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ORIGINAL PAPER

# Molecular characterisation and interpretation of genetic diversity within globally distributed germplasm collections of tall fescue (*Festuca arundinacea* Schreb.) and meadow fescue (*F. pratensis* Huds.)

**Melanie L. Hand · Noel O. I. Cogan · John W. Forster**

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**Abstract** Allohexaploid tall fescue (*Festuca arundinacea* Schreb. syn. *Lolium arundinaceum* [Schreb.] Darbysh.) is an agriculturally important grass cultivated for pasture and turf world-wide. Genetic improvement of tall fescue could benefit from the use of non-domesticated germplasm to diversify breeding populations through the incorporation of novel and superior allele content. However, such potential germplasm must first be characterised, as three major morphotypes (Continental, Mediterranean and rhizomatous) with varying degrees of hybrid interfertility are commonly described within this species. As hexaploid tall fescue is also a member of a polyploid species complex that contains tetraploid, octoploid and decaploid taxa, it is also possible that germplasm collections may have inadvertently sampled some of these sub-species. In this study, 1,040 accessions from the publicly available United States Department of Agriculture tall fescue and meadow fescue germplasm collections were investigated. Sequence of the chloroplast genome-located *matK* gene and the nuclear ribosomal DNA internal transcribed spacer (rDNA ITS) permitted attribution of accessions to the three previously known morphotypes and also revealed the presence of tall fescue sub-species of varying ploidy levels, as well as other closely related species. The majority of accessions were, however, identified as Continental hexaploid tall fescue. Analysis using 34 simple sequence repeat markers was able to further investigate the level of genetic diversity within each hexaploid tall fescue morphotype group. At least two genetically distinct sub-groups of Continental hexaploid tall fescue were identified which are probably associated with palaeogeographic range expansion of this morphotype. This work has comprehensively characterised a large and complex germplasm collection and has identified genetically diverse accessions which may potentially contribute valuable alleles at agronomic loci for tall fescue cultivar improvement programs.

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M. L. Hand · N. O. I. Cogan · J. W. Forster (✉)
Biosciences Research Division, Department of Primary Industries, Victorian AgriBiosciences Centre, 1 Park Drive, La Trobe University Research and Development Park, Bundoora, VIC 3083, Australia
e-mail: john.forster@dpi.vic.gov.au

M. L. Hand · N. O. I. Cogan · J. W. Forster
Dairy Futures Cooperative Research Centre, Bundoora, Australia

M. L. Hand · J. W. Forster
La Trobe University, Bundoora, VIC 3086, Australia

## Introduction

The Poaceae family genus *Festuca* contains over 500 species of temperate grasses, ranging in ploidy level from diploid ($2n = 2x = 14$) to dodecaploid ($2n = 12x = 84$). The most agriculturally important of the *Festuca* species is tall fescue (*Festuca arundinacea* Schreb. syn. *Lolium arundinaceum* [Schreb.] Darbysh.), an outbreeding allohexaploid ($2n = 6x = 42$) which is cultivated world-wide for pasture and turf. Tall fescue is the predominant pasture grass grown in the United States of America, where it is cultivated on greater than 14 million hectares (Buckner et al. 1979; Thompson et al. 2001). Climatic changes are also leading to increased cultivation of tall fescue within cooler temperate

regions such as Europe and southern Australia, where the less drought tolerant species perennial ryegrass (*Lolium perenne* L.) has traditionally been preferred due to ease of grazing management and palatability (Rognli et al. 2010). The agricultural importance of tall fescue also extends to the production of intergeneric hybrids between *Festuca* and *Lolium*. A number of *Festulolium* cultivars have been generated and released by plant breeders, aiming to combine the favourable attributes of each genus (Buckner et al. 1977; Kopecký et al. 2005, 2006; Lewis et al. 1973). As with other crop species, the genetic improvement of tall fescue can potentially be achieved through use of non-domesticated germplasm to diversify breeding populations by the incorporation of novel and superior allele content. However for tall fescue, it is crucial that any such germplasm is initially thoroughly characterised, as following a complex evolutionary history, this species belongs to a group of morphologically similar taxa with differing levels of ploidy and hybrid interfertility.

Classified within the *Schedonorus* sub-genus, tall fescue is a member of a polyploid complex that consists of tetraploid [*F. arundinacea* var. *glaucescens* Boiss syn. *F. arundinacea* subsp. *fenas* (Lag.) Arcang.], octoploid [*F. arundinacea* subsp. *atlantigena* (St. Yves) Auquier] and decaploid [*F. arundinacea* var. *letourneuxiana* (St. Yves) syn. *F. arundinacea* subsp. *cirtensis* (St. Yves) J. Gamisans] taxa (Clayton and Renvoize 1986). Considerable variation also exists within hexaploid tall fescue, with three eco-geographic races (morphotypes) being commonly described within this species. The Continental morphotype is naturally distributed throughout Northern Europe and has contributed to the majority of the traditionally available cultivars, while the Mediterranean morphotype is encountered in Northern Africa and is characterised by lax leaves, incomplete summer dormancy and greater winter growth in comparison to the Continental type (Borrill et al. 1971, 1976; Reed et al. 2004; Robson 1967). Diploid meadow fescue (*F. pratensis* Huds.) and tetraploid tall fescue have previously been identified as the contemporary taxa most closely related to the progenitor species of Continental tall fescue (Humphreys et al. 1995; Pasakinskiene et al. 1998; Xu and Sleper 1994). However, the Mediterranean morphotype appears to have been generated from different, and as yet unknown, progenitor species. This inference is supported by the observation of infertility in $F_1$ hybrids between the two morphotypes (Hunt and Sleper 1981; Jadas-Hecart and Gillet 1973; Lewis 1963) as well as molecular phylogenetic analysis based on use of low copy nuclear gene sequences (Hand et al. 2010). The hypothesis of independent evolution is further supported by the observation that the two morphotypes appear to harbour distinctly different symbiotic fungal endophyte taxa (Clement et al. 2001; Schardl et al. 2007; Tsai et al. 1994).

Rhizomatous tall fescue constitutes the third recognised morphotype. Located predominantly in Portugal and parts of Spain, the rhizomatous morphotype is characterised by rhizomes that are longer and more prevalent than those of the Continental morphotype (Borrill et al. 1971; Jernstedt and Bouton 1985). This trait has established rhizomatous germplasm as the target for turfgrass breeding programs (Charrier and Stewart 2006; Stewart 1995, 1997). Although the relationships between Continental and rhizomatous morphotypes has received less attention, previous study suggests origin from the same progenitor species, but subsequent divergent evolution (Hand et al. 2010).

The United States Department of Agriculture—Agricultural Research Service (USDA-ARS) maintains genebanks for many species, including hexaploid tall fescue and diploid meadow fescue, through the Germplasm Resources Information Network (GRIN). The hexaploid tall fescue germplasm collection contains almost 1,000 accessions, for which the morphotype identity of each accession is currently unknown. This collection undoubtedly provides an important source of diverse germplasm for cultivar improvement, and phenotypic assessment of selected accessions from this collection has previously been performed with the aim of identifying germplasm capable of out-performing current varieties (Burner et al. 1988; Harris et al. 2008). Given the low level of hybrid fertility between the morphotypes, however, the ability to first discriminate between each in a simple, unambiguous manner is important for successful management of this collection, as well as for subsequent tall fescue improvement programs.

Previous work using samples from current cultivars provided the capacity to distinguish each morphotype on the basis of sequence variation in the chloroplast genome-derived *matK* gene and the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA) (Hand et al. 2010). For the purpose of dissecting the USDA tall fescue germplasm collection, DNA sequence from chloroplast DNA (cpDNA) genes can be used as a discriminatory tool, while cpDNA sequence haplotype variation within a population may also be used as a means to quantify higher-order diversity. Analysis of genetic diversity at a finer scale can be achieved through the use of polymorphic nuclear molecular genetic marker systems. Of the available marker types, simple sequence repeats (SSRs) have proven to be highly effective for the evaluation of genetic variation, as they exhibit co-dominant inheritance, multiple allelic complexity and elevated rates of molecular evolution (Vigouroux et al. 2002), which translates to detection of high polymorphism levels within and between populations. For these reasons, SSRs have continued to be the molecular marker system of choice for studies of plant genetic diversity (Balfourier et al. 2007; Blair et al. 2009; Kwak and Gepts 2009; Matsuoka et al. 2002).

This study describes an extensive molecular analysis of the tall fescue and meadow fescue USDA germplasm collections based on variation of both cpDNA and rDNA ITS sequence, as well as a set of 34 SSR molecular markers. The analysis was performed with the aim of: (1) determining the species or morphotype identity of each accession, and (2) characterising the level of genetic diversity present within the collections to identify valuable accessions for breeding programs and to enable definition of a core tall fescue germplasm collection.

## Materials and methods

### Plant material and DNA extraction

Seed was obtained from 851 tall fescue and 189 meadow fescue accessions from the USDA-ARS collection (listed in online resources 1 and 2) and genomic DNA was extracted from 6 to 10 seeds of each accession. Genotypes of *Festuca* and *Lolium* species previously sampled for phylogenetic analysis were used as reference material for the identification of diagnostic nucleotides within the *matK* gene and ITS region (Hand et al. 2010). For these reference genotypes, DNA extracted from leaf material of a single accession was used. Single seeds of accessions selected for the diversity analysis were subsequently grown in a greenhouse for 50 days. Young leaf of a single plant from each accession was harvested and DNA was extracted for the purpose of SSR marker amplification. All DNA extractions were performed using the DNeasy 96 Plant Kit (Qiagen). Cultivar materials of hexaploid tall fescue (all morphotypes), perennial ryegrass (*Lolium perenne*), Italian ryegrass (*Lolium multiflorum* Lam.) and *Festulolium* were also included for SSR amplification. Details of these cultivars are provided in online resource 3.

### Amplification and sequencing of *matK* gene and ITS regions

The cpDNA *matK* gene was amplified, purified and sequenced from each accession as described previously (Hand et al. 2010), except that the primer PO-matK860F (Schneider et al. 2009) was used for sequencing of each amplicon. Further sequencing based on use of the S5-1F amplification primer was then performed to discriminate between accessions initially identified as either *F. mairei*, octoploid tall fescue or decaploid tall fescue. The cpDNA *trnL-F* region was amplified using the trnLc and trnFf primers (Taberlet et al. 1991) following the same protocol as for *matK* amplification, and both the forward and reverse amplification primers were used to sequence the resulting PCR product. The nuclear rDNA ITS region was amplified from those accessions for which attribution to Continental hexaploid tall fescue, rhizomatous hexaploid tall fescue or tetraploid tall fescue could not be unambiguously achieved on the basis of *matK* haplotype. Amplicons were generated as previously described and sequenced using the ITS3 primer (Hand et al. 2010). For those accessions from which fungal rDNA ITS amplification contaminated the sequence, amplification was re-attempted using a primer designed to anneal only to plant-specific targets (GrassITSf = 5′-AGG CTATCGCTTTGGCTACG-3′), in conjunction with the ITSL amplification primer. The plant-specific primer was designed from a 5′-region of the ITS1, in which divergence between plant and fungal sequences was identified. Reaction conditions remained otherwise unchanged and the amplification products were sequenced using the GrassITSf primer. For both the *matK* gene and ITS regions, sequences were aligned using Sequencher 4.7 (Gene Codes), and diagnostic nucleotide variants were scored manually. Geographical distribution of each accession was subsequently mapped using DIVA-GIS v5.2 (Hijmans et al. 2001). If precise longitude and latitude coordinates were not listed for a particular accession, they were estimated based on the provided description of the sampling location. Accessions with a collection location outside the natural distribution range of Europe, Asia and Northern Africa were not included, as they are likely to represent human introductions during recent history.

### SSR genotyping

All accessions within the germplasm collection that were identified as either hexaploid tall fescue (all morphotypes) or meadow fescue from the sequencing of the *matK* gene and ITS region were selected for SSR genotyping. However, only those accessions which produced viable germinants were ultimately used (online resources 1 and 2). A collection of 34 SSR primer pairs designed from perennial ryegrass genomic DNA (Jones et al. 2001) or expressed sequence tag (EST) template sequences (online resource 4) were chosen to perform diversity analysis based upon proven single locus amplification in perennial ryegrass (Wang et al. 2009) and capacity for cross-species amplification in tall fescue. Amplification was performed using 5 ng template genomic DNA extracted from leaf material of a single plant for each cultivar or accession and followed the protocol of Wang et al. (2009) with the modification of reaction volume (6 µl). Cycling conditions included an enzyme activation step of 95°C for 15 min, followed by 30 cycles of 95°C for 1 min, initial annealing temperature for 30 s, 72°C for 1 min with the annealing temperature decreasing 1°C per cycle until the final temperature was attained and a final extension step of 72°C for 10 min. Annealing temperatures differed for each SSR and initial

and final temperatures are detailed in online resource 4. Amplification products were diluted 40-fold with addition of water, and single products labelled with each of the three dyes were pooled. Aliquots of 2 µl from each pool was combined with 8.95 µl of Hi-Di™ formamide (Applied Biosystems) and 0.05 µl of GeneScan™ 500 LIZ® Size Standard (Applied Biosystems) and electrophoresis was performed on the ABI3730xl automated capillary electrophoresis platform (Applied Biosystems).

### Analysis of genetic diversity

SSR alleles were scored using GeneMapper v3.7 (Applied Biosystems) software. Effective performance was evaluated for each marker, and amplification was deemed successful within a given species/morphotype when genotypic data could be recovered from >50% of the samples. All subsequent analyses were performed using only those samples with <10% missing data. As individual alleles are unable to be confidently attributed to a specific sub-genome within the polyploid samples, alleles from all samples were scored in a dominant manner and converted into a binary (presence/absence) matrix. The polymorphism information content of each SSR marker was calculated as a measure of heterozygosity (Nei 1973) within each locus and was calculated as described by George et al. (2006).

A genetic dissimilarity matrix was constructed using the Dice and Jaccard coefficients, both of which consider only presence of alleles rather than shared absence between any two individuals, hence reducing the likelihood of overestimated genetic similarity. The Dice coefficient places emphasis upon alleles shared between two individuals rather than the presence of alleles within one individual or another, while the shared alleles are equally weighted when the Jaccard coefficient is used. As the SSR alleles were scored in a dominant manner, no particular similarity coefficient was considered more appropriate than another for this analysis (Kosman and Leonard 2005), and hence the results of both coefficients were analysed. Construction of the genetic dissimilarity matrix and visualisation via an unweighted neighbour-joining radial tree was performed using the DARwin5 program (Perrier et al. 2003).

The level of genetic diversity within each hexaploid tall fescue morphotype was calculated using data scored from the 28 SSR loci that were successfully amplified from across all three morphotypes. Genetic diversity was measured based on the number of derived alleles observed within each population. Genetic structure within the sub-set of accessions identified as belonging to the Continental and rhizomatous hexaploid tall fescue morphotypes was further explored through Bayesian clustering analysis performed using Structure 2.3.3 (Pritchard et al. 2000). This program has the capacity to accommodate co-dominant marker data from polyploid species through recognition of the property that the copy number of each allele is ambiguous. The number of sub-populations which bests fits the SSR allele variability ($K$) is established, and each individual within the analysis is subsequently assigned to a sub-population. The optimal value was determined after evaluation of $K$ values from 1 to 20, with 10,000 Markov chain Monte Carlo repetitions following a burn-in of 10,000 cycles. An ancestry model of admixture was used, and allele frequencies were assumed to be correlated. Both the ad hoc method (Pritchard et al. 2000) and second order statistics (Evanno et al. 2005) were used to determine the optimal value of $K$.

## Results

### Identification of diagnostic nucleotides within the *matK* gene and ITS region

Sequencing of *matK* amplification products with the S5-1F and PO-matK860F primers yielded sequence reads of c. 880 and 719 nucleotides in length, respectively, and revealed a total of 11 variable base positions that can be used as diagnostic nucleotides to discriminate between each of the reference groupings (Fig. 1a). No SNPs capable of discriminating Continental hexaploid tall fescue from either rhizomatous hexaploid tall fescue or tetraploid tall fescue were identified within the *mat*K gene. A total of 12 diagnostic nucleotides capable of distinguishing these three reference groups were identified by sequencing the ITS region through use of the ITS3 primer (Fig. 1b), which generated a sequence read of c. 307 nucleotides in length. Sequencing of the ITS region with the grass-specific primer yielded sequence reads of c. 590 nucleotides in length, 17 positions being diagnostic for either the Continental or rhizomatous hexaploid tall fescue morphotypes (Fig. 1c).

### Classification of GRIN accessions using the *matK* gene and ITS region

The *matK* gene was successfully amplified and partially sequenced from all accessions included in the study. Of these, 110 accessions were further classified following sequencing of the *matK* gene with the S51F primer, while the ITS region was then amplified and sequenced from 652 accessions initially identified as either Continental hexaploid tall fescue, rhizomatous hexaploid tall fescue or tetraploid tall fescue. Using the previously determined diagnostic nucleotides (Fig. 1), the majority of accessions (71%) within the tall fescue collection were identified as belonging to the Continental morphotype, with a lesser proportion (8%) attributed to the Mediterranean morphotype. The remaining accessions were attributed to either

**A**

| Classification | Ploidy | GenBank ID | 33 | 120 | 1051 | 1062 | 1098 | 1106 | 1193 | 1207 | 1228 | 1454 | 1470 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *F. arundinacea* - Continental morphotype | 6x | HM453052 | C | C | A | T | G | T | C | T | G | T | G |
| *F. arundinacea* - Mediterranean morphotype | 6x | HM453057 | C | C | C | G | A | T | C | G | G | T | G |
| *F. arundinacea* - Rhizomatous morphotype | 6x | HM453065 | C | C | A | T | G | T | C | T | G | T | G |
| *F. pratensis* | 2x | HM453059 | C | C | C | T | G | T | C | T | G | T | A |
| *F. pratensis* subsp. *apennina* | 4x | HM453060 | C | C | C | T | G | T | C | T | A | T | A |
| *F. arundinacea* var. *glaucescens* | 4x | HM453051 | C | C | A | T | G | T | C | T | G | T | G |
| *F.arundinacea* subsp. *atlantigena* | 8x | HM453054 | T | C | C | G | A | T | C | T | G | T | G |
| *F.arundinacea* subsp. *letourneuxiana* | 10x | HM453055 | C | C | C | G | A | T | C | T | G | T | G |
| *F. mairei* | 4x | HM453072 | C | T | C | G | A | T | C | T | G | T | G |
| *F. gigantea* | 6x | HM453058 | C | C | C | T | G | T | T | T | G | T | A |
| Fine leaved fescues (*Festuca* subgenus) | 2x | HM453067 | C | C | C | G | A | C | C | T | G | T | G |
| *Lolium* genus | 2x | HM453061 | C | C | C | T | G | T | C | T | G | G | A |

**B**

| Classification | Ploidy | GenBank ID | 377 | 391 | 405 | 425 | 426 | 428 | 447 | 458 | 540 | 542 | 548 | 574 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *F. arundinacea* - Continental morphotype | 6x | HM453174 | C | C | G | T | C | T | G | C | G | C | G | T |
| *F. arundinacea* - Rhizomatous morphotype | 6x | HM453187 | A | A | T | T | A | C | T | T | A | A | A | C |
| *F. arundinacea* var. *glaucescens* | 4x | HM453192 | A | A | T | C | A | C | T | C | A | A | A | C |

**C**

| Classification | Ploidy | GenBank ID | 12 | 25 | 32 | 34 | 37 | 57 | 59 | 72 | 87 | 88 | 91 | 159 | 160 | 166 | 168 | 171 | 182 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *F. arundinacea* - Continental morphotype | 6x | HM453174 | C | A | T | G | C | C | T | G | A | T | T | G | A | T | T | C | G |
| *F. arundinacea* - Rhizomatous morphotype | 6x | HM453187 | A | C | C | A | T | T | C | T | C | C | C | C | C | C | C | T | A |
| *F. arundinacea* var. *glaucescens* | 4x | HM453192 | A | C | C | A | T | T | C | T | C | C | C | C | C | C | C | T | A |

**Fig. 1** Diagnostic nucleotides identified within the *matK* gene (**a**) and ITS region (**b**, **c**) that were used for the classification of each accession within the germplasm collections. The full length reference sequence of each tall fescue morphotype or species were deposited in GenBank following a prior study (Hand et al. 2010). The base position of each diagnostic nucleotide refers to these sequences

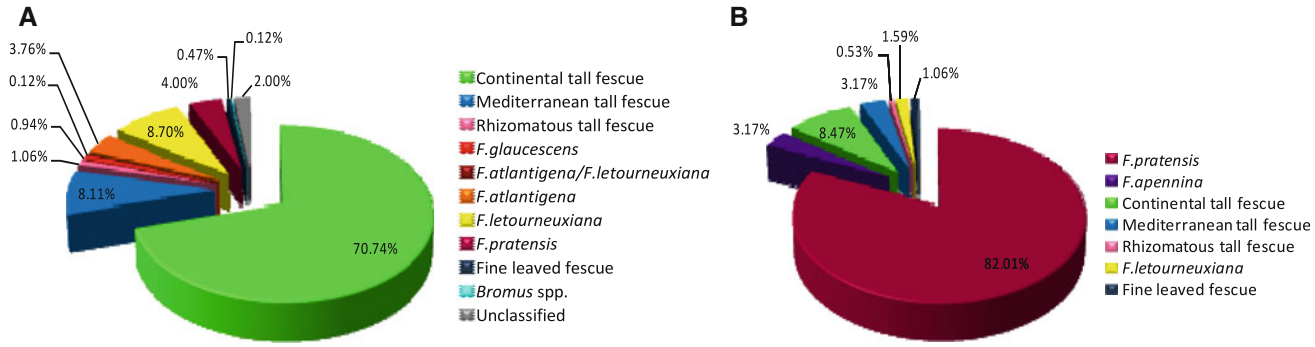


**Fig. 2** The proportion of each tall fescue morphotype and other species identified within the USDA tall fescue (**a**) and meadow fescue (**b**) germplasm collections

rhizomatous hexaploid tall fescue, tetraploid tall fescue, meadow fescue, octoploid tall fescue, decaploid tall fescue or fine-leaved *Festuca* species (Fig. 2a). Conclusive classifications failed to be achieved using any of these methods for 17 accessions which were consequently excluded from further analysis. A single accession produced a *matK* sequence haplotype that failed to match any of the reference groups. In an attempt to identify this species, the sequence was used to query the GenBank non-redundant database in which perfect identity ($E$ value = 0) was detected with the *mat*K gene region of the grass species *Bromus erectus* (GenBank accession AM234570). The meadow fescue GRIN collection largely contains diploid meadow fescue (82%), although representatives of the tetraploid meadow fescue (*F. pratensis* subsp. *apennina*) were also identified (3%). The remaining accessions within this collection were attributed to either Continental, Mediterranean or rhizomatous hexaploid tall fescue, decaploid tall fescue or members of fine-leaved *Festuca* species (Fig. 2b). Details of the final classification for each accession are listed in online resources 1 and 2.

## Sequence haplotype analysis

Following classification of each accession according to previously defined diagnostic nucleotide variants, the diversity of *matK* and ITS sequence haplotypes present within each population of species/morphotype was investigated. For *matK*, haplotype variation was only observed within hexaploid Continental tall fescue, for which two haplotypes were identified with a single nucleotide differentiating the major (95% of samples) from the minor (5% of samples) class. Given this observation, cpDNA variation within Continental hexaploid tall fescue was further investigated by amplification and sequencing of the *trnL-F* region from 12 accessions each from the two *matK* haplotype classes. No haplotype variation was observed within this sub-set (data not shown), and so sequence analysis of the *trnL-F* region was not pursued further. Ambiguous bases that were identified from mixed sequence reads complicated the haplotype analysis of the sequenced ITS regions. However, a single alternative haplotype, which more closely resembled that of a *Lolium* species, was identified from the Continental

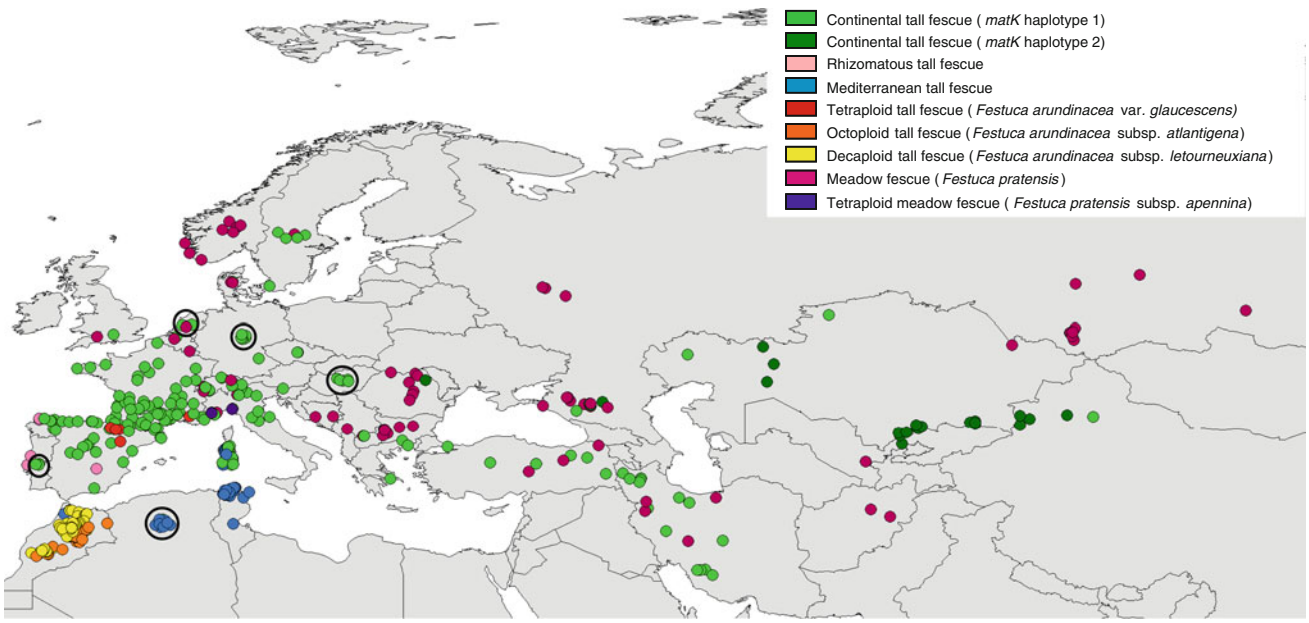


**Fig. 3** Map showing the collection locations of each accession analysed in this study. Those accessions with an attributed country of origin, but no specific collection location are grouped within *circles*. Continental 2 (*dark green*) represents the alternate minor *matK* haplotype

hexaploid tall fescue accession PI 235125. No further haplotype variation was identified.

## Geographical distributions of species

The distributions of each tall fescue morphotype, as well as other species represented within the germplasm collections, were analysed through mapping of the collection site for each accession (Fig. 3). Definite geographical boundaries were apparent between the Continental and Mediterranean hexaploid tall fescue morphotypes, as almost all Mediterranean accessions were derived from Northern Africa, (principally Algeria and Tunisia), while the Continental germplasm was distributed throughout Europe and Asia. The only zone of geographical overlap between the two morphotypes occurs on the island of Sardinia. All accessions of the rhizomatous hexaploid tall fescue morphotype were collected from the Iberian Peninsula (either Spain or Portugal) and share a distribution range with the Continental morphotype. Of the remaining species identified, the octoploid and decaploid tall fescue accessions are both localised to Morocco, and the tetraploid tall fescue accessions were collected mostly in Spain, with a single accession obtained from France. The distribution of the meadow fescue accessions is broad, and similar in range to that of the Continental hexaploid tall fescue morphotype, while the limited number of tetraploid meadow fescue cytotypes present were all collected from northern Italy. Within Continental hexaploid tall fescue accessions, geographic differentiation corresponding to the two chloroplast DNA haplotypes was evident; those accessions possessing the minor haplotype being derived from the eastern extreme of the distribution range (predominantly within Russia, Kazakhstan and northern China).

## Analysis of population structure

Of the 34 SSR primer pairs employed, 20 obtained successful amplification from all of the hexaploid tall fescue morphotypes and other species included in this study (Table 1). Using allele scores from these 20 markers, a phenetic tree was constructed to visualise relationships between the hexaploid tall fescue morphotypes, as well as their respective relationships to meadow fescue and the ryegrass species. Differences between trees generated from use of either the Dice or Jaccard coefficients were considered negligible, both trees displaying the same major groupings. The Dice coefficient-based phenogram was hence used for further analysis (Fig. 4). The Jaccard coefficient-based phenogram is provided as online resource 5.

Four distinctive groups were identified, representing Continental hexaploid tall fescue, Mediterranean hexaploid tall fescue, meadow fescue and the ryegrass species. The group of Continental hexaploid tall fescue accessions is composed of a number of sub-groups, one of which largely, but not exclusively, contains those accessions with the minor *matK* haplotype. Conversely, the largest sub-group is dominated by accessions with the major *matK* sequence haplotype. Smaller sub-groups and individual hexaploid Continental tall fescue accessions are also located as

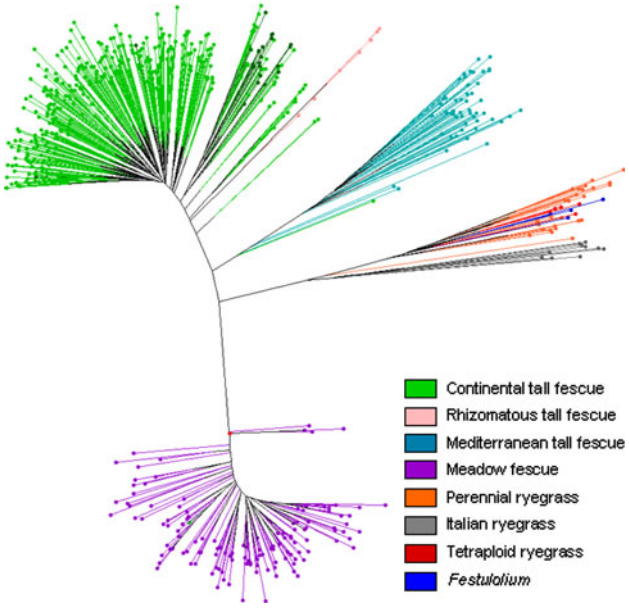


**Fig. 4** Radial unweighted neighbour-joining tree constructed using allelic phenotypes of 595 individual samples. Allele scores are based on data from 20 SSR markers. The tree is based on a dissimilarity matrix formed using the Dice coefficient

outliers to this main group of accessions. SSR polymorphism revealed limited diversity within seven accessions of the rhizomatous morphotype, which all form a single tight cluster that appears closely related to Continental hexaploid tall fescue accessions, particularly those from Spain and France. The phenogram further depicts the Mediterranean morphotype, which forms a single distinct group, as genetically distinct from the other two hexaploid tall fescue morphotypes. Within the ryegrass grouping, the perennial and Italian ryegrasses are closely related, although the two species may still be differentiated. The *Festulolium* cultivar that was included in the analysis is closely related to the perennial ryegrass cultivars, as are the tetraploid perennial ryegrass cultivars, demonstrating the negligible effect of artificial ploidy changes on this form of genetic similarity analysis. Inconsistent attributions were obvious for two accessions, including a Continental accession (PI 315430) which clusters with the meadow fescue group, and a Continental accession (PI 235125) which clusters with the group of Mediterranean accessions. The degree of incongruity of the latter was, however, reduced in analysis based on use of the Jaccard coefficient (online resource 5). Sequencing of the *matK* gene region from these two individual plants revealed identity with meadow fescue for accession PI 315430 (data not shown), suggesting derivation from a collection of inadvertently mixed seed. Identity with Continental hexaploid tall fescue was, however, confirmed for the individual from accession PI 235125 (data not shown).

A similar level of genetic diversity was observed in the Continental and Mediterranean hexaploid tall fescue groups, as measured by the number of observed alleles (Table 2). Lower diversity was apparent within the rhizomatous hexaploid tall fescue population, but a genuine comparison is difficult due to the discrepancies of population sizes between groups. Genetic diversity and the relationships of accessions within the Continental and rhizomatous hexaploid tall fescue groups was further analysed through use of the program Structure, based on allelic data from the 31 SSR markers that were successfully amplified from both of these morphotype groups. Both the ad hoc methodology and the second order statistics suggested that two sub-groups were present within this dataset (online resource 6). Accessions were classified based on the given inferred ancestry, when >50% of the genome was arbitrarily considered sufficient for classification into either of the two sub-groups. Using this method, the major sub-group to be identified consisted of 338 accessions, and the minor group contained 62 accessions (Fig. 5). The minor sub-group contained all seven rhizomatous accessions, and all but one of the Continental accessions that possessed the minor *matK* haplotype. Some geographical differentiation was also apparent, the majority of those accessions collected from Kazakhstan, Iran and Turkey belonging to the minor sub-group.

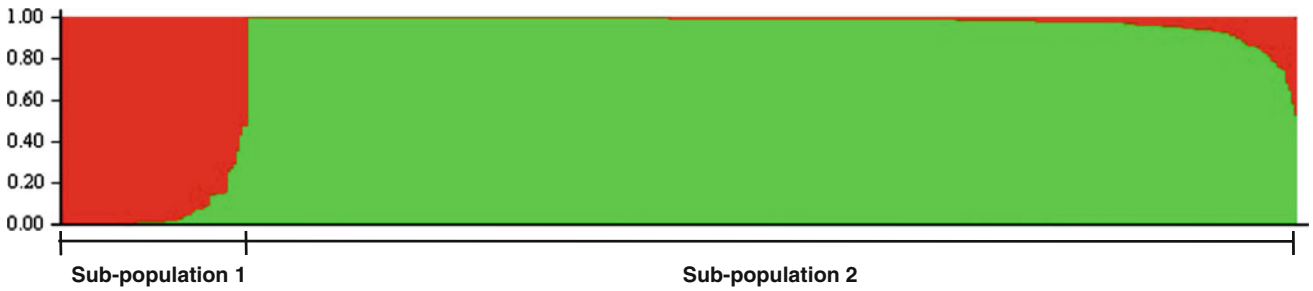


**Fig. 5** Population structure of Continental tall fescue as determined by the program Structure. The *bar plot* details the 421 individuals included in the analysis (*x* axis) and the genome proportion attributed to either of the two identified sub-populations (*y* axis)

**Table 1** Properties of the SSR markers used in this study

| Marker | Subset amplified | PIC | Total no. of alleles |
|---|---|---|---|
| LPSSRH01H06 | TFc, TFr, TFm, MF, RG | 0.79 | 20 |
| LPSSRH02C11 | TFc, TFr, TFm, RG | 0.87 | 40 |
| LPSSRH02H04 | TFc, TFr, TFm, MF, RG | 0.73 | 23 |
| LPSSRH02H05 | TFm, MF, RG | 0.74 | 15 |
| LPSSRH04F12 | TFc, TFr, MF, RG | 0.76 | 39 |
| LPSSRH05D11 | TFc, TFr, TFm, MF, RG | 0.88 | 24 |
| LPSSRH05F02 | TFc, TFr, TFm, MF, RG | 0.79 | 24 |
| LPSSRK01A09 | TFc, TFr, TFm, MF, RG | 0.76 | 28 |
| LPSSRK03A02 | TFc, TFr, TFm, MF, RG | 0.58 | 22 |
| LPSSRK03B03 | TFc, TFr, TFm, MF, RG | 0.93 | 56 |
| LPSSRK03G05 | TFc, TFr, TFm, MF, RG | 0.84 | 11 |
| LPSSRK07C11 | TFc, TFr, TFm, MF, RG | 0.71 | 20 |
| LPSSRK07D12 | TFc, TFr, TFm, MF, RG | 0.66 | 15 |
| LPSSRK07F08 | TFc, TFr, TFm, MF, RG | 0.55 | 5 |
| LPSSRK07H08 | TFc, TFr, TFm, RG | 0.81 | 18 |
| LPSSRK09G12 | TFc, TFr, TFm, RG | 0.67 | 12 |
| LPSSRK10B07 | TFc, TFr, TFm, RG | 0.89 | 30 |
| LPSSRK10B10 | TFc, TFr, TFm, MF, RG | 0.06 | 5 |
| LPSSRK11G12 | TFm, MF, RG | 0.77 | 9 |
| LPSSRK12B02 | TFc, TFr, TFm, MF, RG | 0.68 | 25 |
| LPSSRK12D11 | TFc, TFr, TFm, MF, RG | 0.76 | 15 |
| LPSSRK14F07 | TFc, TFr, TFm, RG | 0.85 | 17 |
| LPSSRK14F12 | TFm, RG | 0.86 | 20 |
| LPSSRK15A07 | TFc, TFr, TFm, RG | 0.44 | 12 |
| pps0040 | TFc, TFr, TFm, MF, RG | 0.42 | 17 |
| pps0049 | TFc, TFr, TFm, MF, RG | 0.85 | 20 |
| pps0122 | TFc, TFr, TFm, MF, RG | 0.87 | 25 |
| pps0164 | TFc, TFr, TFm, MF, RG | 0.82 | 31 |
| pps0187 | TFc, TFr, TFm, RG | 0.78 | 15 |
| pps0211 | TFc, TFr, TFm, MF, RG | 0.10 | 7 |
| pps0255 | TFc, TFr, MF, RG | 0.28 | 11 |
| pps0447 | TFc, TFr, TFm, MF, RG | 0.48 | 12 |
| pps0463 | TFc, TFr, TFm, RG | 0.51 | 8 |
| pps0473 | TFc, TFr, RG | 0.17 | 6 |

*TFc* Continental tall fescue morphotype, *TFr* rhizomatous tall fescue morphotype, *TFm* Mediterranean tall fescue morphotype, *MF* meadow fescue, *RG* ryegrass (perennial ryegrass and Italian ryegrass)

**Table 2** Basic measures of intra-population diversity for each tall fescue morphotype

| | No. of samples | Alleles seen across all loci | Average alleles per individual |
|---|---|---|---|
| Continental tall fescue | 455 | 351 | 12.54 |
| Mediterranean tall fescue | 75 | 316 | 11.29 |
| Rhizomatous tall fescue | 9 | 127 | 4.54 |

## Discussion

### Composition of the tall fescue and meadow fescue germplasm collections

This study describes the investigation of a comprehensive collection of tall fescue germplasm and has succeeded in classifying each accession with respect to a species, subspecies or tall fescue morphotype. The extent of species and sub-species variation that was identified within both the tall fescue and meadow fescue germplasm collections demonstrates the close, complex relationships between broad-leaved *Festuca* species, given that each accession was initially classified on the basis of phenotype as one of only two species (meadow fescue and hexaploid tall fescue). Classification of the majority of hexaploid tall fescue accessions as belonging to the Continental morphotype was unsurprising, given the widespread distribution of this taxon (Borrill et al. 1971, 1976) and the history of domestication into the most commonly cultivated tall fescue varieties. Although ITS sequence variation clearly identified rhizomatous hexaploid tall fescue as a genetically distinct third morphotype, only a limited number of accessions were attributed to this class. Nonetheless, these accessions will provide valuable sources of novel germplasm for tall fescue breeding programs and in particular, for turf-type applications. Although the initial aim of the screening process was to classify each accession as belonging to one of the three hexaploid tall fescue morphotypes, the presence of other *Festuca* species as contaminants fully justified the process of species-identity verification prior to the SSR diversity analysis. This process of classification represents an important initial step in characterising this germplasm collection to enable the development of a core collection of tall fescue germplasm.

Mapping the collection location of each accession has also provided further support for the correct classification of each accession, as the findings resemble those of Borrill et al. (1971, 1976) who studied the geographical distribution of *Festuca* species. Borrill et al. (1971) classified each collected sample using a combination of cytological and morphological criteria and clearly identified the three hexaploid tall fescue morphotypes and their distinct geographical boundaries, which correspond to the distributions observed in this study. The shared distribution of Continental and Mediterranean hexaploid tall fescue upon the island of Sardinia, as noted in this study, has also been previously observed, following analysis of the resident fungal endophyte within 60 natural Sardinian tall fescue populations (Piano et al. 2005).

Clustering based on SSR polymorphisms also supports the initial classification of each accession through clear

separation of all Mediterranean individuals from Continental tall fescue. However, the anomalous accession PI 235125 provided an exception to this rule. Clustering of this accession as an outlier to the major Mediterranean population, along with conflicting *matK* and ITS sequence-based classification, creates uncertainty over its origin and breeding history. Little relevant information is available, but the GRIN listing describes a wild population collected from near Wageningen in the Netherlands in 1956. There is a long history of agriculturally focused plant breeding in this location, and in particular, that of producing *Lolium* × *Festuca* hybrids (Dijkstra and Vos 1975a, b; Wit 1959). A *Festulolium* origin of this accession could account for the *matK* and ITS sequencing results, and possibly explain the unusual allelic composition revealed by the SSR markers. To test this hypothesis, the genomic constitution of the accession could be investigated further through the use of marker arrays designed from *Festuca* and *Lolium* species, as designed by Kopecký et al. (2009).

## Genetic diversity within tall fescue morphotype groups

This study also aimed to assess the level of genetic diversity within each identified morphotype group and to identify potentially novel germplasm for breeding purposes. As anticipated, the SSR marker-derived amplicons designed from perennial ryegrass templates were able to be efficiently amplified from tall fescue samples. The two species are closely related in evolutionary terms (Charmet et al. 1997; Darbyshire 1993; Inda et al. 2008) and successful cross-amplification of SSR markers between the two species has previously been observed (Jones et al. 2001; Saha et al. 2004, 2006). When using SSR markers to estimate diversity, it is important to acknowledge the possibility of size homoplasy, where multiple alleles of the same molecular weight are not distinguished (Estoup et al. 2002). Alleles of identical size are not necessarily identical due to common descent and may represent hidden convergent mutation events. Sequencing of the SSR locus can reveal identical allele sizes resulting from the same number of repeat motifs, as well as indels and other mutations within or flanking the repeat region (Barkley et al. 2009; Symonds and Lloyd 2003; Xie et al. 2006). These effects, however, are likely to be most concerning for intra-specific estimates of diversity. Within this study, SSR markers were used to analyse the diversity of each hexaploid tall fescue morphotype, where all individuals within a morphotype group likely to have evolved from common ancestors. Nevertheless, it is still possible that size homoplasy has reduced the estimates of genetic diversity observed here.

Variation within the Continental tall fescue morphotype was readily apparent due to identification of multiple *matK* haplotypes as well as SSR analysis which identified at least two sub-groups within the studied accessions. This diversity was also associated with patterns of geographical distribution, such that the minor sub-group may represent populations of tall fescue that have subsequently expanded eastwards from the proposed geographic origin of the Continental morphotype. The greater incidence of Continental hexaploid tall fescue within southern France and Spain observed here and in prior studies support this area as a probable site of origin for the morphotype. Furthermore, the two putative progenitor species (meadow fescue and tetraploid tall fescue) overlap in distribution in this region (Borrill et al. 1971). The range expansion from this point of origin may have contributed to the increase in diversity observed for both *matK* haplotypes and the nuclear SSR marker loci, due to adaptation to newly encountered conditions and barriers to gene flow, particularly in mountainous regions. Alternatively, it is possible that genetic variation within Continental hexaploid tall fescue may be the result of multiple hybridization events. Geographically structured cpDNA variation has also been reported for meadow fescue (Fjellheim et al. 2006), for which where western European, eastern European/Asian and isolated Caucasian haplotypes were observed. These authors proposed that a glacial refugium within the region between the Black Sea and Caspian Sea was responsible for the evolution of the Caucasian meadow fescue haplotype. It is possible that the unique Iranian and central Asian hexaploid tall fescues arose following hybridization between the Caucasian meadow fescue and tetraploid tall fescue. Divergence between central European and Asian tall fescue populations has previously been reported following the analysis of a number of phenotypic traits including water soluble carbohydrate concentration, yield and disease resistance (Burner et al. 1988). Particular attention has also been paid to Iranian tall fescue populations, which have been described as highly distinct from accessions of other geographical regions, and have been the subject of multiple genetic diversity studies (Majidi and Mirlohi 2010; Sharifi Tehrani et al. 2009). These diversity results suggest that tall fescue accessions from this minor sub-group may provide novel and desirable alleles to Continental hexaploid tall fescue breeding programs, particularly in terms of adaptation to climatic and edaphic conditions that are not typical of Western Europe.

The presence of genetically diverse sub-groups within the Mediterranean morphotype accessions is less apparent, possibly reflecting the lower representation of this morphotype within the collection or, alternatively, a relatively more recent origin. Accurate comparisons of genetic diversity between the Continental and Mediterranean tall fescue accessions are also complicated by the hexaploid genome architecture, and also by the varying sizes of candidate morphotype groups. However, using ad hoc measures of diversity, a similar level of genetic diversity was revealed

within the Continental and Mediterranean morphotypes. Also, the unique genotype-derived phenotypes observed from accessions of both morphotypes are consistent with large amounts of intra-population variation, as expected for an obligate outbreeding plant species.

Effective use of this rich structure within the GRIN tall fescue collection for varietal improvement relies upon the characterisation of each accession. This process will further allow reduction into a more manageable core collection. From this study, such core collections may now be established for each hexaploid tall fescue morphotype, which is crucial for targeted crossing programs given the observed level of hybrid interfertility between specific morphotypes. Furthermore, the diverse sub-groups identified within Continental hexaploid tall fescue, which are probably associated with palaeogeographic range expansion of this morphotype, provide a source of novel germplasm for future breeding programs. Improvement of tall fescue for use as both pasture and turf is critically reliant on exploitation of functional genetic diversity. This study has provided a foundation for the future assessment of non-domesticated germplasm for favourable phenotypic traits, based on extension of methodology from anonymous genetic diversity to specific haplotype variation in genes associated with known agronomic characters such herbage quality (Ponting et al. 2007) and disease resistance (Dracatos et al. 2008; Dracatos et al. 2009).

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