RESEARCH ARTICLE

Comparison of old and new wheat cultivars in Iran by measuring germination related traits, osmotic tolerance and ISSR diversity

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Abstract A primary concern of modern plant breeding is that genetic diversity has decreased during the past century. This study set out to explore changes in genetic variation during 84 years of breeding by investigating the germination-related traits, inter-simple sequence repeat (ISSR) fingerprinting and osmotic stress tolerance of 30 Iranian wheat (*Triticum aestivum* L.) cultivars. Seeds were planted under control and osmotic stress (−2, −4 and −6 bar) in three replications. The ISSR experiment was carried out using 32 different primers. Genotypes were divided into two groups (old and new) each containing 15 members. The results of ANOVA showed that highly significant differences existed among genotypes and among growth conditions. The results showed that during breeding in some traits such as coleoptile length and seedling vigor index, a significant decrease has been occurred. New cultivars had a mean coleoptile length of 33 mm, shorter than that of old cultivars (42 mm) under osmotic stress of −6 bar. Genetic variance of root length, shoot length and seedling vigor index for old cultivars were 1.59, 1.93 and 45,763, respectively, significantly higher than those for new cultivars (0.55, 1.08 and 27,996, respectively). This

difference was also verified by ISSR results as the polymorphism information content was 0.28 in old cultivars, higher than that of new cultivars (0.26). These results prove this claim that during breeding, genetic diversity has decreased for many germination-related traits and breeders are better to pay more attention to genetic diversity.

Keywords *F* test · Molecular marker · Old wheat cultivars · Polymorphism information content (PIC)

Introduction

Common wheat (*Triticum aestivum* L.) is one of the most important crops in Iran. It is the natural hybrid of tetraploid *T. turgidum* and diploid *Aegilops tauschii* (Yang et al. 2009). A primary concern of modern plant breeding is that genetic diversity has decreased during the past century (Reif et al. 2005). Maintenance and availability of genetic diversity is necessary for preventing disease epidemics (Strange and Scott 2005). During the past several decades, there has been a dramatic increase in use of improved cultivars instead of landraces. Several surveys have shown that many farmers do not cultivate landraces any longer and therefore modern plant breeding has led to the decline in the frequency of landraces (Khlestkina et al. 2004; Reif et al. 2005; Bonnin et al. 2014). However, the claim of decrease in genetic diversity needs to be proven and the much debated question is whether new cultivars are genetically less diverse than old cultivars. Also the trend change in many important traits such as germination related traits during a long time of breeding has not been investigated.

As water resources are likely to decline in the coming decades, drought will become increasingly important in

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many regions of the world and will be a common stress (Chaves and Oliveira 2004). The past erosion of genetic diversity may have decreased the drought tolerance of newer wheat cultivars vis-à-vis old cultivars, which were mostly landraces. To date, there has been no reliable evidence on whether or not osmotic stress tolerance of modern cultivars in Iran is less than that of old cultivars. Modern wheat genotypes in the UK have been shown to have greater stem reserve storage capacity, which is correlated with tolerance to drought (Shearman et al. 2005).

Genetic diversity has been studied by many researchers using morphological and molecular methods (Dreisigacker et al. 2005; Fufa et al. 2005; Reif et al. 2005; Akbari et al. 2006; Hao et al. 2008). In comparison with other molecular markers, the inter-simple sequence repeat (ISSR) markers are useful from standpoints of reduced technical difficulties, good reproducibility and low cost. The ISSRs are amplified from single primer polymerase chain reactions (PCR) where the primer sequence is determined according to the di-, tri-, tetra-, or penta-nucleotide repeat motifs. Also a random anchoring sequence of one to three nucleotides is added to the 3' terminal of the primer (Reddy et al. 2002).

While many investigations on genetic diversity of Iranian wheat have been conducted (Moghaddam et al. 1997; Naghavi et al. 2004; Eivazi et al. 2008; Khodadadi et al. 2011), none has considered changes in genetic variation during a long period of wheat breeding in Iran. However, a few studies have addressed temporal trend of genetic diversity in other countries (Donini et al. 2000; Reif et al. 2005; Bonnin et al. 2014).

The aim of this research was to explore changes in genetic variation during a breeding period of 84 years considering germination-related traits, ISSR fingerprinting and osmotic stress tolerance. Also another objective of this study was to investigate the trend of changes in different germination-related traits during this period of breeding.

Materials and methods

Plant material

Thirty bread wheat genotypes were selected from the cultivars released by Seed and Plant Improvement Institute, Karaj, Iran or landraces that had been commercial cultivars in the past in Iran (Table 1). These cultivars were selected from nearly 100 varieties cultivated in different parts of Iran. From each decade the most successful genotypes which had been cultivated in vast areas of the country were selected. The oldest cultivar (Sardari) has been released in 1931 and the newest cultivar (Zare) has been released in 2011. Thus, the cultivar release spanned 8 decades.

Genotypes were divided into two groups each containing 15 members. Those which were released in 1982 or earlier were designated as “old genotypes” and those released in 1987 or later were designated as “new cultivars”.

Osmotic stress tolerance at germination stage

To measure important traits at germination stage under osmotic stress and normal conditions, a factorial experiment using a randomized complete-block design with three replications of plates of 20 seeds was carried out in laboratory. Factors were cultivars (Table 1) and osmotic stress simulated by PEG6000 (at four levels: 0, −2, −4 and −6 bar). Each Petri dish was lined with a soaked filter paper and contained 10 ml of PEG solution (for 0 level of PEG, only distilled water was used). Healthy seeds of the same size were surface sterilized with sodium hypochlorite (0.5 %) and were placed in Petri dishes. Petri dishes were transferred to a germinator ($24 \pm 1^\circ\text{C}$). Number of germinated seeds was recorded every 12 h during the first 3 days. From fourth day onwards until the end of experiment (8 days total), germination was recorded daily. Finally, number of roots, root length, coleoptile length and seedling length were measured for all germinated seeds in each Petri dish. The shoot and roots were cut from seeds and fresh weight was determined. Weight was also determined after drying the root and shoots at 70°C for 48 h. Data measured on single seedlings were averaged across all seedlings in a Petri dish.

ISSR experiment

Foliar DNA was extracted following Murray and Thompson (1980). The DNA was electrophoresed using agarose gel and was quantified by visual comparison with a molecular size marker (1 kb ladder). High quality DNA samples were then eluted using distilled water to a final concentration of 50 ng/ μl .

PCR amplification

Thirty two ISSR primers, purchased from Sinaclon Company (Tehran, Iran), were used for the PCR amplification (Table 4). Amplification reactions and electrophoresis were performed according to Thomas and Bebeli (2010). Briefly, PCR reactions were carried out in a 25 μl volume containing 1X PCR buffer, 0.4 mM dNTPs, 2 mM MgCl_2 , 0.5 μM ISSR primer, 1.0 U Taq DNA polymerase and 50 ng genomic DNA. The temperature profile for amplification included one cycle of 5 min at 94°C , followed by 45 cycles of amplification. Each cycle of amplification had a denaturing step at 94°C for 30 s, an annealing step at $56\text{--}59^\circ\text{C}$ (depending on primer sequence) for 45 s and an extension step at 72°C for 2 min. After the final cycle,

Table 1 Name, pedigree, growth habit and year of release of 30 bread wheat genotypes used in this study

Name	Growth habit	Pedigree/cross name	Year of release
Sardari	Winter	Local variety	1931
Shahpasand	Winter	Local variety	1943
Tabasi	Spring	Local variety	1952
Omid	Winter	Local variety	1957
Sholeh	Spring	Local variety	1958
Roshan	Spring	Local variety	1958
Khalij	Spring	Local variety	1961
Inia	Spring	Lr64/Sn64	1969
Karaj1	Spring	200H/Vfn//Rsh	1974
Karaj 2	Facultative	Fa*Th–Mt (Omid)	1974
Moghan2	Spring	Choti Lerma	1975
Adl	Spring	Shahpasand/Turky	1977
Karaj 3	Winter	Drc/Mxp//Son64/Tzpp-Y54/3/Nai60	1977
Azadi	Spring	4820/1-32-15409//Mxp	1980
Sabalan	Winter	908/FnA12/2*1-32-438	1982
Golestan	Spring	Alandora“s”	1987
Qods	Facultative	Rsh/5/Wt/4/Nor10/2*K54//Fn/3/Ptr/6/Omid/Kal/Bb	1990
Navid	Facultative	63-112/66-2*7C (Kirkpinar 79)	1991
Darab 2	Spring	Maya”s’/Nac	1996
Alvand	Facultative	1-27-6275/CF1770	1996
Alamut	Winter	Kvz/Ti71/3/Maya“s”//Bb/Inia/4/Karaj2/5/Anza/3/Pi/Nar//Hys	1996
Zarrin	Facultative	PK15841	1996
Chamran	Spring	Attila	1998
Pishtaz	Spring	Alvand//Aldan/Ias58	2003
Toos	Facultative	Spn/Mcd//Cama/3/Nzr	2003
Shahriyar	Winter	Kvz/Ti71/3/Maya“s”//Bb/Inia/4/Karaj2/5/Anza/3/Pi/Nar//Hys	2003
Darya	Spring	SHA4/CHIL	2008
Parsi	Spring	Dove“s”/Buc“s”//2*Darab	2009
Pishgam	Facultative	Bkt/90Zhong87	2009
Zare	Winter	130L1.11//F35.70/Mo73/4/Ymh/Tob//Mcd/3/Lira	2011

samples were held for an extra 5 min at 72 °C. Amplification products were analyzed via electrophoresis in 2 % agarose gel containing ethidium bromide in 1× TAE buffer. The ISSR bands were detected and photographed using Geldoc (UVP). For estimation of molecular size of each band, 1 Kb DNA ladder was used.

Data analysis

Seedling vigor index (SVI) was calculated via the following formula (Abdul-Baki and Anderson 1973):

$$SVI = (RL + SL) \times G(\%) \quad (1)$$

where RL is root length, SL is shoot length and G(%) is the germination percentage. Mean germination time (MGT) was obtained via the following formula (Ellis and Roberts 1981):

$$MGT = \frac{\sum Dn}{\sum n} \quad (2)$$

where D is days from the start of experiment and n is the number of germinated seeds. Days to 50 % germination for each Petri dish was calculated with calculating the median of days of germination. Genetic variance component was estimated using the result of ANOVA (Hallauer 1970). The genetic variance of old and new genotypes were named as V_1 and V_2 , respectively. To assess the genetic variance change, F statistic (V_1/V_2) was calculated and its significance was determined referring to F distribution. To test the difference between old and new cultivars for the measured traits, *t* test was performed using SAS (Version 9.00, SAS Institute Inc. 2002). To compare drought tolerance of old and new set of cultivars, the stress susceptibility index (SSI) was calculated with the following equation (Abdolshahi et al. 2012):

Table 2 *F* test of comparison of genetic variances estimated from ANOVA for traits measured at germination stage for old and new cultivars

	Root number	Root length	Coleoptile length	Shoot length	Root-to-shoot length ratio	Root dry weight	Shoot dry weight
V ₁	0.14	1.65	0.178	1.85	0.0098	2.2e−5	4.4e−5
V ₂	0.11	0.55	0.192	1.08	0.0080	1.9e−5	1.98e−5
F (V ₁ /V ₂)	1.25	2.99**	0.96	1.70**	1.21	1.12	2.2**
	Root-to-shoot weight ratio	Biomass weight	Mean germination time	Germination percentage	Seed vigor index	Time to 50 % germination	
V ₁	0.0088	8.5e−5	0.0049	3.69	45,763	0.013	
V ₂	0.0048	6.3e−5	0.0159	8.62	27,996	0.012	
F (V ₁ /V ₂)	1.83**	1.32	0.30	0.41	1.63*	1.07	

V₁ and V₂ are genetic variance of old and new genotypes, respectively. The significant value of *F* indicates that genetic variance of old genotypes is significantly higher than that of new genotypes

*, ** Significant at 0.05 and 0.01 probability level, respectively

$$SSI = \frac{1 - \frac{SVI_s}{SVI_p}}{1 - \frac{\overline{SVI}_s}{\overline{SVI}_p}} \quad (3)$$

where, SVI_s and SVI_p are seedling vigor index for each cultivar under stress (–6 bar) and control, respectively. The stress level of –6 was selected as for other stress levels the differences were not high enough to be statistically significant compared to control condition. The $\overline{SVI}_s$ and $\overline{SVI}_p$ represent mean seedling vigor index under stress (–6 bar) and control, respectively. Data management and analysis were performed using SAS (Version 9.00, SAS Institute Inc. 2002). The mean scores for the three replications were used for construction of a distance matrix using SPSS (Version 19, SPSS Inc. 2010).

The ISSR amplicons were scored for presence or absence for each genotype, and the data were entered into a binary matrix as discrete variables (1 = presence and 0 = absence). Polymorphism information content (PIC) of each marker was calculated (De Riek et al. 2001). Prior to principal coordinates (PCO) analysis, genetic distance matrix was generated using the following formula:

$$E = n \left[1 - \left(\frac{2n_{xy}}{2n} \right) \right] \quad (4)$$

where *E* is the genetic distance between each pair of genotypes, *n* is the total number of polymorphic bands and 2*nxy* is the number of markers shared by two individuals. The PCO analysis was performed using GenAlEx6.501 (Peakall and Smouse 2012).

Results

Osmotic stress had high significant effect on all measured traits. Variation among cultivars was highly significant for the measured traits. The genotype × stress interaction was

highly significant for most traits, indicating that cultivars performance changed from one growth condition to another. Hence, some genotypes were more tolerant to osmotic stress than others. Osmotic stress had negative effect on all traits except root/shoot length ratio and root/shoot weight ratio. Seedling vigor index decreased by 29 % under osmotic stress of –6 bar compared with control condition. Root/shoot length ratio increased by around 40 % under osmotic stress of –6 bar compared with control condition (data not shown).

Comparisons of genetic diversity of old and new set of cultivars are shown in Table 2. Genetic variance of old (V₁) and new (V₁) cultivars were used to calculate *F* statistic. For many traits genetic diversity of old genotypes was significantly higher than that of new cultivars. Cultivars released 84 years ago were significantly more diverse when root length, shoot length, shoot dry weight, root to shoot weight ratio and seedling vigor index were considered. For other traits, genetic diversity of old set of cultivars was not significantly higher than that of new cultivars. Distance matrix (Euclidian function) results were consistent with the results of variance comparison, as the mean pair-wise distance of old cultivars was 10.09, whereas for new cultivars it was 9.9. The biggest decrease in genetic variance expressed by *F* statistic was observed for root length where the variance for old and new cultivars was 1.65 and 0.55, respectively and hence, the *F* statistic was 2.99.

The mean differences between old and new sets of cultivars are highlighted in Table 3. There were no significant differences between the means of two sets for root number, root length, root dry weight, root/shoot weight ratio, mean germination time, germination percentage, time to 50 % germination and SSI for drought tolerance. The differences between two sets of cultivars were significant for coleoptile length, shoot length, root/shoot length ratio, shoot dry weight, biomass weight and seedling vigor index.

Table 3 *t* test for difference between old and new set of genotypes considering different traits measured at germination growth stage

	Root number	Root length	Coleoptile length	Shoot length	Root-to-shoot length ratio	Root dry weight	Shoot dry weight
Mean of old	4.3	10.7	4.29	12.8	0.86	0.045	0.061
Mean of new	4.34	10.1	3.36	11.1	0.97	0.041	0.052
<i>t</i> statistic	0.52 ^{ns}	−1.59 ^{ns}	−5.26 ^{**}	−3.8 ^{**}	2.61 [*]	−1.73 ^{ns}	−4.14 ^{**}
	Root-to-shoot weight ratio	Biomass weight	Mean germination time	Germination percentage	Seedling vigor index	Time to 50 % germination	SSI for drought susceptibility
Mean of old	0.76	0.106	1.26	95.4	2252	0.708	0.98
Mean of new	0.82	0.093	1.31	94.6	1987	0.781	0.96
<i>t</i> statistic	2 ^{ns}	−3.4 ^{**}	1.06 ^{ns}	−0.67 ^{ns}	−3.1 ^{**}	1.46 ^{ns}	−0.14 ^{ns}

Means were calculated across three replications and four levels of stress treatments

^{ns} non-significant

^{*}, ^{**} Significant at 0.05 and 0.01 probability level, respectively

Fig. 1 Coleoptile length change during 84 years of breeding. Coleoptile length is useful in relation to drought tolerance in wheat especially at seedling stage

All significant differences, except for root/shoot length ratio, were negative, implying that trait values were higher in older cultivars than newer ones. The value for root/shoot length ratio was higher in new set of cultivars than in older set of cultivars.

The second objective of this research was to understand the trend of changes in germination-related traits of Iranian wheat genotypes during breeding. A decreasing trend was found for some traits during 84 years. One of these traits was coleoptile length (Table 3; Fig. 1). The mean coleoptile length under osmotic stress of −6 bar for old and new set of genotypes was 42 and 33 mm, respectively. The highest value for this trait was for Roshan (50 mm); Pishgam had the shortest coleoptile (28 mm). Roshan is an old cultivar whereas Pishgam is a newer one (Table 1). For the root to shoot length ratio there was a significant increase in new set of genotypes, implying the breeders have favored drought-resistant genotypes over sensitive ones in recent years. The

highest root to shoot length ratio was for Sabalan under osmotic stress of −6 with 1.48. Considering the stress susceptibility index (SSI), it is obvious that old genotypes were not more tolerant than new set of genotypes (Table 3).

Out of a total of 32 ISSR primers used for fingerprinting, six showed repeatable and visible bands. Results of primer amplification in PCR for old and new sets of cultivars are shown in Table 4. In old and new sets of cultivars, respectively, 82 and 85 bands were produced. The percentage of polymorphism in old cultivars (71 %) was slightly higher than that in the new cultivars (70 %). The mean PIC for older cultivars was 0.28, whereas it was 0.26 for new cultivars, which confirmed the higher diversity in old cultivars compared to newer cultivars. Therefore, molecular data verified the higher diversity of old cultivars in comparison to new cultivars. The result of PCO analysis is presented in Fig. 2. Also an example of ISSR diversity is shown in Fig. 3 which is the electrophoresis of PCR

Table 4 Primer sequence, number of bands, number of polymorphic bands and PIC (polymorphism information content) values for two sets of old and new cultivars

Primer	Sequence	Old			New		
		No. of bands	Polymorphism %	PIC	No. of bands	Polymorphism %	PIC
ISSR5	(AC)8-TG	13	46	0.17	13	46	0.16
ISSR18	(CA)8-A	19	68	0.2	19	63	0.2
ISSR19	(CA)8-G	9	53	0.26	9	66	0.29
ISSR21	(AC)8-G	21	81	0.25	21	81	0.32
ISSR28	(AC)8-YG	12	91	0.35	12	83	0.28
ISSR29	(ACTG)4	8	87	0.44	11	82	0.36
	Total	82			85		
	Average	13.6	71	0.28	14.1	70	0.26

Fig. 2 Two dimensional plot resulting from principal coordinate (PCO) analysis of all cultivars evaluated. New and old cultivars are shown with different symbols. The names of cultivars in graph correspond to those given in Table 1. The first and second axes accounted for 13.8 and 11 % of total variation, respectively

products amplified by primer ISSR28. Altogether it can be deduced that genetic variation of old cultivars was slightly higher than that of new cultivars based on PIC value (Table 4). Average genetic distance among old cultivars and among new cultivars was 23 and 21, respectively; implying there was little difference in their genetic diversity.

Discussion

Osmotic stress had negative effect on all traits except root/shoot length ratio and root/shoot weight ratio. Seedling vigor index decreased by 29 % under osmotic stress of −6 bar compared with control condition. Dhanda et al. (2004) reported a decrease of 85 % for this trait under severe osmotic stress (−10 bar). Root/shoot length ratio increased by around 40 % under osmotic stress of −6 bar compared with control condition. There may be many

factors responsible for this increase. Firstly, root tissue is less sensitive to drought than shoot tissue. Secondly, abscisic acid (ABA), which is synthesized under drought stress, promotes root growth but inhibits shoot growth (Blum 2011).

Donini et al. (2000) in a study of genetic diversity trend in winter wheat varieties released from 1934 to 1994 in UK reported non-significant narrowing of genetic diversity. This differs from the findings presented here which show significant decrease of genetic diversity in Iranian wheat varieties. The main reason is that in UK, breeders have clearly been able to maintain diverse sources of germplasm within their nurseries and introduced it into commercial varieties. Also the necessity of introducing novel disease resistance genes into breeding germplasm has been an important driver of diversity turnover in UK varieties (Donini et al. 2000). On the other hand a recent research showed that at least 50 % of in situ wheat genetic diversity has decreased during twentieth century in France (Bonnin

Fig. 3 Example of ISSR diversity among 30 wheat cultivars released during 1931–2011. This gel image was produced by PCR reaction with primer ISSR28. –C is PCR negative control. Three cultivars of Chamran, Karaj2 and Golestan are presented in both gels to help accurate scoring

et al. 2014). In current research for many traits genetic diversity of old genotypes was significantly higher than that of new cultivars. These results further support the notion that plant breeding decreases trait variability. A possible explanation for this result is that most of the new cultivars have been developed from a narrow based gene pool. The pedigrees of new cultivars show some limited genotypes have been utilized in development of several cultivars while many of old cultivars are landraces (Table 1). These findings were confirmed by molecular data in which old cultivars were more diverse than new ones.

The second objective of this research was to understand the trend of changes in germination-related traits of Iranian wheat genotypes during breeding. A decreasing trend was found for some traits during 84 years. One of these traits was coleoptile length (Table 3; Fig. 1). We can infer from this that germination ability of newer genotypes had been reduced. Under dry conditions, such being the case with most farms in Iran, deeper sowing provides seeds with more moisture for germination. Usually in this situation, topsoil moisture disappears after a short period of warm weather. Also deeper sowing will saves seeds from removal by birds and rodents. There exists a strong association between coleoptile length and plant number with

deep sowing (Rebetzke et al. 2007). These findings demonstrate that development of varieties with longer coleoptile length would benefit growers in arid conditions. Shorter coleoptile length in new cultivars is mainly associated with dwarfing genes, such as *Rht-B1b* and *Rht-D1b* which decrease both plant height and coleoptile length. Alternative dwarfing genes (e.g. *Rht8*) have been reported, which decrease plant height without reducing coleoptile length. Such genes can be used for breeding more adapted varieties to dry condition (Ellis et al. 2004). Iranian wheat cultivars do not seem to have this gene as the genotypes with longest coleoptiles, such as Roshan, Shahpasand, Adl, Tabasi, Omid, Karaj3, Sabalan and Sholeh are tall genotypes. The highest root/shoot length ratio was belonged to Sabalan under osmotic stress of -6 with 1.48. This cultivar has previously been reported to be drought resistant (Sio-Se Mardeh et al. 2006).

In summary, these results showed that regarding some important features of germination behavior, the genetic diversity has been decreasing during this period of breeding. It seems that breeders paid attention to yield during the past 84 years of breeding and ignored some important secondary traits, such as coleoptile length, which play an important role in drought tolerance during germination period. These findings have important implications for developing new

strategies in plant breeding and using more diverse genotypes in starting breeding plant material. Future work is required to focus more on other important traits. The strength of this study is using cultivars released during a long period of breeding. This is the first study to investigate the changes in genetic diversity during plant breeding in Iran. The most obvious finding to emerge from this study is that germination had been affected adversely during 84 years of breeding. However, the decrease was shown by ISSR was not high. Although the current study is based on a single stage of plant growth, the findings suggest that these trends in genetic diversity during breeding may be observed in other important traits that are worth exploring further. More research is required to better understand trends of genetic diversity in other traits at adult plant growth stage, such as yield components, biotic resistance, abiotic tolerance and other morpho-physiological traits.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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