RESEARCH ARTICLE

Interspecific molecular variation of Tunisian complex *Lolium perenne* L. and *Festuca arundinacea* Schreb. based on the internal transcribed spacer locus (ITS)

Salma Ghariani¹ · Amina Charfeddine¹ · Marwen Amari¹ · Mohamed Chakroun² · Trifi-Farah Neila¹

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Abstract To establish phylogenetic relationships and estimate the intra and interspecific divergence, the amplification and the sequencing of the internal transcribed spacers of ribosomal DNA (ITS = ITS1 + 5.8S + ITS2) were analyzed in Tunisian complex *Lolium–Festuca* DNA. These spacer regions have evolved mainly by point mutations. Results revealed a high level of polymorphism within studied species. Significant similarities were observed between these two species and showed the existence of an important phylogenetic relationship. Besides, this molecular approach has revealed two new clusterings, with a homologous ITS gene namely: *Bromus hordeaceus* and *Hordeum murinum* subsp. This could be explained by the conservation of an ancestral ITS gene in some fescue plants. Thus, Tunisian tall fescue and perennial ryegrass may derive from *Bromus hordeaceus* and *Hordeum murinum* subsp. Considerable morphology and bioclimatic distribution similarities were discovered in ITS sequences within the same species. This study can be of great help to identify suitable accessions that could be used in local fescue and ryegrass improvement program.

Keywords Genetic diversity · Intra and interspecific phylogeny · ITS · Ryegrass · Tall fescue · Tunisia

Introduction

Being of high importance in the agricultural fields and exposed to degradation of Tunisian natural resources, biodiversity was subjected to several research studies (Chakroun et al. 1995; Neffati et al. 1999). Several strategies were adopted to face up this ecological menace by the conservation and the development of plant genetic resources. Perennial forages present a wide range of distribution in the world (Charmet et al. 1997; Ghariani et al. 2004). Among perennial crops, *Festuca* and *Lolium* genera (*Poaceae*) are important germplasm plants that need to be preserved and enhanced (Abdelkefi and Marrakchi 2000). These species are closely related and commonly known to be present in a complex *Festuca–Lolium* (Mallett and Orchard 2002; Wheeler et al. 2002) *Festuca arundinacea* Schreb.; i.e. tall fescue is a hexaploid species ($6n = 42$) and well adapted to abiotic stress (Sharifi Tehrani et al. 2009). *Lolium perenne* L.; i.e. perennial ryegrass is a diploid species ($2n = 14$) and known by its adequate yield of green material and good nutritional value (Ghariani et al. 2003). Both species are autogamous and present important roles in pastures, lawns, hay, feed and soil protection (Ghariani et al. 2003; Hemming et al. 1994; Slepser 1985).

In northern Tunisia, spontaneous tall fescue and perennial ryegrass are more adapted to cool seasons. Due to numerous agronomic and ecological interests, the preservation of these phytoresources is highly recommended. To reduce the genetic extinction (domestication, urbanization, overgrazing) and to improve forage production of spontaneous species, the introduction of foreign cultivars is notably recommended (Ghariani et al. 2004; Trifi-Farah et al. 2002). Collection, preservation, and improvement of genetic phytoresources are among Tunisian research priorities. Silage and grazing productivity, leave length and

✉ Salma Ghariani
salma.ghariani@fst.rnu.tn

¹ Laboratoire de Génétique Moléculaire, Immunologie et Biotechnologie, Faculté des Sciences de Tunis, Université de Tunis El Manar, 2092 Tunis, Tunisia

² Laboratoire des Productions Animales et Fourragères, Institut National de Recherche Agronomique de la Tunisie, Université de Carthage, Rue Hédi Karray, 2049 Ariana, Tunisia

tiller development, persistence, sustainability, and quality are examples of agronomic and morphological parameters that are in continual improvement (Chtourou-Ghorbel et al. 2011, 2012; Elazreg et al. 2011a; Ghariani et al. 2003).

The use of molecular approaches enlarges genetic diversity, conservation and improvement of crop productions. Several genetic markers were used to qualify *Festuca-Lolium* complex such as Simple Sequence Repeats (SSR), Amplified Fragment Length Polymorphism (AFLP), Random Amplification of Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP) (Charmet et al. 1997; Elazreg et al. 2011b; Guthridge et al. 2001; Huff 1997; Roldan-Ruiz et al. 2000; Stammers et al. 1995; Xu and Sleper 1994; Yamada and Kishida 2003). The use of nuclear rDNA polymorphism as a powerful approach to specifying either genetic diversity or phylogenetic relationships (Chennaoui et al. 2007). It is worth noting that rDNA organization presents high copies of tandem units in a single or multiple loci, offers several advantages over other parts of the genome (1) It is useful for phylogenetic studies at the intraspecific level (2) This gene family undertakes rapid concerted evolution (iii) Its detection, amplification, and sequencing are made easy due to the presence of highly conserved sequences flanking each of the two spacers (Baldwin et al. 1995; David and Michael 1991; Hamby and Zimmer 1988). In the present study, the use of nuclear rDNA ITS spacers helped to establish the phylogenetic relationships and to estimate the inter- and intra-specific divergences between the two closely related Tunisian species *Lolium perenne* L. and *Festuca arundinacea* and specifically between introduced and local cultivars.

Materials and methods

Plant material

Four spontaneous populations of Tunisian perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinacea* Schreb.) were collected. Introduced and local cultivars were also included. Ten plants were considered for each accession. Geographic origins of the studied spontaneous and cultivated populations were shown in Table 1. Seeds were collected from the North of Tunisia and conserved at 8 °C in a core-collection created in 1994 at the Institute of Agronomic Research of Tunisia (INRAT) (Chakroun et al. 1995; Cunningham et al. 1997).

DNA extraction

Total cellular DNA was extracted from fresh leaves using Qiagen kit. Obtained DNA was quantified by

spectrophotometer Gene-Quant (Pharmacia) and the quality was checked by electrophoresis on 1% agarose gel using Sambrook protocol (Sambrook et al. 1989).

Primers, ITS assays, and sequencing

Two oligonucleotides specific of the 18S and 26S ribosomal DNA conserved regions were chosen to amplify the ITS region (ITS1, 5.8S rDNA and ITS2). They correspond to ITS1 [5'-AAGGTTTCCGTAGGTGAAC-3'] for the 3' end of 18S rDNA and ITS2 [5'-TATGCTTAAACT-CAGCGGG-3'] for the 5' end of 26S rDNA (White et al. 1990).

PCR amplifications were carried out in a total volume of 25 µl including 20 ng of template DNA, 200 µM of dNTP, 1.5 U of *Taq* polymerase (Qbiogene, France), 2.5 µl of *Taq* polymerase buffer (10 ×) and 6 µM of primers of each ITS1 and ITS2. The amplification was performed in a thermocycler (Biometra Professional TRIO) according to the following program; 35 cycles of 94 °C for 45 s, 56 °C for 60 s, and 72 °C for 90 s. These cycles were preceded by a denaturation at 94 °C for 3 min and followed by a final elongation step for 10 min at 72 °C. Electrophoresis performed in 1.5% agarose gel running in a 0.5 X TBE buffer was used to display the PCR efficiency.

Amplified sequences were purified using “QIAquick PCR purification kit protocol (GmbH, Hilden, Germany) according to the manufacturing instructions. Then, the obtained PCR products were directly sequenced by a semi-automatic sequencer using the ready reaction “BigDye Terminator 3.1 cycle sequencing kit” with a fluorescent label (Genetic Analyzer 3130, Applied Biosystems).

Sequence analyses

The sequences were deposited and coded in the GenBank database (accession nos. KC478364–KC478383). The ITS nucleotide sequences were aligned and analyzed using the MEGA version 5.10 software (Tamura et al. 2011). A manual check of the aligned sequences was carried out to avoid the *taq* polymerase mismatches.

Length and GC content were estimated for each sequence. Phylogenetic analyses were conducted with the “Maximum Composite Likelihood” method (MCL) (Tamura et al. 2011). The Neighbor-joining (NJ) phylogenetic trees were drawn based on a genetic distance matrix (Saitou and Nei 1987). The transition/transversion ratio was estimated using the following formula: $R = [A \times G \times k1 + T \times C \times k2] / [(A + G) \times (T + C)]$ with A, G, C and T as the corresponding frequencies of the nucleotides (Tamura et al. 2011). The number of nucleotide substitutions/site is also given.

Table 1 The studied accessions of Tunisian perennial ryegrass and tall fescue

Species	Accessions	Ploidy	Abbreviations
<i>Festuca arundinacea</i> Schreb.	Sedjnene	6X	FSd
	Hammem Bourguiba	6X	FHb
	Menzel Temime	6X	FMt
	Boussaleme	6X	FBs
	Grombalia (Local cultivar)	6X	FcG
	Jbibina (Local cultivar)	6X	FcJ
<i>Lolium perenne</i> L.	Sedjnene	2X	LSd
	Hammem Bourguiba	2X	LHb
	Menzel Temime	2X	LMt
	Nefza	2X	LNf
	Ain Melliti (Local cultivar)	2X	LcA
	Talbot (Introduced cultivar)	2X	LcT

Genetic diversity parameters were calculated using DnaSP version 4.0 software (Rozas et al. 2003) (Table 2). The test (D) of Tajima and the Fu test (Fs) were estimated using MEGA software version 5.01 beta 8 (Fu 1997; Jukes and Cantor 1969; Nei and Tajima 1983; Tajima 1989; Tamura et al. 2011).

Results

Nucleotide composition and length variation of ITS sequences

The amplified ITS region (ITS1, ITS2, and 5.8S) exhibited an approximate length of 600 bp for perennial ryegrass and 650 bp for tall fescue. The BLAST step confirmed the authenticity and identity of each sequence.

Table 2 Evaluation of the ITS sequences polymorphism in *Festuca arundinacea* (Fa) and *Lolium perenne* (Lp)

	ITS1			5.8S			ITS2			ITS		
	Fa	Lp	Fa & Lp	Fa	Lp	Fa & Lp	Fa	Lp	Fa & Lp	Fa	Lp	Fa & Lp
Number of sequences (N)	60	60	120	60	60	120	60	60	120	60	60	120
Number of polymorphic sites (S)	65	0	66	76	1	80	37	0	28	206	1	203
Number of informative sites (Is)	43	0	45	44	0	47	23	0	18	130	0	132
Number of unique sites (Us)	22	0	21	32	1	33	14	0	10	76	1	71
Number of haplotypes (h)	9	0	10	7	2	9	8	0	8	11	2	13
Haplotype diversity (Hd)	0.879	0	0.863	0.846	0.333	0.874	0.824	0	0.832	0.956	0.333	0.926
Nucleotide diversity (π)	0.175	0	0.1516	0.09881	0.00125	0.09085	0.266	0	0.18376	0.137	0.00062	0.12467
Differences between pairs of nucleotides (k)	23.692	0	20.474	24.703	0.333	22.621	13.318	0	8.637	72.659	0.333	62.084
Tajima's (D)	0.0203	0	− 0.28089	− 0.186	− 0.933	− 0.31758	− 0.256	0	− 0.28181	0.0406	− 0.933	− 0.15872
Fu's (Fs)	3.238	0	4.715	6.754	− 0.003	6.819	2.482	0	2.807	3.941	− 0.003	6.506

All amplified sequences of both tall fescue and ryegrass showed nearly similar length and composition. The sequence length of tall fescue spontaneous populations fluctuates from 631 to 652 bp. It varies from 646 to 651 bp in spontaneous perennial ryegrass populations. A constant length (650 pb) of cultivar sequences was observed.

When examining each ITS region separately, we noticed no variation in sequence length: for ITS1 region, the size varied from 219 to 243 bp in *Festuca arundinacea* and reached about 238 bp in *Lolium perenne*; for ITS2 region, a constant mean size of 142 bp was observed in all studied populations of both species; and for 5.8S coding region a constant length of 267 bp was observed.

Almost a similar GC base content in the ITS region was observed 62% and 61% for tall fescue and ryegrass respectively. Concerning the ITS1 sequence, it showed 59.5% and 56.5% for tall fescue and ryegrass respectively. When examining the ITS2 sequence GC content, it was observed that 62.5% and 63% for tall fescue and ryegrass respectively.

Regarding the 5.8S gene, 63.3% and 62.2% GC composition were obtained for tall fescue and ryegrass respectively.

In both species, the intraspecific sequence length and GC content analysis of 5.8S RNA revealed several fluctuations in some tall fescue samples. These individuals were clustered into two groups characterized by sequence length (270 bp and 269 bp) and GC content (60.7% and 57.6%).

Genetic diversity within *Festuca arundinacea*

Mutational events, nucleotide composition and length variation of ITS sequences

Ten sequences of the six considered *Festuca arundinacea* accessions were analyzed. The multiple sequence alignment was based on a matrix of 679 nucleotides. A total number of 151 gaps were obtained and considered as missing data. These gaps were eliminated in next statistical analyses. Out of all the analyzed sites, 206 were variable. Among them, there were 130 informative sites and 76 singletons. Indeed, 11 haplotypes were observed. The haplotype (Hd) and the nucleotide (π) diversity were 0.956 and 0.137 respectively. The average differences between nucleotides pairs (K) were calculated (72.659). A positive test D of Tajima was obtained for ITS regions contrary to that obtained in the 5.8S gene and ITS2 region. Fu's test was also positive (Table 2).

The multiple alignments of the 60 ITS sequences showed that a nucleotide composition was 22.17% for Adenine (A), 19.68% for Thymine (T), 28.79% for Cytosine (C), and 29.36% for Guanine (G). Transition/transversion ratios were $K1 = 2.768$ and $K2 = 5.408$

for purine and pyrimidine bases respectively. The ratio of quotient transition/transversion (R) was 1.957 for all bases (Table 3).

Phylogenetic relationships

The 60 analyzed sequences of tall fescue showed that 46 of them presented 100% homology so only one sequence was considered. Only 14 sequences were examined. The estimated genetic distance according to the "Maximum Composite Likelihood" method ranged from 0 to 0.379 with an average of 0.189. The lowest genetic distance (0.000) was recorded among representative plants of each homology group of the same accession (FSd, FMt, FHb, FBs, FcG, and JcF). These samples had very high similarity with their ITS sequences. However, the highest distance (0.379) was recorded between Hammam Bourguiba accessions (FHb10) and those of Menzel Temime (FMT10). All the remaining individuals showed moderate genetic distances.

A clustering analysis of the 14 selected plants from a tall fescue was established. Figure 1 showed distribution of the studied accessions independently of their geographic origin. Two main groups were observed: group I included five *Festuca* plants (FSd6, FHb7, FHb8, FHb10, and FMt3) and group II contained all remaining accessions. The latter was subdivided into two subgroups: the first one (III) included individuals of homologous groups of tall fescue and showed a rapprochement between cultivated and spontaneous forms. The second subgroup (IV) included FHb6, FMt9, and FMT10. When separately analyzed, the dendrogram organization based on ITS1, 5.8S, and ITS2 sequences were compared to those obtained from the ITS gene (Data not shown).

Genetic diversity within *Lolium perenne*

Mutational events, nucleotide composition and length variation of ITS sequences

The multiple alignment sequence of the ITS region was based on the 639 nucleotides matrix. It was worth noting that 114 gaps were eliminated. Only two haplotypes were observed. The haplotype (Hd) and nucleotide (π) diversity were estimated at 0.333 and 0.00062 respectively. Similarly, the average difference between nucleotides pairs (K) was calculated (0.333) revealing a low level of polymorphism within ITS gene. A significant negative value of the test D of Tajima was obtained for the ITS region and the 5.8S gene in opposition to those obtained in ITS1 and ITS2 regions where all values were equal to zero. Negative values of the Fu's test (Fs) were also obtained (Table 2).

Table 3 Rates of transition/transversion calculated for *Lolium perenne* (Lp) and *Festuca arundinacea* (Fa)

	ITS1			5.8S			ITS2			ITS		
	Fa	Lp	Fa & Lp	Fa	Lp	Fa & Lp	Fa	Lp	Fa & Lp	Fa	Lp	Fa & Lp
K1	2.35	1	2.286	12.559	0	2.897	0.609	1	0.671	2.768	0	2.655
K2	2.73	1	2.842	14.1	0	6.74	2.929	1	34.331	5.408	0	5.419
R	1.236	0.49	1.243	6.378	0	2.25	0.863	0.49	7.673	1.957	0	1.91

K1 rate of transition/transversion for purine bases; K2 rate of transition/transversion for pyrimidine bases; R rate of the quotient transition/transversion

Fig. 1 NJ dendrogram representing tall fescue accessions based on ITS sequences. F, *Festuca* accessions; FSd, Sedjnene; FHb, Hammem Bourguiba; FMt, Menzel Temime; FBs, Boussalem; FcG, Grombalia Local cultivar; FcJ, Jbibina Local cultivar

The nucleotide composition of the whole ITS region (ITS1 + 5.8S + ITS2) showed 20.78% for Adenine (A), 18.34% for Thymine (T), 29.90% for Cytosine (C), and 30.98% for Guanine (G). Transition/transversion ratios K1, K2 and R, were equal to zero. The K1, K2, and R ratios for the ITS1 region were identical to those obtained in the ITS2 region. However, there were twice as many transversions as transitions in these regions (Table 3).

In both ITS1 and ITS2 regions, substitutions were absent. A null ratio R was obtained in the 5.8S gene and ITS (ITS1 + 5.8S + ITS2) region (Table 3).

Phylogenetic relationships

Only 6 over 60 sequences were retained. The “Maximum Composite Likelihood” genetic distance matrix (Tamura et al. 2011, 2004), showed null values among all studied accessions. The clustering analysis based on the genetic distance matrix of *Lolium perenne* ribosomal ITS gene retained sequences using Neighbor-Joining method (NJ) showed a high degree of similarity among the studied sequences (Fig. 2).

The dendrogram organization based on the ITS region has a similar structure to that based on 5.8S gene.

Fig. 2 NJ dendrogram representing perennial ryegrass accessions based on ITS sequences. L, *Lolium* accessions; LSd, Sedjnene; LHb, Hammem Bourguiba; LMt, Menzel Temime; LNF, Nefza; LcA, Ain Melliti Local cultivar; LcT, Talbot Introduced cultivar

Genetic diversity in the *Lolium*–*Festuca* complex

Mutational events, nucleotide composition and ITS sequences length variation of the complex *Lolium*–*Festuca*

Twenty fescue-*Lolium* complex sequences out of 120 were retained. Nucleotide composition analyses of the entire ITS region (ITS1 + 5.8S + ITS2) revealed 21.83% for Adenine (A), 18.99% for Thymine (T), 29.33% for Cytosine (C), and 29.86% for Guanine (G). The transition/transversion ratios for K1, K2, and R were 2.655, 5.419, and 1.91 respectively (Table 3). These values are comparable to those obtained in *Festuca arundinacea* intraspecific analyses. It is worth noting that the gaps observed in studied sequences were not considered. Particularly, the substitutions of C into T and T into C were 26.95 and 17.13 respectively (Table 4).

The multiple alignments of the 20 retained sequences were based on a matrix of 680 nucleotides from which 182 gaps were eliminated. This analysis showed the existence of 203 variable sites in the ITS region classified into 132 informative sites and 71 singleton sites.

Also, a large nucleotide variation at the intergenic ITS spacer level was detected. Indeed, 13 haplotypes were observed, of which 11 were generated by tall fescue and two by *Lolium perenne*. The Hd and π were estimated to 0.926 and 0.12467 respectively. Similarly, the average difference between nucleotides pairs K is 62.084. A

Table 4 Substitution frequencies of ITS regions in *Lolium perenne* (Lp) and *Festuca arundinacea* (Fa)

	A			T			C			G		
	Fa	Lp	Fa & Lp	Fa	Lp	Fa & Lp	Fa	Lp	Fa & Lp	Fa	Lp	Fa & Lp
A	–	–	–	3.25	9.17	3.16	4.84	15.49	4.97	13.14	0	12.97
T	3.66	10.39	3.63	–	–	–	26.19	0	26.95	4.75	14.95	4.88
C	3.66	10.39	3.63	17.56	0	17.13	–	–	–	4.75	14.95	4.88
G	10.12	0	9.69	3.25	9.17	3.16	4.84	15.49	4.97	–	–	–

negative and significant Tajima's D test and a positive Fs test of Fu were obtained (Table 2).

Genetic relationships

The matrix of “Maximum Composite Likelihood” genetic distances showed that genetic distances vary from 0 to 0.407 with an average of 0.203. We note that the genetic distances registered among individual representatives of the homology groups of both studied species indicated a high homology of sequence. However, Hammam Bourguiba tall fescue accession (FHb10) was the most divergent (0.407).

The hierarchical classification dendrogram (NJ) presented two main groups (Fig. 3). The first group I consisted of five tall fescue plants (FSd6, FHb7, FHb8, FHb10, and FMT3). When analysing group I, it was observed that FHb10 was detached from the remaining spontaneous individuals. The second group II comprised the remaining individuals and hence it was divided into two subgroups. Subgroup III was composed of only three Tall Fescue plants (FHb6, FMT9, and FMT10). Subgroup IV comprised all the remaining sequences. It is worth noting that *Lolium perenne* plants were separated from those of *Festuca arundinacea*. A similar dendrogram organization was obtained either when we analyzed the entire ITS region or ITS1, 5.8S and ITS2 regions separately (Data not shown).

When examining the sequence of the ITS2 spacer in both species, a large number of substitutions were obtained compared to the ITS1 spacer. The ITS2 region evolved 6 times faster than the ITS1, 3.4 times than the 5.8S gene and 4 times than the entire ITS gene.

The structure analysis of *Lolium* and *Festuca* populations allowed the separation of the two species except eight individuals belonging to fescue accessions (FHb, FMT, and FSd).

Unexpected results were obtained using the Blast of ITS sequences from these distinguished individuals. FSd6, FHb7, FHb8, FHb10, and FMT3 Fescue plants from group I had similar ITS sequences to those in the “*Bromus hordeaceus*” species. Also, FHb6, FMT9 and FMT10 plants

Fig. 3 NJ dendrogram representing tall fescue and perennial ryegrass accessions based on ITS sequences. L, *Lolium*; LSd, Sedjène; LHb, Hammam Bourguiba; LMT, Menzel Temime; LNF, Nefza; LcA, Ain Melliti Local cultivar; LcT, Talbot Introduced cultivar; F, *Festuca*; FSd, Sedjène; FHb, Hammam Bourguiba; FMT, Menzel Temime; FBs, Boussalem; FcG, Grombalia Local cultivar; FcJ, Jibina Local cultivar

from group III, had similar ITS sequences to those of “*Hordeum murinum subsp.*” species.

Discussion

The phylogeny study within and among spontaneous and cultivated accessions of *Festuca arundinacea* and *Lolium perenne* was performed using the ribosomal DNA intergenic transcribed spacers (ITS). Comparison of the primary structure showed that all the studied ITS regions (ITS1, 5.8S, ITS2) were typical characteristics of forage species

(GC composition, total length, ITS1 length compared to that of ITS2). The *Festuca arundinacea* amplified ITS spacers size was in agreement with previous research (Baraket et al. 2013; Cerbah et al. 1998; Chennaoui et al. 2007; Hand et al. 2010; Moody and Les 2007). The comparison among ITS1 and ITS2 spacers and 5.8S gene showed different haplotype numbers, suggesting the existence of unequal evolutionary events among the three studied regions. These results were expected since ITS2 and 5.8S genes are conserved sequences. It is worth mentioning that this gene presents a high level of conservation which can be explained by its coding nature.

In all spontaneous and cultivated populations of both species, the length of ITS1 sequences was almost twice taller than those of ITS2. These findings were confirmed by research carried out on several families of *Fabaceae*, *Poaceae*, and *Rosaceae* (Baraket et al. 2013; Campbell et al. 1995; Cerbah et al. 1998; Wojciechowski et al. 1993). A similar GC composition was observed in some eukaryotic genomes. Both ITS1 and ITS2 regions display several evolutionary origins and suggest a co-evolution mechanism (Baldwin et al. 1995; Torres et al. 1990; van der Sande et al. 1992).

In *Festuca arundinacea*, the average differences between nucleotides pairs (K) were calculated (72.659) showing a high level of polymorphism within the ITS region. A positive test D of Tajima was obtained for ITS regions showing a non-stabilizing selection contrary to that obtained in the 5.8S gene and ITS2 region. Fu's test was also positive which confirms the non-stabilizing selection.

Previous studies considering *Lolium* and fescue accessions revealed that R was lower (0.94) than that in the present study (Charmet et al. 1997). The highest values were obtained when analyzing the 5.8S region among tall fescue, showing that transitions were more abundant than transversions mainly in the substitution of C into T and G. The ITS1 spacer showed high substitution events compared to ITS2, suggesting that ITS1 evolves about 1.43 times faster. Therefore ITS1 shows less homoplastic characters. Indeed, the recorded ratio R for all sequences showed a dominance of the transitional mutations for ITS1. In the 5.8S region, transversions were more important than those in the ITS2 spacer. Frequent mutational events were recorded and showed faster evolution for the 5.8S gene compared to intergenic spacers ITS1 and ITS2. The ratio R (6.378) suggested that the 5.8S gene evolves 5 and 7 times faster than the ITS1 and ITS2 spacers respectively. The estimated genetic distance supported a considerable level of polymorphism.

When examining perennial ryegrass, a total lack of transition was noted showing the existence of a single variable site in this region. A significant nucleotide relationship at the intergenic ITS spacer level was detected. A

significant negative value of D parameter of Tajima was obtained for the ITS region and the 5.8S gene suggesting a stabilizing selection in opposition to those obtained in ITS1 and ITS2 regions where all values were equal to zero. This result showed a high conservation level. Negative values of the Fu's test (Fs) confirmed the effect of a recent mutation or a rare allele ($U_s = 1$). The 5.8S gene was a consequence of this low polymorphism rate. A strong genetic similarity between spontaneous and cultivated populations of Tunisian perennial ryegrass in the ribosomal ITS gene was noted. This can be explained by gene exchange since the studied accessions occupied very close prospection areas. These spontaneous and cultivated forms suggested the possibility of their use in agricultural exploitation. This result was confirmed by ISSR, SSR, and AFLP molecular markers where the same accessions have a non-significant divergence and can be exploited in Northern Tunisia breeding programs (Elazreg et al. 2011b; Ghariani et al. 2003). A null ratio R was obtained in the 5.8S gene and ITS (ITS1 + 5.8S + ITS2) region, implying the absence of transitions. Hence, the absence of evolution was observed. Given that the 5.8S gene is coding and naturally conserved region, a highly natural conservation of ITS spacer was also obtained.

When both species were examined, the recorded ratio R for all database showed a dominance of transitions compared to transversions in the ribosomal studied region. Thus, the percentage of similarity was highly variable. It reached 98.58% within *Lolium perenne* and 84.25% within *Festuca arundinacea*. However, the genetic similarity between the two species was rather similar to that in tall fescue (86.05%). It is worth noting that both species have a strong related evolutionary history that resulted in two independent gene pools (Jauhar 1993). The important Hd, π and K showed a high level of polymorphism within ITS spacer. These results were confirmed by a negative and significant Tajima's D test and a positive Fs test of Fu. This revealed a stabilizing selection. We note that the genetic distances registered among individual representatives homology groups of both studied species indicated a high sequence homology. This can be explained by high spacer conservation level and a strong relationship between tall fescue and ryegrass. However, the Hammam Bourguiba tall fescue accession (FHB10) was the most divergent (0.407).

It is worth noting that *Lolium perenne* plants were separated from those of *Festuca arundinacea*. Given the fact that they have adjacent prospection sites, despite the allogamous mating system, cultivated forms of these two species were closely related to spontaneous forms of the corresponding species and can exchange genes (Ghariani et al. 2004).

When examining the ITS2 spacer sequence in both species, a large number of substitutions were obtained

compared to the ITS1 spacer. This can be explained by a difference in speed evolution. The ITS2 region evolved 6 times faster than the ITS1, 3.4 times than the 5.8S gene and 4 times than the entire ITS gene. Consequently, the ITS2 spacer had fewer homoplastic characters than the remaining regions. This disproves the coevolution hypothesis of the three ITS spacer regions.

Previous results claimed that FHB6, FHB7, and FHB8 tall fescue plants were grouped with LHB2 ryegrass plant (Elazreg et al. 2011a). Being of the same region can explain the natural hybridization between the two studied species. This hypothesis was supported by specific molecular markers (SSR and AFLP) used to examine both species.

Concerning phylogenetic structure and when compared to results based on molecular markers such as SSR and RFLP (Elazreg et al. 2011b), groups were formed exclusively by individuals of the same accession.

Unexpected results were obtained using the BLAST of ITS sequences from these distinguished individuals. FSD6, FHB7, FHB8, FHB10, and FMT3 Fescue plants from group I had similar ITS sequences to those in the “*Bromus hordeaceus*” species. Also, FHB6, FMT9 and FMT10 plants from group III, had similar ITS sequences to those of “*Hordeum murinum subsp*” species. Such results can be explained by the fact that these species descended from the same common ancestor “*Bromus hordeaceus*” more probably than “*Hordeum murinum*” and they probably preserved an ancestral gene over time.

On the whole, the phylogenetic study based on ITS sequences showed very high genetic similarity between these two species indicating the existence of a strong phylogenetic relationship. However, a remarkable variability in some tall fescue plants (FSD6, FHB6, FHB7, FHB8, FHB10, FMT3, FMT9, and FMT10) was observed. This variability was independent of studied accessions geographical origin. Besides, two groups identified by BLAST showed ITS homologous gene to “*Bromus hordeaceus*” and “*Hordeum murinum subsp*”. This can be explained by conservation of an ancestral ITS gene during evolution. Tall fescue and ryegrass may be derived from “*Bromus hordeaceus*” and “*Hordeum murinum subsp*”. Previous studies using molecular markers (RFLP, SSR, and ISSR) showed that both species *Festuca arundinacea* and *Lolium perenne* were divided into two different phylogenetic branches, that is why they were considered until recently as a complex *Festuo-Lolium* (Elazreg et al. 2011a, b; Ghariani et al. 2004). This polymorphism level between tall fescue and *Lolium* identified by ribosomal DNA intergenic spacer analysis reflected the existence of an appropriate gene pool that can be used in improvement and valorization programs of these two local forage species.

Conclusion

The present study provides molecular markers reliable for genetic diversity surveying and differentiation among cultivars and spontaneous accessions. ITS sequences constitute an attractive approach to generate a useful tool for genetic diversity study. The relationship between *F. arundinacea* and *L. perenne* is difficult to deduce conclusively, however, this study demonstrates a comprehensive comparison of rDNA variation among *Lolium* and *Festuca* species that may provide some resolution to existing taxonomic uncertainties.

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