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Transferability of Genomic and EST-Microsatellites from *Festuca arundinacea* Schreb. to *Lolium persicum* Boiss. and Hohen. ex Boiss.

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Abstract: This study was primarily aimed to assess the applicability of genomic and EST-microsatellite markers for genetic study of *Lolium persicum*, through investigating the transferability of publicly available SSR markers. The transferability of 12 genomic and 20 EST-SSR primer pairs from *Festuca arundinacea* to representatives of Iranian natural populations of *Lolium persicum* was assessed. The percentage of polymorphic loci and PIC (Polymorphism Information Content) values of utilized markers showed that (i) genomic SSRs were as transferable as EST-SSRs (75%) and (ii) level of polymorphism revealed by EST-SSRs (average PIC = 0.278) was similar to that of the genomic-SSRs (average PIC = 0.219). PCO analysis of data matrix showed that the studied specimens could be genetically divided into three groups (N, NW and SW), related to their geographic origin. This study showed that the cross species transferable SSRs are valuable molecular tools, for genetic studies of the *L. persicum* for which limited number of molecular markers are available.

Key words: EST-SSR, *Festuca arundinacea*, *Lolium persicum*, ryegrass, SSR

INTRODUCTION

The genus *Lolium* L. consists of about eight diploid (2n = 2x = 14) species native to Europe, North of Africa and West of Asia; though widely distributed in all temperate regions of the world due to human activities. The genus is grouped into in-breeding and out-breeding species; with the inbreeding group consists of *L. temulentum* L. and *L. remotum* Shrank (distributed in Europe and Mediterranean region) and *L. persicum* Boiss. and Hohen. ex Boiss. (restricted to West of Asia). Close relationship between *L. temulentum* and *L. persicum* was proposed based on morphological traits and a common ancestor was demonstrated for these two resembling self-compatible species (Terrell, 1968; Bennett, 1997; Balfourier *et al*., 2000; Catalan, 2006). The out-breeding group, however, consists of most economically important forage grass species of the genus, *L. perenne* L., *L. multiflorum* Lam. and *L. rigidum* Gaud. which are self-incompatible species, readily hybridizing in nature with offsprings of different levels of viability (Jenkin, 1954; Naylor, 1960).

Malik (1967) and Borrill (1976) agreed that the Mediterranean basin is the center of origin of the genus *Lolium* and that 'true self-pollinating' species of this genus (*L. temulaentum*, *L. persicum* and *L. remotum*), ancestors of *L. perenne* and *L. rigidum*, are known only as weeds of cultivated crops. Therefore, *Lolium temulentum* is being used as a model species for genetic and genomic studies in forage and turf grasses (Wang *et al*., 2005). The closest relative of *L. temulentum*, (i.e., *L. persicum*), which is restricted to the West of Asia (center of origin and probably center of diversity of the genus), has similar advantages (such as simplicity of the biology, easy to grow, self-fertility, short life cycle, diploid, close relationship to major grass species in the *Festuca-Lolium* complex) for being investigated as a model grass. For that reason, the genetic diversity present within *Lolium persicum* is of considerable interest for finding new genes for exploitation in agriculture and grasses improvement. However, due to the limited molecular markers available, the genetic diversity of the *L. persicum* has not yet been extensively studied (Bennett 1997; Balfourier *et al*., 1998; Balfourier *et al*., 2000).

Among several molecular marker systems developed so far, microsatellites have become the marker of choice in many recent investigations due to their high reproducibility and polymorphism. However, despite the high labor, time and fund required to design microsatellite markers, they have the drawback of limited transferability to species of other genera, though they are transferable to close congener species (Ellis and Burke, 2007). The new class of microsatellites, which is derived from EST libraries (EST-SSRs) is more readily transferable to other species but sometimes in cost of less polymorphism.

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Saha *et al*. (2004) developed a number of EST-SSR markers with transferability across members of *Lolium-Festuca* complex and Kirigwi *et al*. (2008) studied them for their cross-species transferability for genetic diversity assessment of *L. temulentum*.

In the current study, we were aimed to assess the applicability of these polymorphic and reproducible molecular markers for genetic study of *L. persicum*, through investigating the transferability of publicly available SSR markers. Assessing the magnitude of selected genomic and EST microsatellite markers in revealing both the polymorphism and phylogenetic signals were the secondary aims of this study.

MATERIALS AND METHODS

Plant material: Fresh leaves from fifteen samples of *Lolium persicum* from nine localities in the Northwest (NW), North (N) and Southwest (SW) of Iran were collected during May-June 2006, dried using silica gel and stored frozen until the DNA was extracted (Table 1). The molecular researches were conducted from June 2006 to March 2008. Herbarium voucher specimens for each individual were prepared and identified taxonomically using identification keys (Bor, 1968, 1970).

DNA extraction and SSR analysis: A small amount of silica dried leaves of each individual was ground to fine powder in liquid N_2. DNA was extracted following the CTAB DNA isolation protocol described by Doyl and Doyl (1987) with minor modifications. Twelve SSR and twenty EST-SSR markers from published primer sequences (Saha *et al*., 2004; Lauvergeat *et al*., 2005) were used to assess their transferability to *L. persicum* (Table 2). PCR reactions were performed at a final volume of 10 mL, using 0.3 unit of Taq DNA polymerase (Fermentas Life Sciences), 1x supplied Taq-buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.1 mM of each primer pair. For SSR markers, after initial denaturation at 94°C for 3 min, the touchdown program consisted of (i) 10 cycles at 94°C for 30 sec, 65°C (-1°C per cycle) for 30 sec and 72°C for 30 sec, followed by (ii) 30 cycles at 94°C for 30 sec, 65°C for 30 sec and 72°C for 30 sec and (iii) a final elongation step at 72°C for 5 min. For EST-SSR markers, after 1 min at 95°C, 40 cycles were performed with 50 sec at 95°C, 50 sec at the optimum annealing temperature for the respective primer pair, 90 sec at 72°C and a final extension step of 10 min at 72°C. PCR products were separated on 6% denaturing polyacrylamide gel in a Sequi-Gen GT sequencing gel apparatus (BioRad Laboratories Inc., Hercules, CA, USA) and visualized by silver staining (Bassam *et al*., 1991). Gels were scanned and scored manually (twice and independently) using Photoshop software package.

Data analysis: Bands were scored as presence (1) or absence (0) of dominant bi-allelic loci (Saha *et al*., 2004; Kirigwi *et al*., 2008) and raw data were recorded in a scoring matrix generated by Microsoft Excel. PIC (Polymorphic Information Content) values were computed using formula $PIC = 2P_iQ_i$, in Microsoft Excel where P_i is the frequency of presence and Q_i is the frequency of absence of a particular band. In order to calculate PIC value for a primer pair, PIC values for all the polymorphic bands produced by a primer pair were averaged (Rana and Bhat, 2004). POPGENE ver. 1.32 (Yeh *et al*., 2000) was used to calculate the number and percentage of polymorphic loci across groups of specimens (Table 3). Genetic similarity among the genotypes was calculated according to Dice coefficient (Dice, 1945) using SIMQUAL module in NTSYS-pc software version 2.1 (Rohlf, 2000) and was subsequently used for principal coordinates analysis (Fig. 1).

Table 1: Sample code and geographic origin of samples studied

Sample	Region and province	Locality	Altitude
Khyrd1	N: Guilan	Rasht, Kheyrood-kenar Forest	550
R20001	N: Guilan	Rasht, Tonekabon, Road 2000	160
Pareh1	N: Guilan	Rasht, Near Parehsar	30
Jvahr1	N: Guilan	Rasht, Ramsar to Javaherdeh	750-900
Khlkh1	NW: Azerbayjan	Khalkhal, 60 km to Ardabil via Sarab	2030
Khlkh2	NW: Azerbayjan	Khalkhal, 60 km to Ardabil via Sarab	2030
Khlkh3	NW: Azerbayjan	Khalkhal , 60 km to Ardabil via Sarab	2030
Mishi1	SW: Kuhgiluyeh and Boyerahmad	Yasouj, Mt. Dena, Near Cheshmeh Mishi	2150
Mishi2	SW: Kuhgiluyeh and Boyerahmad	Yasouj, Mt. Dena, Near Cheshmeh Mishi	2150
Mishi3	SW: Kuhgiluyeh and Boyerahmad	Yasouj, Mt. Dena, Near Cheshmeh Mishi	2150
Siskt1	SW: Kuhgiluyeh and Boyerahmad	Yasouj, Mt. Dena, Sisakht	1790
Siskt2	SW: Kuhgiluyeh and Boyerahmad	Yasouj, Mt. Dena, Sisakht	1790
Siskt3	SW: Kuhgiluyeh and Boyerahmad	Yasouj, Mt. Dena, Sisakht	1850
Maleh1	SW: Kuhgiluyeh and Boyerahmad	Yasouj, After Sepidan, Gardaneh Malehshooreh	2300
Arzhn1	SW: Fars	Shiraz, Dasht-e Arzhan	1880

Table 2: Primer sequences of SSR (Lauvergeat *et al*., 2005) and EST-SSR (Saha *et al*., 2004) markers, band size ranges, number of bands and Polymorphism Information Content (PIC) of primers used in this study

Marker name	Forward primer sequence	Reverse primer sequence	T_a (°C)	Band size Expected	Band size Observed	No. of bands	PIC
Genomic SSRs							
B1A2	5'-GTGCAGCAGTTTGAATTGGA-3',	5'-AGCATCGGGAGCTATGAATG-3'	65-55	218	220-270	6	0.258
B1A8	5'-GACTTTCAGGCATCGGTCAT-3'	5'-CCCAGCTCCATTCTTAATGC-3'	65-55	295	280-295	2	0.356
B2G6	5'-CCAACTAGACAAAGGGGATTG-3'	5'-GGAGAGCACCATTCATCCAT-3'	60-50	212	-	-	-
B1A11	5'-CCGGCCTATACCTGATGCT-3'	5'-GGAACACCGTTCCAGGTATG-3'	65-55	226	230-240	1	0.231
B1B6	5'-GGAGCTGCATCTTTCTTGCT-3'	5'-GCAAACCCAGACACCCATTA-3'	65-55	291	270-320	7	0.279
B1B3	5'-AGGTGTCCTGTTGCTTTGGA-3'	5'-TTTACCCCCAGGGATCAAAT-3'	65-55	207	-	-	-
B4D9	5'-GACGTCATACCTGCGTGCTA-3'	5'-GCGAATCAAAGAAGCATGTG-3'	65-55	251	230-250	2	0.258
B1C8	5'-TTCTGGCCATGTTGATTTGC-3'	5'-GTCTACGGGTTGGAGCAGTG-3'	65-55	198	195-210	3	0.415
B3A2	5'-CCAGAACAAAATCAGTGTCACC-3'	5'-TACCAGGCACCTTCACCTTC-3'	65-55	144	145-160	4	0.204
B1A9	5'-ACCCATGTCTTCGGATTTGA-3'	5'-ACTACTCCAGCTCCGGGATT-3'	65-55	208	-	-	-
B3B8	5'-TGTCATGTCCGCTGTCTACG-3'	5'-GAGAGTGGGCGATCATCTTC-3'	65-55	307	300-340	4	0.342
B1C9	5'-GAGCCGATGCACAGGTTACT-3'	5'-AAAGGAAGCCGGCTAATCAC-3'	65-55	193	180-240	7	0.279
EST-SSRs							
NFA132	5'-CAATAATGGAGGAAGTGGAGGA-3'	5'-CTTGGCTCTAGGATGGCTTACT-3'	58	212	120-220	7	0.363
NFA085	5'-CAACTTCAGAGCCTTGTTGATG-3'	5'-AACCCTAACCCTAAAACCTCCA-3'	58	200	-	-	-
NFA154	5'-GTTTTGCTCCCTCCTGTCC-3'	5'-AGGGTCCTTCTGCAAGTCCT-3'	59	179	255- >1031	7	0.185
NFA124	5'-CAGAGAGGGTTTGGGCTTT-3'	5'-ACTTGAGGTTGGTGCAGGAG-3'	57	250	190- >1031	9	0.168
NFA002	5'-GCTCCAGCTTCTCCATCATC-3'	5'-ACCAAGTCGTCCAAGTCAGC-3'	59	213	300-590	6	0.290
NFA020	5'-GCACGAGGCTCTTTCCTCTA-3'	5'-GGTGCTTGGCCTTCTTCC-3'	59	209	550-600	2	0.364
NFA106	5'-CTCTCCTCGAGCCGCATC-3'	5'-CTCTCCTCGAGCCGCATC-3'	61	116	-	4	0.227
NFA108	5'-ATCAAACCCTGAGAAGCAAAAC-3'	5'-GTTCCGTCGCGTACATTCTT-3'	56	251	-	-	-
NFA116	5'-TGTGTTCATGCAAGTGTGTGTT-3'	5'-CCTGAAGGTTTGGAATATCGAC-3'	57	258	210-1031	2	0.258
NFA143	5'-TTTTCTACGTTTGTGCAGGTTC-3'	5'-ACTGAAGACAGGCCATACATGC-3'	57	297	-	-	-
NFA146	5'-GGAAGCAAAATGATAGGATTGG-3'	5'-ATAGCGCATGACTGTTGATGAT-3'	57	226	210-230	3	0.444
NFA021	5'-CACAGCTCGTATAGGCGTCA-3'	5'-CTTGTCGAAGAGCGGGAAC-3'	59	226	-	-	-
NFA030	5'-AGTCGGTGGTGAAGCTGAAG-3'	5'-ACAACTAGGGGGCTGGTCA-3'	59	200	175-190	6	0.320
NFA070	5'-TCCAGTTCAACTTCCAGCAA-3'	5'-CCCGTATTTTATCCTCGATCTG-3'	57	207	-	-	-
NFA111	5'-CATCCATCTCCAAAATACGTCA-3'	5'-AGGCTTCTCCTTCCTTTCCAT-3'	56	214	550- >1031	4	0.373
NFA153	5'-ATCAGCATTCCACCAGTTGTTC-3'	5'-CACTACCAGCAAGGCTCTAACC-3'	58	192	170-190	4	0.298
NFA089	5'-CTCTCTATCCGCTTGATCTCG-3'	5'-CTCTCCCCTCCTCTCCTTTC-3'	59	203	155-185	9	0.275
NFA105	5'-TCGGTTAGCATCACCATATTCA-3'	5'-CGAGCGTTCCGGTTGG-3'	56	265	>1031	3	0.160
NFA076	5'-ACGAGGGGATCTCCTACACC-3'	5'-TGGTCACAGAGCTTGTCAGG-3'	61	189	>1031	3	0.267
NFA103	5'-GCAAGACATCACATAGCTGTCG-3'	5'-CTCGTACCGCCCCACAAG-3'	59	238	100-240	4	0.173

T_a: Annealing temperature

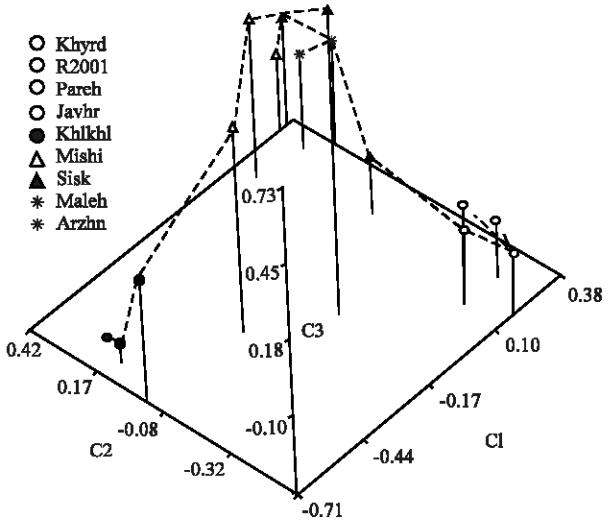

Fig. 1: 3D-projection of three first axes in PCO analysis (using Dice coefficient). Percentages of polymorphism extracted by each of the three first axes and Eigen values of each axis were: 1:21.52 (23.45); 2:13.96 (15.22) and 3:9.38 (10.22)

Table 3: Number and percentage of polymorphic loci in each site or geographic region

Site	Samples	No. of polymorphic loci (%)
Mishi (SW)	Mishi1, Mishi2, Mishi3	42 (38.53)
Siskt (SW)	Siskt1, Siskt2, Siskt3	46 (42.20)
Arzhn (SW)	Arzhn1, Maleh1	21 (19.27)
Guilan (N)	Jvahr1, R20001, Khyrd1, Pareh1	43 (39.45)
Khlkhl (NW)	Khlkh1, Khlkh2, Khlkh3	26 (23.85)
Northern Iran	(N+NW)	73 (66.97)
Southern Iran	(Mishi+Siskt+Arzhn)	72 (66.06)

RESULTS AND DISCUSSION

From 32 primer pairs used, 22 primer pairs (75%) amplified clean repeatable PCR bands in *L. persicum*, indicating high transferability of SSR markers from *Festuca arundinacea* to this species. The average PIC for each locus, the number of bands per locus, band size range and some other details of these primers and those did not amplified any PCR bands are shown in Table 2. A total of 371 bands (127 genomic and 244 EST-SSR bands) from 109 loci (36 genomic and 73 EST-SSR loci) were scored. The number of polymorphic loci was ranged from 1 (B1A11) to 9 (NFA124, NFA089) with an average of

4.54 for all utilized primers. Both the highest and lowest number of polymorphic loci (Table 3) were observed in Sisakht and Arzhan (SW localities) respectively, however, dividing the samples into two major Northern and Southern groups, showed a similar percent of polymorphic loci for each group (Table 3). PIC values for genomic SSRs ranged from 0.204 (B3A2) to 0.415 (B1C8) with an average of 0.219; while, for EST-SSRs it ranged from 0.160 (NFA105) to 0.444 (NFA146) with an average of 0.278 (Table 2).

The used EST-SSR primers, revealed a high level of polymorphism similar to that of the genomic-SSR primers; although, the average PIC of EST-SSRs (0.278) was slightly higher than that of the genomic-SSRs (0.219).

In a recent study, genetic diversity of *Lolium temulentum* using microsatellite markers showed that tall fescue genomic and EST-SSRs were useful in assessing genetic diversity of the species and could benefit the genetic improvement of members of the *Festuca-Lolium* complex (Kirigwi *et al*., 2008). Similarly, present study showed the applicability of these markers for *L. persicum* which is a congener and close relative to *L. temulentum* and also a potential genetic resource for improvement of cool season grasses. In contrast to the results of Pashley *et al*. (2006), in which the EST-SSRs were three times as transferable across sunflower species as compared with the anonymous SSRs, almost-equal transferability of genomic- and EST-SSRs between tall fescue and Persian ryegrass may reflects the close genetic relationships between *L. persicum* and *F. arundinacea* as was previously indicated between other species of the *Festuca-Lolium* complex (Catalan *et al*., 2004; Catalan, 2006).

In PCO analysis, the samples were clearly divided into three groups, which could be related to their geographic origin (N, NW and SW). These groupings were supported by superimposition of MST (Minimum Spanning Tree) on the PCO projection (Fig. 1). The first three axes in PCO analysis collectively extracted (21.52+13.96+9.38%) = 44.86% of the total variation (Fig. 1) and taking the forth axis into account, the cumulative percent of variation became 52.64%.

High levels of polymorphism revealed by both the genomic and EST-SSR markers used in this study indicate the potential of these markers for estimating the genetic diversity, study of population genetics, marker assisted selection programs and tracking the quantitative traits of *L. persicum*. Of course, the results of polymorphism and genetic diversity of *L. persicum* as revealed in this study can be correlated with the low number of samples. It can be suggested that a greater sampling throughout the geographic range of the species would be of high value to measure the genetic diversity of this valuable species.

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