*Genetics and Molecular Biology*, 36, 2, xxx-xxx (2013)

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Short Communication

# Genetic diversity in a world germplasm collection of tall fescue

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## Abstract

*Festuca arundinacea* Schreb., commonly known as tall fescue, is a major forage crop in temperate regions. Recently, a molecular analysis of different accessions of a world germplasm collection of tall fescue has demonstrated that it contains different species from the genus *Festuca* and allowed their rapid classification into the three major morphotypes (Continental, Mediterranean and Rhizomatous). In this study, we explored the genetic diversity of 161 accessions of *Festuca* species from 29 countries, including 28 accessions of INTA (Argentina), by analyzing 15 polymorphic SSR markers by capillary electrophoresis. These molecular markers allowed us to detect a total of 214 alleles. The number of alleles per locus varied between 5 and 24, and the values of polymorphic information content ranged from 0.627 to 0.840. In addition, the accessions analyzed by flow cytometry showed different ploidy levels (diploid, tetraploid, hexaploid and octaploid), placing in evidence that the world germplasm collection consisted of multiple species, as previously suggested. Interestingly, almost all accessions of INTA germplasm collection were true hexaploid tall fescue, belonging to two eco-geographic races (Continental and Mediterranean). Finally, the data presented revealed an ample genetic diversity of tall fescue showing the importance of preserving the INTA collection for future breeding programs.

*Keywords*: SSR markers, genetic diversity; flow cytometry, tall fescue, genetic resources.

Received: November 14, 2012; Accepted: March 20, 2013.

The genus *Festuca*, which belongs to the family *Poaceae*, contains over 500 species of grasses, varying in ploidy level from diploid (2n = 2x = 14) to dodecaploid (2n = 12x = 84) (Hand *et al.*, 2012a). The most relevant species of *Festuca* in agriculture is *Festuca arundinacea* Schreb., commonly known as tall fescue (Hand *et al.*, 2010). Tall fescue is a forage grass widely grown throughout temperate regions. This crop plays a critical role in forage and livestock systems, forming the plant basis for beef and milk production worldwide. This species is also used as turf grass and to preserve the soil (Smarda *et al.*, 2008). *Festuca arundinacea* Schreb. is an allohexaploid (2n = 6x = 42) species which has a gametophytic self-incompatibility system controlled by two genes designated S and Z (Lundquist, 1962). Tall fescue is evolutionarily close to other *Festuca* species, including diploid and hexaploid meadow fescue (*Festuca pratensis*) (Hand *et al.*, 2010). This important crop is also member of a polyploid set of taxa, this including a tetraploid (*Festuca arundinacea* var. glaucescens Boiss syn. *Festuca arundinacea* subsp. fenas -Lag.-Arcang.), an octaploid (*Festuca arundinacea* subsp. atlantigena -St. Yves- Auquier) and a decaploid one (*Festuca arundinacea* var. letourneuxiana -St. Yves- syn. *Festuca arundinacea* subsp. cirtensis -St. Yves- J. Gamisans) (Hand *et al.*, 2010). Additionally, there are three major eco-geographic races (morphotypes) commonly described within tall fescue: Continental, Mediterranean and Rhizomatous (Hand *et al.*, 2010).

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The phylogenetic relationship among accessions from a world germplasm collection of tall fescue has been recently analyzed using the *matK* gene and rDNA ITS region (Hand *et al.*, 2012a). These robust analyses allowed classifying the accessions into the three morphotypes and showed the presence of tall fescue sub-species of varying ploidy levels, in addition to other closely related species within this germplasm collection (Hand *et al.*, 2012a). In addition, numerous molecular tools have been developed to explore the genetic diversity of these related plants (Xu *et al.*, 1994; Pasakinskiene *et al.*, 2000; Jones *et al.*, 2002;

Mian *et al.*, 2002; Momotaz *et al.*, 2004; Saha *et al.*, 2004; Lauvergeat *et al.*, 2005; Saha *et al.*, 2006; Tehrani *et al.*, 2009; Hand *et al.*, 2012a,b). Particularly, large genetic diversity of tall fescue has been observed using simple sequence repeats (SSR) and single-nucleotide polymorphism (SNP) molecular markers (Hand *et al.*, 2012a,b). In this study, we estimated the genetic diversity and ploidy level of tall fescue from the publicly available National Institute of Agricultural Technology (INTA) germplasm collection (Argentina).

The material used consisted of 133 accessions from the United States Department of Agriculture (USDA) and 28 populations collected in the Pampa Region of Argentina, an area located between 34º10' S 59º03' W and 36º11' S 62º46' W. The latter are available in the Active Germplasm Bank (AGB) at the National Institute of Agricultural Technology of Argentina. Accessions "718" and "791" were collected in Unquera, Santander, Spain (43º37' S 5º54' W) and Ortigueira, La Coruña, Spain (43º65' S 7º88' W), respectively.

For the analysis of genetic variability, genomic DNA (75 mg) was extracted from 30 young leaves of the populations (bulk), according to the methodology described by Puecher *et al.* (2001). We analyzed a set of 10 genomic SSRs from *Lolium*: LPSSRK10F08, LPSSRK10H05, LPSSRH01A07, LPSSRH07G05, LPSSRH03F03, LPSSRK02E02, LPSSRK03A02, LPSSRK03B03, LPSSRH01H06, and LPSSRK02E08 (Jones *et al.*, 2001; Forster *et al.*, 2004), and a set of 30 EST-SSRs from tall fescue: NFFa002, NFFa004, NFFa009, NFFa015, NFFa019, NFFa021, NFFa023, NFFa024, NFFa027, NFFa030, NFFa031, NFFa034, NFFa036, NFFa039, NFFa041, NFFa045, NFFa047, NFFa048, NFFa049, NFFa052, NFFa058, NFFa059, NFFa061, NFFa064, NFFa066, NFFa068, NFFa069, NFFa073, NFFa074, and NFFa075 (Saha *et al.*, 2004). PCR amplifications were performed in a final volume of 20 µL in the presence of 75 ng DNA, 1 U of *Taq* polymerase (Platinum Taq DNA Polymerase, Invitrogen), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 2 µL of 10 X PCR Buffer (Invitrogen) and 0.5 mM of each primer. The PCR protocol was: 1 cycle at 94 °C for 3 min, 40 cycles at 94 °C for 30 s, 55-65 °C (see Table 1) for 1 min, and 72 °C for 2 min. SSR fragments were detected by a Genetic Analyzer ABI 3130 (Applied Biosystems). Each PCR was performed six times.

Phylogenetic and genetic diversity analyses were done using Genemapper 3.4 (Applied Biosystems). A phylogenetic tree was constructed as previously described (Ayub *et al.*, 2007; Soto *et al.*, 2010, 2011, 2012a,b). The root tip squash method described by Ahloowalia (1965) was used to determine chromosome number. Three plants were studied with this protocol: Ryegrass (*Lolium perenne*) CV. Florida (Gentos) = 2x, *Festuca arundinacea* subsp. Fenas (Lagsca) Arcangeli Segovia- Spain (GenBank accession number: AF532951) = 4x, and *Festuca arundinacea* Encore variety (Marathon II®) = 6x. These plants were used as controls for the flow cytometry assays. The ploidy level of all accessions was determined using a flow cytometer (Partec, CA) according to Hand *et al.* (2010).

We selected 12 EST-SSRs derived from *F. arundinacea* and 3 SSRs derived from *Lolium* due to their high levels of polymorphism. The 15 SSRs selected showed 214 alleles, with band sizes of 122 bp-380 bp and multiple products per SSR (ranging from 5 to 24), at an average of 14.26 alleles per locus (Table 1). In addition, we observed high Polymorphic Information Content (PIC) values: 0.627-0.84 (Table 1). In the dendrogram, Ryegrass was used as an external control (outgroup) because this species is related to the genus *Festuca*. Ryegrass was the most divergent cluster, with a genetic distance of 1 (+ 1). As described by Hand *et al.* (2012a), the dendrogram showed an association between the accessions previously assigned to *Festuca arundinacea* var. letourneuxiana, *Festuca pratensis* and tall fescue (Continental, Mediterranean and Rhizomatous) (Figure 1).

Although using a set of SRR molecular markers different from that used by Hand *et al.* (2012a) our evolutionary analysis supports the current classification. Only few accessions were in incongruent position: Pi384873 (Iran) Continental tall fescue, Pi198088 (Morocco) *Festuca arundinacea* var letourneuxiana, Pi229947 (Iran) Continental tall fescue, Pi512315 (Spain) Rhizomatous accessions within *Festuca arundinacea* var. letourneuxiana, Continental tall fescue, Mediterranean tall fescue and Continental tall fescue groups, respectively (Figure 1). In addi-

**Table 1** - SSR marker properties following screening of 161 tall fescue accessions.

| Locus | Alleles per locus | Allele size (bp) | PIC | T (°C) |
|---|---|---|---|---|
| NNFa002 | 13 | 280-316 | 0.627 | 62 |
| NNFa015 | 17 | 143-215 | 0.729 | 60 |
| NNFa019 | 13 | 122-182 | 0.671 | 60 |
| NNFa023 | 13 | 156-207 | 0.81 | 62 |
| NNFa024 | 5 | 190-208 | 0.743 | 62 |
| NNFa031 | 16 | 220-380 | 0.709 | 65 |
| NNFa034 | 15 | 161-236 | 0.744 | 62 |
| NNFa041 | 11 | 175-210 | 0.751 | 60 |
| NNFa048 | 16 | 244-310 | 0.761 | 60 |
| NNFa058 | 18 | 85-246 | 0.764 | 60 |
| NNFa064 | 11 | 145-204 | 0.794 | 60 |
| NNFa066 | 12 | 274-329 | 0.721 | 62 |
| LPSSRK03B03 | 24 | 241-320 | 0.791 | 55 |
| LPSSRK10H05 | 16 | 200-310 | 0.7 | 55 |
| LPSSRK02E08 | 14 | 153-227 | 0.84 | 55 |

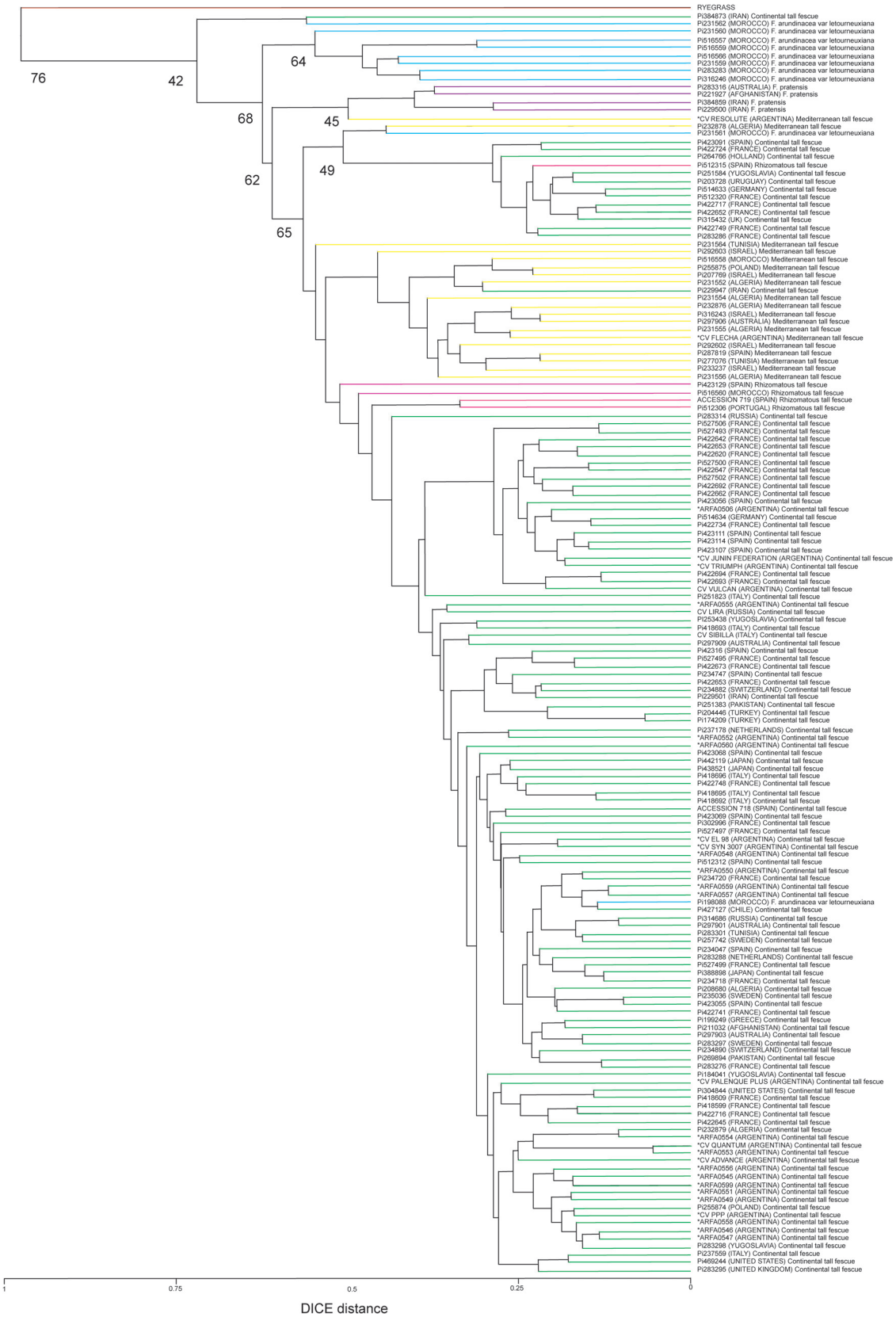


**Figure 1** - UPGMA dendrogram showing the relationships among 161 accessions of *Festuca* from different countries. Bootstrap percentages are indicated at the branch points. Tree topologies obtained using UPGMA, Neighbor joining, Minimum evolution and Maximum parsimony methods were identical. Ryegrass (red), Continental tall fescue (green), Mediterranean tall fescue (yellow), Rhizomatous tall fescue (pink), *Festuca arundinacea* var letourneuxiana (blue) and *Festuca pratensis* (violet). Accessions from INTA germplasm collection (*).

tion, CV. RESOLUTE (Argentina) Mediterranean tall fescue, Pi232878 (Algeria) Mediterranean tall fescue and Pi231561 (Morocco) *Festuca arundinacea* var. letourneuxiana, previously described by Hand *et al.* (2012a), were not found within a defined cluster (Figure 1). With the exception of CV. RESOLUTE, the accessions of tall fescue belonging to the INTA germplasm collection formed a cluster with Continental or Mediterranean tall fescue (Figure 1), suggesting that these populations can be used in breeding programs.

The genetic variation within and between populations was analyzed using 22 populations representing different clusters, and employing the 15 polymorphic SSRs listed in Table 1. We found 153 alleles (85-380 bp) with 14 alleles per locus. The DICE similarity coefficient computed for the 660 individuals (30 individuals per population) was 0.12 to 0.8. The cophenetic correlation was r = 0.75. AMOVA (Excoffier *et al.*, 1992) analysis revealed a 71% variance within populations and a 29% variance among populations, with a statistically significant variance in heterogeneity among populations ($p < 0.0001$). Our data place in evidence a large genetic diversity among and within the populations tested.

Ploidy level information is also important for biodiversity and evolutionary studies, and flow cytometry is an efficient, rapid and convenient method to estimate DNA content in plants. The flow cytometry data obtained herein were validated by chromosome counts from root tip cells. Our results suggest that the tall fescue accessions analyzed have four ploidy levels: Pi384873 (Iran) Continental tall fescue (diploid), Pi423129 (Spain) Rhizomatous tall fescue (tetraploid), *Festuca pratensis* and the three major morphotypes of tall fescue (hexaploid), and *Festuca arundinacea* var. letourneuxiana (octaploid) (Figure 2). *Festuca pratensis*, the three major morphotypes of tall fescue, and *Festuca arundinacea* var. letourneuxiana were classified as diploid, hexaploid and decaploid, respectively (Hand *et al.*, 2012a). Our results, thus, indicate that the ploidy pattern within *Festuca* accessions is more complex than expected.

The selection of *Festuca arundinacea* Schreb. varieties was intensely based on phenotypic appearance, and this breeding method is accompanied with problems, such as decreased forage and seed yield. To overcome this, a supplementary study based on molecular markers for a more precise evaluation of genetic variability at the genotype level is desireable. The estimation of genetic variation among and within populations is, therefore, a useful tool to predict potential genetic gain in breeding programs which make use of a genetic structure analysis of germplasm collections. Among DNA markers, SSRs or microsatellites, which are abundant, codominant and hypervariable, are being extensively used in genetic mapping, phylogenetic studies, and marker-assisted selection (Hand *et al.*, 2012a).

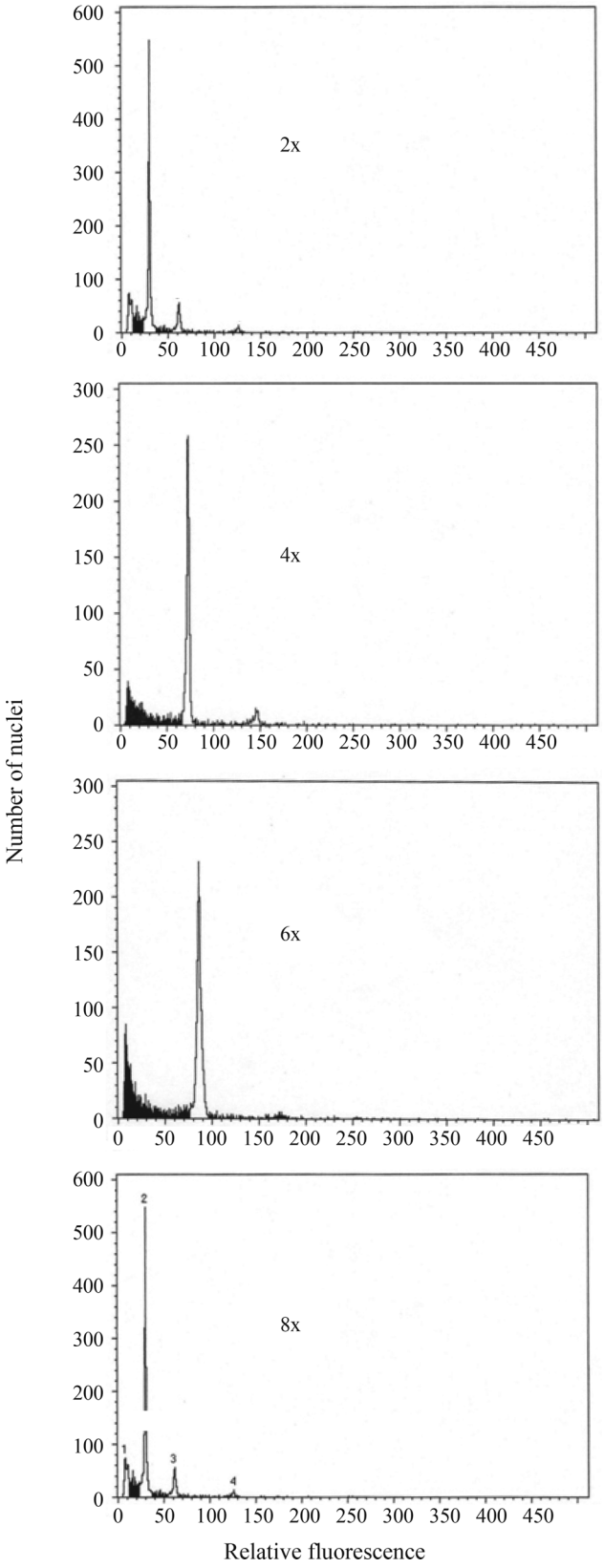


**Figure 2** - Flow cytometry fluorescence intensities of Pi384873 from Iran (2x), Pi423129 from Spain (4x), Pi221927 from Afghanistan (6x) and Pi231562 from Morocco (8x). Ploidy levels were analyzed for all accessions. The figure shows only one representative example of each ploidy level.

In this study, we used SSR markers and capillary electrophoresis as tools to assess the genetic variation and determine the relationships among different *Festuca* accessions from a wide range of geographical origins. These accessions are available in international collections (USDA and AGB), and the high quality and easily reproducible data presented in this work can be used to select diverse parents in breeding programs. They are also useful for maintaining the genetic variation in germplasm, which is crucial in utilizing the genetic potential of these genotypes for improvement of traits needed for adaptation to different conditions. In addition, the results from the flow cytometric analysis of DNA content in the *Festuca* accessions suggest that the genetic structure of fescue is more complex than previously thought. Thus, analyzing the DNA content of plants should be an easy way for creating new cultivars in this economically important forage grass. Furthermore, the recent identification and characterization of SNP markers in tall fescue morphotypes suggests that the SNP collection could be used for cultivar identification, genetic linkage map construction, genome-wide association studies and genomic selection in this important crop (Hand *et al.*, 2012b). Finally, the results shown in this study indicate that *Festuca arundinacea* Schreb. has a high level of genetic diversity within the INTA germplasm collection and, as a consequence, represents valuable material for future breeding programs.

## Acknowledgments

We thank Dr. John Foster for the information about the efficiency of cross-amplification and ortholocus detection by perennial ryegrass genomic DNA-derived SSR markers in tall fescue. In memory of Ing. Agr. Raul Rios (Buenos Aires, Argentina, 1952-2010).

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*Associate Editor: Dario Grattapaglia*