenne*; H. *L. perenne*; I. *L. rigidum*; J. *L. rigidum*; K. *L. persicum*; L. *Vulpia myuros*; M. *V. myuros*; N. *F. gigantea*; O. *F. alaica*.

Festuca arundinacea L. (syn.: *Lolium arundinaceum* (L.) Darb.): 10 distinct spots were identified (profile A) for this species which R*fs* ranged from 0.13 to 0.8. The most prominent spot had R*f* = 0.63 which contained 26.6% of the total flavonoids in the corresponding extract. *Festuca pratensis* Hudson (syn.: *Lolium pretense* (Hudson) Darb.): 10 distinct spots were identified (profiles B, D) for this species which R*f*s ranged from 0.06 to 0.84. The most prominent spot had R*f* = 0.66 (profile D) which contained 21.7% of the total flavonoids in the corresponding extract. *Festuca sclerophylla* Boiss. et Hohen. (syn. *Leucopoa sclerophylla* (Boiss. et Hohen.) Krecz. et Bobr.): 10 distinct spots were identified (profile C) for this species which R*fs* ranged from 0.11 to 0.93, and the most prominent spot had R*f* = 0.59 which contained 33.3% of the total flavonoids in the corresponding extract. *Lolium multiflorum* Lam. (syn. *L. italicum* Braun): 9 distinct spots were identified

(profile F) for this species which R*fs* ranged from 0.12 to 0.78. The most prominent spot was R*f* = 0.6 which contained 33.2% of the total flavonoids in the corresponding extract. *Lolium perene* L. (syn. *L. marschallii* Steven): Up to 11 distinct spots were identified (profiles G, H) for this species which R*f*s ranged from 0.1 to 0.8. The most prominent spot was R*f* = 0.6 (profile G) which contained 38.4% of the total flavonoids in the corresponding extract. *Lolium rigidum* Gaudin: Up to 9 distinct spots were identified (profiles E, I, J) for this species which R*f*s ranged from 0.1 to 0.83. The most prominent spot was R*f* = 0.6 (profile I) which contained 59.6% of the total flavonoids in the corresponding extract. *Lolium persicum* Boiss. and Hohen. ex Boiss.: 4 distinct spots were identified (profile K) for this species which R*fs* ranged from 0.14 to 0.46. The most prominent spot was R*f* = 0.19 which contained 27.7% of the total flavonoids in the corresponding extract. *Vulpia myuros* (L.) C.C.Gmel.: 4 distinct spots were identified (profiles L, M) for this species which R*f*s ranged from 0.05 to 0.68. The most prominent spot was R*f* = 0.05 (profile M) which contained 27.8% of the total flavonoids in the corresponding extract.

Festuca gigantea (L.) Vill. (syn. *Lolium giganteum* (Linnaeus) Darb.): 4 distinct spots were identified (profile N) for this species which R*fs* ranged from 0.11 to 0.59. The most prominent spot was R*f* = 0.4 which contained 49.6% of the total flavonoids in the corresponding extract. *Festuca alaica* Drobow: 5 distinct spots were identified (profile O) for this species which R*fs* ranged from 0.06 to 0.61. The most prominent spot was R*f* = 0.45 which contained 44.6% of the total flavonoids in the corresponding extract.

Morphology

Phenetic relationships between species belonging to *Lolium* s. str. plus those *Festuca* spp. routinely hybridize them (i. e. *Lolium* s.l., excluding *F. gigantea*) were studied using quantitative morphological characters. Measurements were performed using digital images taken from different parts of specimens, while a millimeter paper was in background of each photo. After calibration of images in ImageJ software, measurements were performed and a scale bar (white on black background) was superimposed on each photo and backgrounds were replaced with black color. Fertile parts of florets in six *Lolium* s.l. species are shown in Figure 3.

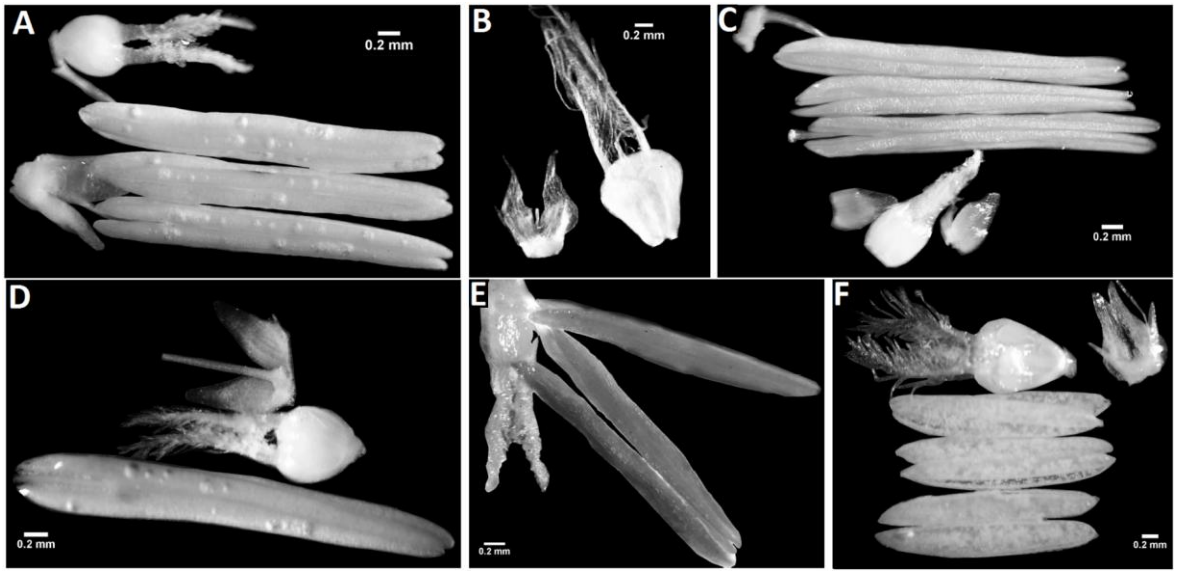

Figure 3. Fertile parts of florets in six *Lolium* s.l. species. Scale Bars length = 0.2 mm. A. *Festuca pratensis*; B. *L. multiflorum*; C. *L. rigidum*; D. *L. perenne*; E. *F. arundinacea*; F. *L. persicum*

Results (Table 4) showed that quantitative morphological characters contained variations that could be used both for description of taxa and multivariate analysis to elucidate the phenetic relationships between them. Table 4 summarizes the data, as the first least- and most-variable characters for each taxon are reported, with annotations for their min, max, SD, and P-values (P is the standardized value, and is defined here as: range divided by the mean).

Table 4. Measurement of morphological characters. The unit of all measures is mm

Less variable characters						**More variable characters**					
Character	**Min**	**Max**	**Mean**	**SD**	**P**	**Character**	**Min**	**Max**	**Mean**	**SD**	**P**
F. arundinacea											
Awn pubescent length	0.05	0.06	0.05	0.00	0.20	Rachilla internode length	2.75	8.15	5.33	1.88	1.01
Lemma thickness	0.08	0.09	0.09	0.01	0.11	Upper glume width in CS	1.37	5.31	2.00	1.19	1.97
Lemma width	1.23	1.55	1.40	0.11	0.23	Auricle length	0.60	2.84	1.79	0.88	1.25
Glume base cilia length	0.07	0.41	0.15	0.12	2.27	Palea length	5.03	6.54	5.83	0.76	0.26
Style width	0.17	0.74	0.35	0.17	1.63	Lemma width in CS	1.12	2.13	1.63	0.71	0.62
F. pratensis											
Base of glume width	0.50	0.53	0.52	0.02	0.06	Sheath width in CS	3.26	6.91	4.76	1.91	0.77
Ovary width	0.50	0.58	0.52	0.04	0.15	Lower glume number of veins	1	3	1.5	1	1.33
Upper glume thickness	0.09	0.20	0.13	0.05	0.85	Lower glume length	1.50	3.59	2.75	0.81	0.76
L. multiflorum											
Upper glume thickness	0.16	0.18	0.17	0.01	0.12	Upper glume length	6.14	9.00	7.57	2.02	0.38
Anther width	0.37	0.40	0.39	0.02	0.08	Leaf width	4.88	5.74	5.31	0.61	0.16
Style width	0.26	0.35	0.31	0.06	0.29	Anther length	2.70	3.54	3.12	0.59	0.27
L. perenne											
Upper glume width in CS	2.13	2.21	2.17		0.04	Leaf thickness	0.12	0.20	0.16		0.50
Stem width adjacent to node	1.55	1.63	1.59		0.05	Anther width	0.49	0.57	0.53		0.15
Stem node length	1.48	1.56	1.52		0.05						
L. persicum											
Lemma thickness	0.08	0.09	0.09	0.01	0.11	Upper glume length	3.56	13.50	9.52	5.26	1.04
Leaf thickness	0.16	0.34	0.23	0.08	0.78	Upper glume width	0.80	2.19	1.64	0.62	0.85
						Palea width in CS	1.14	2.09	1.53	0.50	0.62
L. rigidum											
Palea width	1.30	1.46	1.39	0.08	0.12	Auricle length	1.38	2.84	2.16	0.73	0.68
Stem node length	1.50	1.69	1.62	0.11	0.12	Upper glume length	9.00	10.00	9.50	0.71	0.11
Floret length	7.23	7.40	7.32	0.12	0.02	Ovary+stigma length	1.33	2.30	1.70	0.53	0.57

Multivariate analysis

Multivariate analysis (cluster and PCO analyses) of six *Lolium* s.l. based on quantitative morphological characters was performed to elucidate the phenetic relationships between them. Resultant dendrogram using Cosine coefficient and UPGMA method (Figure 4A) showed that *L. persicum* specimens were grouped together and distantly separated from the rest of specimens. Specimens belonging to *F. pratensis* were also grouped together (except for *F. pratensis* acc.# 833; a specimen collected from Mt. Dena in central Zagros region). Specimens belonging to *F. arundinacea* were divided into two well defined groups. Geographical location of populations from which specimens of *F. arundinacea* were collected, did not support the sub-grouping of these specimens. However, the partitioning

of *F. arundinacea* specimens based on quantitative morphological data was interesting. Another interesting result was the misplacement of one specimen belonging to *L. persicum* (acc.#799, collected from Ardabil province, Khalkhal) which was grouped with *F. pratensis* specimens. Cophenetic analysis (Figure 4B) showed that there was moderate levels (r = 0.58) of correlation between resultant dendrogram and the underlying distance matrix. However, the results were strongly confirmed with resultant plot from PCO analysis (Figure 4C) which showed the separation of *L. persicum*, partitioning of *F. arundinacea* and misplacement of *L. persicum* acc.#799. Results of PCO analysis confirmed that the first three axes had collected and expressed 82 percent of the variation held in raw data matrix (Table 5).

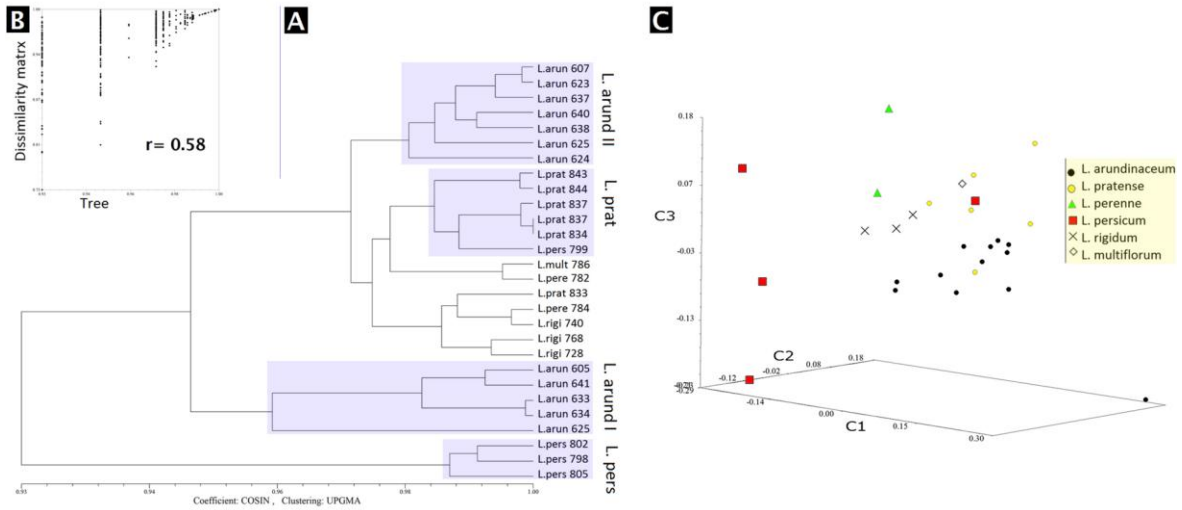

Figure 4. Multivariate analyses. A. Cluster analysis based on Cosine dissimilarity coefficient, and UPGMA as sorting method; B. Co-phenetic plot; C. Principal Coordinates analysis based on Cosine dissimilarity coefficient for quantitative data

Table 5. Axes loadings in PCO analysis. More than 82 percent of variation was expressed by three first axes

Axis	Eigen value	Percent	Cumulative%
1	1.70	33.70	33.70
2	1.41	27.89	61.59
3	1.05	20.78	82.37
4	0.77	15.19	97.56

Partitioning of *Lolium persicum* was reported in a previous work by Sharifi-Tehrani *et al.* (2008), so that the population Ardabil, Khalkhal (the population from which acc.#799 was collected), was distantly separated from other populations. Although population Khalkhal was clearly identified as *L. persicum* based on diagnostic characters and available keys, however, quantitative morphological studies and microsatellites provided evidence supporting for its separation from other *L. persicum* populations. To elucidate the taxonomic status of this population, more extensive studies are required.

SDS-PAGE analysis of seed protein profiles of taxa belonging to genera *Festuca* and *Lolium* proved to be useful for classification of festucoids (Aiken *et al*., 1998). Their method consisted scoring of each protein band identified based on R*f* values, and analyzing the qualitative data using Jaccard's coefficient. Their resultant dendrogram showed that members of *Festuca* subgen. *Schedonorus* (*F. arundinacea* and *F. pratensis*), had constructed a major group with *Lolium* spp., within which *F. pratensis* specimens were sub-grouped together, and

F. arundinacea was closer to *L. rigidum* than to other outbreeding *Lolium* spp.

Results obtained from analysis of quantitative morphological characters in our study were concordant to Aiken's results. *F. arundinacea* although divided into two separate groups, showed close relationships with *L. rigidum*. Analysis of quantitative morphological data provided more resolution in *F. arundinacea*, and the separation of *L. persicum* Acc.#799 (Ardabil, Khalkhal) from other populations in this study was also in concordance with the molecular analysis by Sharifi-Tehrani *et al.* (2008). The application of flavonoids spot profiles for classification of *Festuca* and *Lolium* species in this study was comparable to SDS-PAGE profiles of seed proteins Aiken *et al.* (1998). Flavonoids spot Profiles belonging to the members of *F.* subgen. *Shedonorus* were similar to profiles of their relatives in genus *Lolium*. Presence/absence of spot profiles were not used here as qualitative data to elucidate the relationships, or to classify taxa, as the homology of spots are to be certified. Observed variations in spot profiles of the studied species, specially, in *L. rigidum, L. perenne*, and *F. pratensis* claimed for their applicability for investigating the chemical variation between the populations within the species level. TLC chromatograms of flavonoid extracts in this study provided sharp-enough bands which could be scored and analyzed. Close relationships between *F. pratensis* and *L. multiflorum* which was demonstrated by Pasakinskiene *et al.* (1998) through analysis of GISH bands, was also confirmed by both morphological data (Figure 4C; PCO plot) and flavonoids spot profiles (Figures 2F, 2B and 2D).

Application of quantitative morphological characters for phenetic classification of *Lolium* spp. was reported in a recent work by Oshib-Nataj *et al.* (2011), where the resultant dendrogram clustered the 33 specimens into the 5 species. Relationships between some Iranian members of festucoids (including *Lolium*) using AFLPs (Majidi *et al.*, 2006; Majidi and Mirlohi, 2010) showed the close relationships between *F. arundinacea* and *F. pratensis* specimens, to which group, the specimens belonging to *L. perenne* and *L. rigidum* were connected. Results obtained from analysis of AFLPs were concordant with the previous foundings about relationships in this genus. The study also demonstrated the application of AFLPs for genetic relationships studies in cool season grasses. The phenetic analysis of Iranian species of *Lolium* based on 27 morphological characters, measured on 68 specimens (6 species) dispersed *L. perenne* and *L. multiflorum* (closely related taxa) among other *Lolium* species which claimed for the inapplicability of morphological characters for phenetic analysis of *Lolium*, i. e. (Mirjalili *et al.*, 2008). Relationships between *Lolium* species in the resultant clustering scheme could hardly be accepted (see also Mirjalili and Bennett, 2006) regarding the previous results from many other literatures (see Darbyshire, 1993 and refs. there in for review).

Results of the current study based on quantitative morphological data and flavonoids spot profiles, were in concordance with the previous foundings about species relationships in this group, and produced interpretable groupings in both cluster- and PCO analyses which also further confirmed our previous founding about *L. persicum* population Khalkhal (Ardabil province of Iran). Flavonoid spot profile of *F. gigantea* was different from those of *F. arundinaceae* and *Festuca pratensis*. These results did not support the results of seed protein electrophoresis analysis as they produced similar seed protein profiles (Bulinska-Radomska and Lester, 1985).

Conclusions

For complex plant groups (such as *Lolium* s.l.) misleading characters should be identified and avoided. Many non-reproductive characters and certain reproductive characters (see

Bulinska-Radomska and Lester, 1988) may lead to uninterpretable results. Careful selection of morphological characters, along with adoption of proper methods for analysis, will have great impact on resulting phenograms. Measurements of morphological characters reported in Table 4 may be of interest for those researchers working on gene pools of these taxa using molecular markers or for plant breeders or physiologists who want to know how these taxa may vary in their different morphological characters. The grouping of Iranian *F. arundinacea* populations into two distinct subgroups was an interesting result in our study which supported for existence of two forms in mixed populations. The applicability of quantitative morphological data to reveal phenetic relationships in this taxonomically complex group, and the potential usage of flavonoids to further description of taxa with biochemical data, and to study the variation held in their populations, were reported in this study and are intended for a more extensive study in tribe Poeae.

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References

Aiken, S. G., Gardiner, S. E., Bassett, H. C. M., Wilson, B. L. and Consaul, L. L. (1998) Implications from SDS-PAGE analyses of seed proteins in the classification of taxa of *Festuca* and *Lolium* (Poaceae). Biochemical Systematics and Ecology 26: 511-533.

Bor, N. L. (1970) Gramineae. In: Flora Iranica (Ed. Rechinger, K.H.) vol. 70. Akademische Drucku.-Verlagsanstalt, Graz.

Bulinska-Radomska, Z. and Lester, R. N. (1985) Relationships between three species of *Festuca* sect. *Bovinae* (Poaceae). Plant Systematics and Evolution 149: 135-140.

Bulinska-Radomska, Z. and Lester, R. N. (1988) Intergeneric relationships of *Lolium*, *Festuca*, and *Vulpia* (Poaceae) and their phylogeny. Plant Systematics and Evolution 159: 217-227.

Catalan, P. (2006) Phylogeny and evolution of *Festuca* L. and related genera of subtribe Loliinae (Poeae, Poaceae). In: Plant genome, Biodiversity and evolution (Eds. Sharma, A. K. and Sharma, A.). Science Publishers, Enfield, NH, US.

Catalan, P., Kellogg, E. A. and Olmstead, R. G. (1997) Phylogeny of Poaceae subfamily Pooideae based on chloroplast ndhF gene sequences. Molecular Phylogenetics and Evolution 8: 150-166.

Catalan, P., Torrecilla, P., Rodriguez, J. A. L. and Olmstead, R. G. (2004) Phylogeny of the festucoid grasses of subtribe Loliinae and allies (two Dimoeae, Pooideae) inferred from ITS and trnL-F sequences. Molecular Phylogenetics and Evolution 31: 517-541.

Charmet, G., Ravel, C. and Balfourier, F. (1997) Phylogenetic analysis in the *Festuca-Lolium* complex using molecular markers and ITS rDNA. Theoretical and Applied Genetics 94: 1038-1046.

Darbyshire, S. J. (1993) Realignment of *Festuca* subgenus *Schedonorus* with the Genus *Lolium* (Poaceae). Novon 3: 239-243.

Darbyshire, S. J. and Warwick, S. I. (1992) Phylogeny of North-American *Festuca* (Poaceae) and Related Genera Using Chloroplast DNA Restriction Site Variation. Canadian Journal of Botany 70: 2415-2429.

Davis, P. H., Mill, R. R. and Tan, K. (1988) Poacaee. In: Flora of Turkey and The East Aegean Islands (Ed. Davis, P.H.) vol. 10. Edinburgh University Press, Edinburgh.

Gaut, B. S., Tredway, L. P., Kubik, C., Gaut, R. L. and Meyer, W. (2000) Phylogenetic relationships and genetic diversity among members of the *Festuca-Lolium* complex (Poaceae) based on ITS sequence data. Plant Systematics and Evolution 224: 33-53.

Ghassemi-Dehkordi, N., Ghannadi, A. and Khabbaz-Mehrjardi, A. (2012) Microscopical, macroscopical and chemical investigations and their uses in chemotaxonomy of *Crataegus pontica* C. Koch. Taxonomy and Biosystematics 4(10): 1-16 (in Persian).

Hackel, E. (1882) Monographia Festucarum Europaearum. Verlag von Fischer, Berlin.

Majidi, M. M. and Mirlohi, A. (2010) Genetic similarities among Iranian populations of *Festuca*, *Lolium*, *Bromus* and *Agropyron* using AFLP markers. Iranian Journal of Biotechnology 8: 16-23.

Majidi, M. M., Mirlohi, A. F. and Sayed-Tabatabaei, D. E. (2006) AFLP analysis of genetic variation in Iranian fescue accessions. Pakistan Journal of Biological Sciences 9: 1869-1876.

Markham, K. R. (1982) Techniques of flavonoid identification. Academic Press, London.

Mirjalili, S. A. and Bennett, J. S. (2006) Morphological Variation in Population of the Genus *Lolium* (Poaceae) in Iran. International Journal of Botany 2: 286-292.

Mirjalili, S. A., Bennett, S. J. and Poorazizi, E. (2008) A phenetic analysis on the genus *Lolium* (Poaceae) in Iran. Plant Systematics and Evolution 274: 203-208.

Muller, J. and Catalan, P. (2006) Notes on the infrageneric classification of *Festuca* L. (Gramineae). Taxon 55: 139-144.

Nevski, S. A. (1965) *Lolium*. In: Flora USSR (Eds. Komarov, V. L., Rozhevits, R. Y. and Shishkin, B. K.). Translation for the National Science Foundation and the Smithsonian Institutation Isreal Program for Scientific Translations.

Oshib-Nataj, M. K., M., Shekarchi, H. and Akbarzadeh, M. (2011) A phenetic study of the genus *Lolium* from Poaceae family in Iran. Taxonomy and Biosystematics 6: 1-16 (in Persian).

Pasakinskiene, I., Anamthawat-Jonsson, K., Humphreys, M. W., Paplauskiene, V. and Jones, R. N. (1998) New molecular evidence on genome relationships and chromosome identification in fescue (*Festuca*) and ryegrass (*Lolium*). Heredity 81: 659-665.

Rasband, W. S. (2008) ImageJ, National Institutes of Health, Bethesda, Maryland, USA. Retrieved from http://rsbweb. nih. gov/ij. On: 6 July 2013.

Sharifi-Tehrani, M., and Ghasemi-Dehkordi (2011) *Matricaria* L. (Anthemideae, Asteraceae) in Iran: a chemotaxonomic study based on flavonoids. Taxonomy and Biosystematics 3(8): 25-34.

Sharifi-Tehrani, M., Kazemi, A. and Shabani, L. (2012) Phenetic relationships among natural population accessions of *Glycyrrhiza glabra* L. (Fabaceae) in central Zagros region of Iran, based on quantitative morphology, flavonoids and glycyrrhizin contents data. Taxonomy and Biosystematics 4(13): 59-72.

Sharifi-Tehrani, M., Mardi, M., Saeidi, H., Gharehyazi, B. and Assadi, M. (2008) Transferability of Genomic and EST-Microsatellites from *Festuca arundinacea* Schreb. to *Lolium persicum* Boiss. and Hohen. ex Boiss. International Journal of Botany 4: 476-480.

Soreng, R. J. and Terrell, E. E. (1998) Taxonomic notes on Schedonorus, a segregate genus from *Festuca* or *Lolium*, with a new nothogenus, *xSchedololium*, and new combinations. Phytologia 83: 85-88.

Stace, C. A., Al-Bermani, A. K. K. A. and Wilkinson, M. J. (1992) The distinction between the *Festuca ovina* L. and *Festuca rubra* L. aggregates in the British Isles. Watsonia 19: 107-112.

Torrecilla, P. and Catalan, P. (2002) Phylogeny of broad-leaved and fine-leaved *Festuca* lineages (Poaceae) based on nuclear ITS sequences. Systematic Botany 27: 241-251.

Torrecilla, P., Lopez-Rodriguez, J. A. and Catalan, P. (2004) Phylogenetic relationships of *Vulpia* and related genera (Poeae, Poaceae) based on analysis of ITS and trnL-F sequences. Annals of the Missouri Botanical Garden 91: 124-158.

Torrecilla, P., Rodriguez, J. A. L., Stancik, D. and Catalan, P. (2003) Systematics of *Festuca* L. sects. *Eskia* Willk., *Pseudatropis* Kriv., *Amphigenes* (Janka) Tzvel., *Pseudoscariosa* Kriv. and *Scariosae* Hack. based on analysis of morphological characters and DNA sequences. Plant Systematics and Evolution 239: 113-139.

روابط فنتیک *Lolium* s.l. از تیره Poaceae در ایران بر اساس پروفایل لکه‌های فلاونوئیدی و صفات ریخت‌شناسی کمی

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چکیده

روابط بین گونه‌های *Lolium* و *Festuca* از دیرباز موضوع قابل توجه در تاکسونومی زیرقبیله Loliineae بوده است. مطالعه حاضر به بررسی روابط فنتیک در *Lolium* s.l. (شامل زیرجنس *Schedonorus* از جنس *Festuca*) با استفاده از پروفایل لکه‌های فلاونوئیدی و صفات ریخت‌شناسی کمی می‌پردازد. اندازه‌گیری صفات ریخت‌شناسی کمی و دانسیتومتری لکه‌های فلاونوئیدی و رسم پلات پروفایل آنها با استفاده از تصاویر کالیبره شده دیجیتال و نرم‌افزار ImageJ و تحلیل‌های چند متغیره خوشه‌ای و رسته‌بندی با استفاده از نرم‌افزار NTSYS-pc صورت گرفتند. هر یک از گونه‌های مطالعه شده بر اساس پروفایل لکه‌های توصیف شده و مقادیر R*f* و درصد هر یک از لکه‌ها در پروفایل مربوط گزارش شده‌اند. تنوع موجود در لکه‌های پروفایل گونه‌های *L. rigidum*، *L. perenne* و *F. pratensis* نشان داد که پروفایل لکه‌های فلاونوئیدی را می‌توان به عنوان صفات مفید برای مطالعه بیشتر تنوع درون سطح گونه نیز مطرح نمود. تحلیل خوشه‌ای صفات ریخت‌شناسی کمی، گونه‌ها را در گروه‌هایی مجزا تفکیک نمود و جمعیت اردبیل از گونه *L. persicum* را نیز از سایر جمعیت‌های این گونه تفکیک نمود. تفکیک جمعیت‌های *F. arundinacea* به دو گروه مجزا نیز نتیجه‌ای قابل توجه است که وجود دو شکل مجزا از این گونه در ایران را پیشنهاد می‌کند.

واژه‌های کلیدی: روابط فنتیک، فلاونوئید، ریخت‌شناسی کمی، *Festuca*، *Lolium*

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