Chloroplast Genome Sequencing and Comparative Analysis for Fine Fescue (Festuca L., Poaceae*) Turfgrasses*

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Abstract: Fine fescues (*Festuca* L., Poaceae) are turfgrass species that perform well in low-input environments. Based on morphological characteristics, the most commonly-utilized fine fescues are divided into five taxa: three are subspecies within *F. rubra* L. and the remaining two are treated as species within the *F. ovina* L. complex. Morphologically, these five taxa are very similar, and both identification and classification of fine fescues remain challenging. In an effort to develop identification methods for fescues, we used flow cytometry to estimate genome size, and sequenced the chloroplast genome of all five taxa. Fine fescue chloroplast genome sizes ranged from 133,331 to 133,842 bp and contained 113 to 114 genes. Phylogenetic reconstruction using whole chloroplast genome sequences agreed with previous work based on morphology. Comparative genomics suggested unique repeat signatures for each fine fescue taxon that could potentially be used for marker development for taxon identification.

Keywords: Fine fescue, chloroplast genome, phylogeny, comparative genomics

1. Introduction

With ca. 450 species, Fescues (*Festuca* L., Poaceae) is a large and diverse genus of perennial grasses [1]. Fescue species are distributed mostly in temperate zones of both the northern and southern hemispheres, but most commonly found in the northern hemisphere [2]. Several of the fescue species have been commonly used as turfgrass. Based on both leaf morphology and nuclear ITS sequences, fescue species can be divided into two groups: broad-leaved fescues and fine-leaved fescues [3]. Broad-leaved fescues commonly used as turfgrass include tall fescue (*F. arundinacea* Schreb.) and meadow fescue (*F. pratensis* Huds.). Fine-leaved fescues are a group of cool-season grasses that include fine fescues. Five fine fescue taxa: hard fescue (*F. brevipila* Tracey, 2n=6x=42), sheep fescue (*F. ovina* L., 2n=4x=28), strong creeping red fescue (*F. rubra* ssp. *rubra* 2n=8x=56), slender creeping red fescue (*F. rubra* ssp. *litoralis* (G. Mey.) Auquier 2n=6x=42), and Chewings fescue (*F. rubra* ssp. *fallax* (Thuill.) Nyman 2n=6x=42) are commonly used as perennial turfgrasses [4]. All five taxa share very fine and narrow leaves and have been used for forage, turf, and ornamental purposes. They are highly tolerant to shade and drought, prefer low pH (5.5-6.5) and low fertility soils [5]. Additionally, fine fescues grow well in the shade or sun, have reduced mowing requirements, and do not need additional fertilizer or supplemental irrigation [4].

Based on morphological and cytological features, fine fescues are currently divided into two groups referred to as the *F. rubra* complex (includes *F. rubra* ssp. *litoralis*, *F. rubra* ssp. *rubra*, *F. rubra* ssp. *fallax*) and the *F. ovina* complex (includes *F. brevipila* and *F. ovina*) [4]. While it is relatively easy to identify fine fescue species into their proper complex, it is challenging to identify taxa within the same complex. In the *F. rubra* complex, both ssp.

litoralis and ssp. *rubra* are rhizomatous while ssp. *fallax* is non-rhizomatous. However, the separation of ssp. *litoralis* from ssp. *rubra* using rhizome length is challenging. Species identification within the *F. ovina* complex relies heavily on leaf characters; however, abundant morphological and ecotype diversity within *F. ovina* makes species identification difficult [6]. This is further complicated by inconsistent identification methods between different continents. For example, in the United States, sheep fescue is described as having a bluish gray leaf color and hard fescue leaf blade color is considered green [5], while in Europe, it is the opposite [7]. Beyond morphological classifications, laser flow cytometry has been used to determine ploidy level of fine fescues and some other fescue species [8]. A wide range of DNA contents within each complex suggests that the evolutionary history of each named species is complicated, and interspecific hybridization might interfere with species determination using this approach. Plant breeders have been working to improve fine fescues for turf use for several decades, with germplasm improvement efforts focused on disease resistance, traffic tolerance, and ability to perform well under heat stress [9]. Turfgrass breeders have utilized germplasm collections from old turf areas as a source of germplasm [10]; however, confirming the species identity in these collections has been challenging. A combination of molecular markers and flow cytometry could be a valuable tool for breeders to identify fine fescue germplasm [11].

Due to the complex polyploidy history of fine fescues, sequencing plastid genomes provides a more cost-effective tool for taxon identification than the nucleus genome because it is maternally inherited, lack of heterozygosity, present in high copies and usable even in partially degraded material. Previous studies have developed universal polymerase chain reaction (PCR) primers to amplify non-coding polymorphic regions for DNA barcoding in plants for species identification [12, 13]. However, the polymorphisms discovered from these regions are often single nucleotide polymorphisms that are difficult to apply using PCR screening methods. For these reasons, it would be helpful to assemble chloroplast genomes and identify simple sequence repeat (SSR) polymorphism and tandem repeats. Chloroplast genome sequencing has been simplified due to improved sequencing technology. In turfgrass species, high throughput sequencing has been used to assemble the chloroplast genomes of perennial ryegrass (*Lolium perenne* cv. Cashel) [14], tall fescue (*Lolium arundinacea* cv. Schreb) [15], diploid *Festuca ovina*, *Festuca pratensis*, *Festuca altissima* [16], and bermudagrass (*Cynodon dactylon*) [17]. To date, there is limited molecular biology information on fine fescue species identification and their phylogenetic position among other turfgrass species [16, 18]. In this study, we used flow cytometry to confirm the ploidy level of five fine fescue cultivars, each representing one of the five commonly utilized fine fescue taxon. We then reported the complete chloroplast genome sequences of these five taxa, carried out comparative genomics and phylogenetic inference. Based on the genome sequence we identified unique genome features among fine fescue taxa and predicted taxa specific SSR and tandem repeat loci for molecular marker development.

2. Results

2.1 Species Ploidy Level Confirmation

We used flow cytometry to estimate the ploidy levels of five fine fescue taxa by measuring the DNA content in each cell. DNA content was reflected by the flow cytometry mean PI-A value. Overall, fine fescue taxa had mean PI-A values roughly from 110 to 180 (**Figure 1 and Figure S1**). *F. rubra* ssp. *rubra* cv. Navigator II (2n=8x=56) had the highest mean PI-A value (181.434, %rCV 4.4%). *F. rubra* ssp. *litoralis* cv. Shoreline (2n=6x=42) and *F. rubra*

ssp. *fallax* cv. Treazure II (2n=6x=42) had similar mean PI-A values of 137.852, %rCV 3.7 and 145.864, %rCV 3.5, respectively. *F. brevipila* cv. Beacon (2n=6x=42) had a mean PI-A of 165.25, %rCV 1.9, while *F. ovina* cv. Quatro (2n=4x=28) had a mean PI-A of 108.43, %rCV 2.9. Standard reference *L. perenne* cv. Artic Green (2n=2x=14) had a G1 phase mean PI-A of 63.91, %rCV 3.0. USDA *F. ovina* PI 230246 (2n=2x=14) had a G1 mean PI-A of 52.73 (histogram not shown). The estimated genome size of USDA PI 230246 was 4.67 pg/2C. Estimated ploidy level of *F. brevipila* cv. Beacon was 6.3, *F. ovina* cv. Quatro was 4.11, *F. rubra* ssp. *rubra* cv. Navigator II was 6.9, *F. rubra* ssp. *litoralis* cv. Shoreline was 5.2, and *F. rubra* ssp. *fallax* cv. Treazure II was 5.5 (**Table 1**). All newly estimated ploidy levels roughly correspond to previously reported ploidy levels based on chromosome counts.

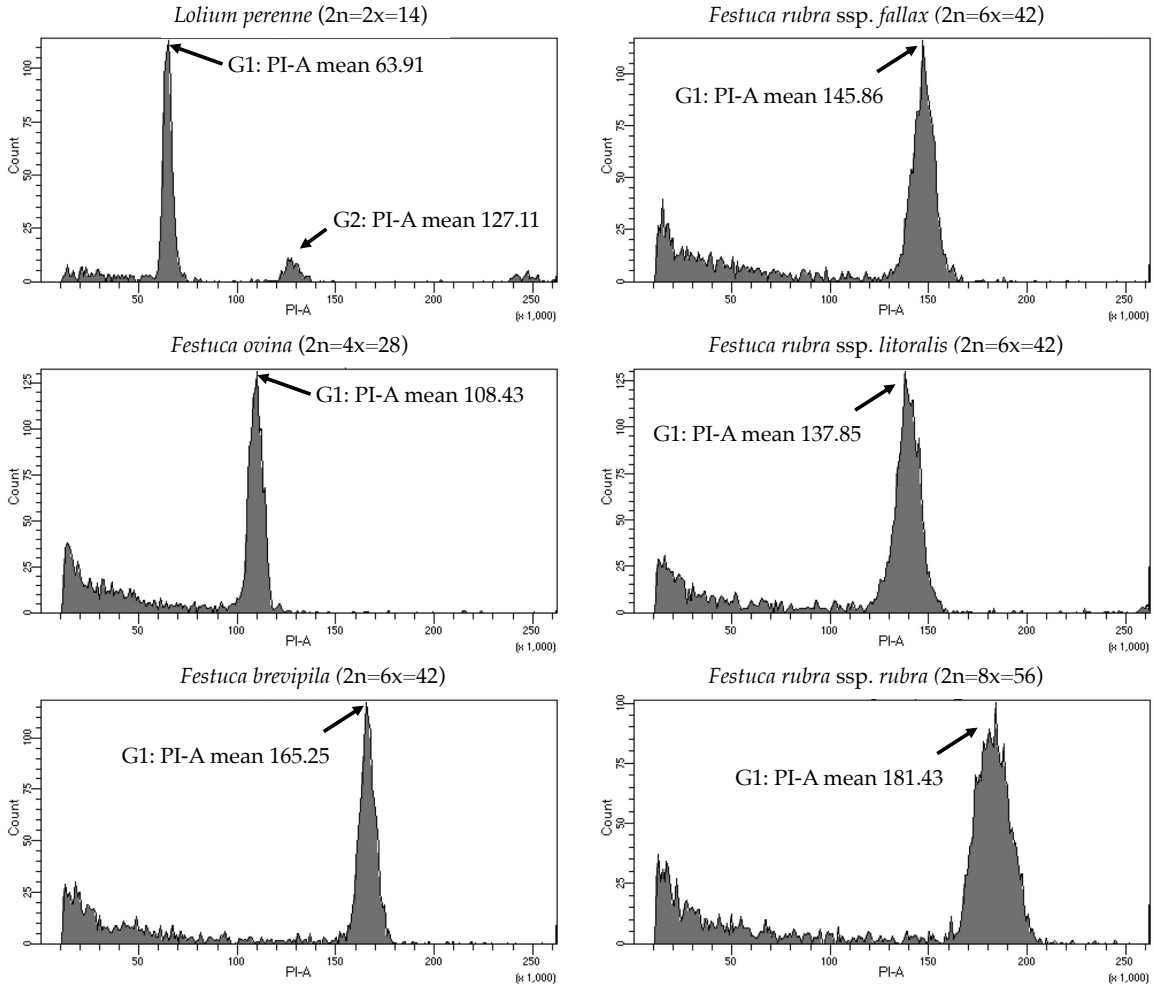


Figure 1. Flow cytometry results for the five fine fescue taxa. *Lolium* (2n=2x=14) was used as the reference. Flow cytometry was able to separate *F. rubra* ssp. *rubra* from the other two subspecies in the *F. rubra* complex. The mean PI-A values of *F. rubra* ssp. *fallax* and *F. rubra* ssp. *litoralis* were similar (145.86 to 137.85).

Table 1: Summary of flow cytometry statistics, genome size estimation, and ploidy level estimation of fine fescue species. *Lolium perenne* 2C DNA content was used to calculate fine fescue and USDA *F. ovina* PI 230246 genome size, calculated PI 230246 DNA content was used as reference to infer fine fescue ploidy level

Species name	Chromosome count	Cultivar name	Mean PI-A	%rCV *	Estimated Genome Size (pg/Nuclei)	Estimated Ploidy Level

F. brevipila	2n=6x=42	Beacon	165.3	1.9	14.6	6.3
F. ovina	2n=4x=28	Quatro	108.4	2.9	9.6	4.1
F. ovina PI 230246	2n=2x=14	NA	52.7	3.1	4.7	1.7
F. rubra ssp. rubra	2n=8x=56	Navigator II	181.4	4.4	16.1	6.9
F. rubra ssp. litoralis	2n=6x=42	Shoreline	137.9	3.7	12.2	5.2
F. rubra ssp. fallax	2n=6x=42	Treazure II	145.9	3.5	12.9	5.5
L. perenne	2n=2x=14	Artic Green	63.9	3.0	5.7	2.0

*** %rCV:** Quality of laser alignment. Low %rCV suggests high resolution sensitity.

2.2 Plastid Genome Assembly and Annotation of Five Fescue Taxa

A total of 47,843,878 reads were produced from the five fine fescue taxa. After Illumina adaptor removal, we obtained 47,837,438 reads. The assembled chloroplast genomes ranged from 133,331 to 133,842 bp. The large single copy (LSC) and small single copy (SSC) regions were similar in size between the sequenced fine fescue accessions (78 kb and 12 kb, respectively). *Festuca ovina* and *F. brevipila* in the *F. ovina* complex had exactly the same size inverted repeat (IR) region (42,476 bp). In the *F. rubra* complex, *F. rubra* ssp. *rubra* and *F. rubra* ssp. *litoralis* had the same IR size (21,235 bp). Species in the *F. rubra* complex had a larger chloroplast genome size compared to species in the *F. ovina* complex. All chloroplast genomes shared similar GC content (38.4%) (**Figure 2**, **Table 2**). The fine fescue chloroplast genomes encoded for 113-114 genes, including 37 transfer RNAs (tRNA), 4 ribosomal RNAs (rRNA), and 72 protein-coding genes (**Table 2**). Genome structures were similar among all five fine fescue taxa sequenced, except that the pseudogene *accD* was annotated in all three subspecies of *F. rubra,* but not in the *F. ovina* complex (**Table 3**).

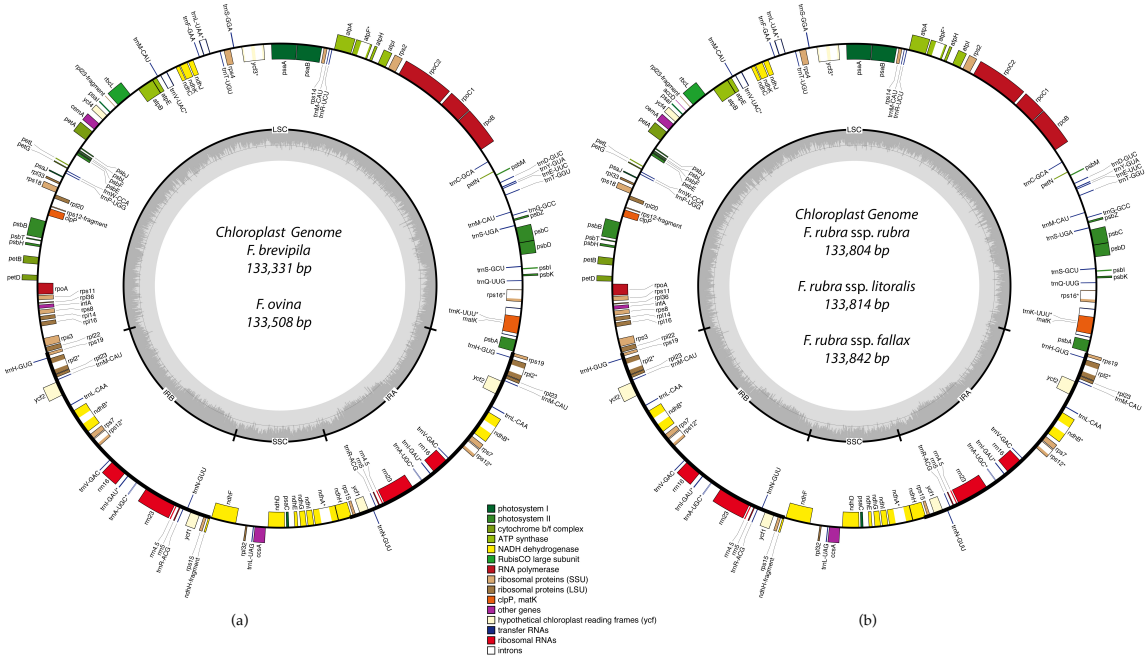


Figure 2: Whole chloroplast genome structure of *F. ovina* complex (a) and *F. rubra* complex (b). Genes inside the circle are transcribed clockwise, genes outside are transcribed counter-clockwise. Genes belong to different functional groups are color coded. GC content is represented by the dark gray inner circle, the light gray corresponded to the AT content. IRA(B), inverted repeat A(B); LSC, large single copy region; SSC, small single copy region.

134 **Table 2**: Characteristics of fine fescue chloroplast genomes.

	F. brevipila cv. Beacon	*F. ovina* cv. Quatro	*F. rubra* ssp. *rubra* cv. Navigator II	*F. rubra* ssp. *litoralis* cv. Shoreline	*F. rubra* ssp. *fallax* cv. Treazure II
Total Genome Size (bp)	133,331	133,508	133,804	133,814	133,842
Large Single Copy (bp)	78,462	78,632	78,888	78,909	78,882
Small Single Copy (bp)	12,393	12,400	12,446	12,435	12,452
Inverted Repeat (bp)	42,476	42,476	42,470	42,470	42,508
Ratio of LSC (%)	58.85	58.9	58.96	58.97	58.94
Ratio of SSC (%)	9.29	9.29	9.3	9.29	9.3
Ratio of IRs (%)	31.86	31.82	31.74	31.74	31.76
GC content (%)	38.4	38.4	38.4	38.4	38.4

135 **Table 3.** Fine fescue chloroplast genomes gene content by gene category.

Category	Group of Gene	Name of gene			
Self-replication (58/77)	Ribosomal RNA genes (4/8)	*rrn4.5* [a]	*rrn5* [a]	*rrn16* [a]	*rrn23* [a]
	Transfer RNA genes (27/38)	*trnA-UGC* *[a]	*trnC-GCA*	*trnD-GUC*	*trnE-UUC*
		trnF-GAA	*trnG-GCC*	*trnH-GUG* [a]	*trnI-GAU* *[a]
		*trnK-UUU**	*trnL-CAA* [a]	*trnL-UAA**	*trnL-UAG*
		trnM-CAU [c]	*trnN-GUU* [a]	*trnP-UGG*	*trnQ-UUG*
		trnR-ACG [a]	*trnR-UCU*	*trnS-GCU*	*trnS-GGA*
		trnS-UGA	*trnT-GGU*	*trnT-UGU*	*trnV-GAC*[a]
		*trnV-UAC**	*trnW-CCA*	*trnY-GUA*	
	Small subunit of ribosome (12/16)	*rps2*	*rps3*	*rps4*	*rps7* [a]
		rps8	*rps11*	*rps12* *[ab]	*rps14*
		rps15 [a]	*rps16**	*rps18*	*rps19* [a]
	Large subunit of ribosome (9/11)	*rpl2**[a]	*rpl14*	*rpl16*	*rpl20*
		rpl22	*rpl23*[a]	*rpl32*	*rpl33*
		rpl36			
	RNA polymerase subunits (4)	*rpoA*	*rpoB*	*rpoC1*	*rpoC2*
Photosynthesis (45/46)	Subunits of Photosystem I (6)	*psaA*	*psaB*	*psaC*	*psaI*
		psaJ	*ycf3***		

	Subunits of Photosystem II (15)	*psbA*	*psbB*	*psbC*	*psbD*
		psbE	*psbF*	*psbH*	*psbI*
		psbJ	*psbK*	*psbL*	*psbM*
		psbN	*psbT*	*psbZ*	
	Subunits of cytochrome (6)	*petA*	*petB*	*petD*	*petG*
		petL	*petN*		
	Subunits of ATP synthase (6)	*atpA*	*atpB*	*atpE*	*atpF**
		atpH	*atpI*		
	Large subunit of Rubisco (1)	*rbcL*			
	Subunits of NADH Dehydrogenase (11/12)	*ndhA**	*ndhB*[a]*	*ndhC*	*ndhD*
		ndhE	*ndhF*	*ndhG*	*ndhH*
		ndhI	*ndhJ*	*ndhK*	
	Translational initiation factor (1)	*infA*			
	Maturase (1)	*matK*			
	Envelope membrane protein (1)	*cemA*			
Other genes (5)	C-type cytochrome (1)	*cssA*			
	Protease (1)	*clp*			
	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	*accD*[$]			
Unknown function (5)	Conserved open reading frames (3/5)	*ycf1*[a]	*ycf2*[a]	*ycf4*	

[a] Two gene copies in IRs; [b] Gene divided into two independent transcription units; [c] Gene that has five copies; [*] One intron-containing genes; [**] Two intron-containing genes. [$] Gene annotated in *F. rubra* spp. only. Fine fescue species chloroplast genomes share high structure similarity and gene content. Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta pseudogene is annotated in *F. rubra* ssp.

2.3 Chloroplast Genome IR Expansion and Contraction

Contraction and expansion of the IR regions resulted in the size variation of chloroplast genomes. We examined the four junctions in the chloroplast genomes, LSC/IRa, LSC/IRb,

SSC/IRa, and SSC/IRb of the fine fescue and the model turfgrass species *L. perenne*. Although the chloroplast genome of fine fescue species was highly similar, some structural variations were still found in the IR/LSC and IR/SSC boundary. Similar to *L. perenne*, fine fescue species chloroplast genes *rpl22-rps19, rps19-psbA* were located in the junction of IR and LSC; *rps15-ndhF* and *ndhH-rps15* were located in the junction of IR/SSC. In the *F. ovina* complex, the *rps19* gene was 37 bp into the LSC/IRb boundary while in the *F. rubra* complex and *L. perenne*, the *rps19* gene was 36 bp into the LSC/IRb boundary (**Figure 3**). The *rsp15* gene was 308 bp from the IRb/SSC boundary in *F. ovina* complex, 307 bp in *F. rubra* complex, and 302 bp in *L. perenne*. Both the *ndhH* and the pseudogene fragment of the *ndhH* (*ψndhH)* genes panned the junction of the IR/SSC. The *ψndhH* gene crossed the IRb/SSC boundary with 10 bp into SSC in *F. brevipila* and *F. ovina*, 21 bp in *F. rubra* ssp. *rubra*, 4 bp in *F. rubra* ssp. *litoralis* and *F. rubra* ssp. *fallax*, and 7 bp in *L. perenne*. The *psbA* gene was 87 bp apart from the IRa/LSC boundary into the LSC in *L. perenne* and *F. ovina* complex species but 83 bp in the *F. rubra* complex.

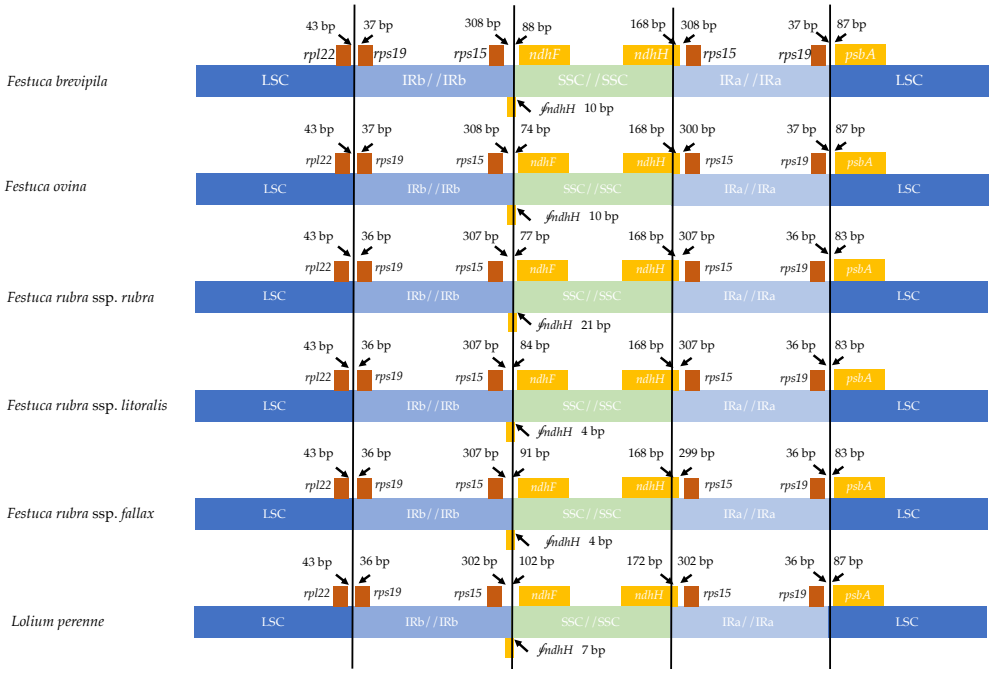
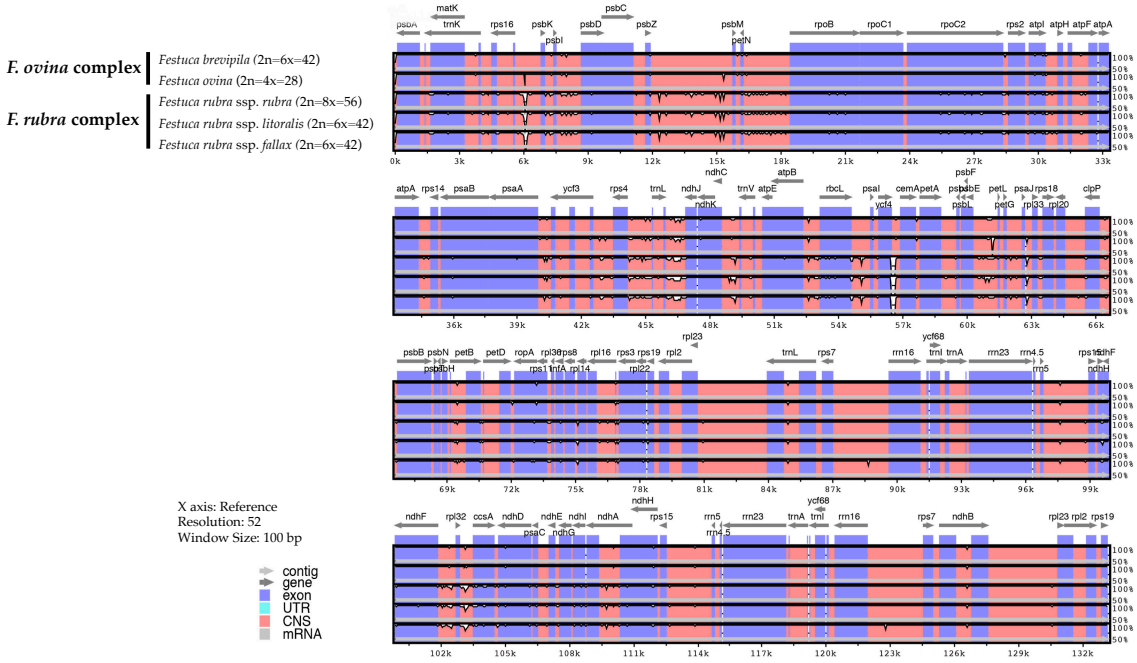


Figure 3. Comparison for border positions of LSC, SSC and IR regions among five fine fescues and *L. perenne*. Genes are denoted by boxes, and the gap between the genes and the boundaries are indicated by the number of bases unless the gene coincides with the boundary. Extensions of genes are also indicated above the boxes.

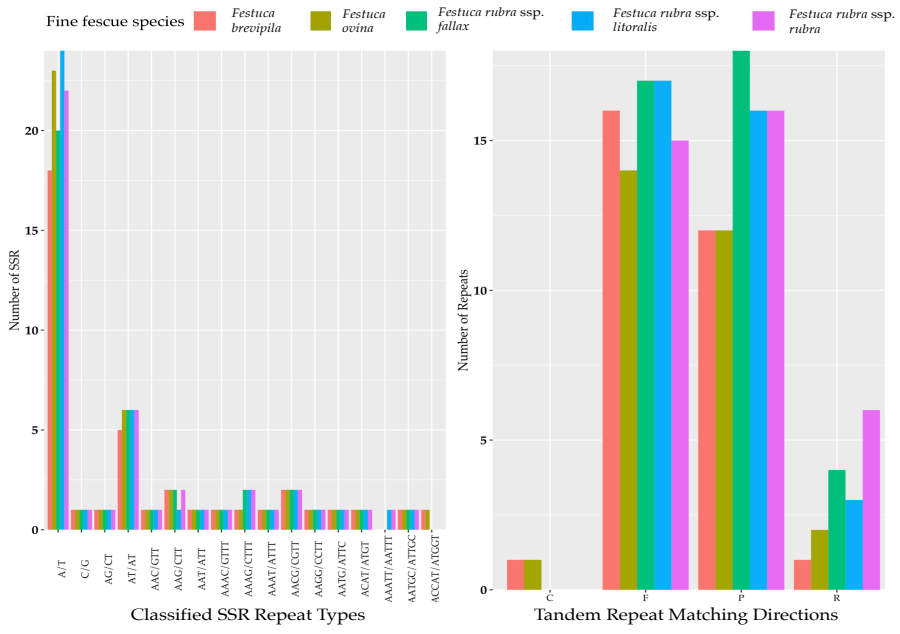
2.4 Whole Chloroplast Genome Comparison and Repetitive Element Identification

Genome-wide comparison among five fine fescue taxa showed high sequence similarity with most variations located in intergenic regions (**Figure 4**). To develop markers for species screening, we predicted a total of 217 SSR markers for fine fescue taxa sequenced (*F. brevipila* 39; *F. ovina* 45; *F. rubra* ssp. *rubra* 45; *F. rubra* ssp. *litoralis* 46; *F. rubra* ssp. *fallax* 42) that included 17 different repeat types for the fine fescue species (**Figure 5a, Table S1**). The most frequent repeat type was A/T repeats, followed by AT/AT. The pentamer AAATT/AATTT repeat was only presented in the rhizomatous *F. rubra* ssp. *litoralis* and *F. rubra* ssp. *rubra*, while ACCAT/ATGGT was only found in *F. ovina* complex species *F. brevipila* and *F. ovina*. Similar to SSR loci prediction, we also predicted long repeats for the fine fescue species and identified a total of 171 repeated elements ranging in size from 20 to 51 bp (**Figure 5b, Table S2**). Complementary (C) matches were only identified in *F. brevipila* and *F. ovina*. *F. rubra*

173 species had more palindromic (P) and reverse (R) matches. Number of forward (F) matches
174 were similar between five taxa.



175
176 **Figure 4**. Sequence identity plot of fine fescues chloroplast genome sequences with *F. ovina* (2x) as the
177 reference using mVISTA. A cut-off of 70% identify was used for the plots, and the percent of identity
178 varies from 50% to 100% as noted on the y-axis. Most of the sequence variation between fine fescues
179 were in intergenic regions. Species in the *F. ovina* complex, *F. brevipila* and *F. ovina* showed high
180 sequence similarity. Similarly, subspecies within *F. rubra* complex also showed high sequence
181 similarity.



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183 **Figure 5**. (a) SSR repeat type and numbers in the five fine fescue taxa sequenced. Single nucleotide
184 repeat type has the highest frequency. No hexanucleotide repeats were identified in the fine fescue
185 chloroplast genomes sequenced. One penta-nucleotide repeat type (AAATT/AATTT) is unique to *F.*

rubra ssp. *rubra* and *F. rubra* ssp. *litoralis*; One penta-nucleotide repeat type (ACCAT/ATGGT) is unique to *F. brevipila* and *F. ovina* (b) Long repeats sequences in fine fescue chloroplast genomes. Complement (C) match was only identified in the *F. ovina* complex; Reverse (R) match has the most number variation in fine fescues.

2.5 SNP and InDel Distribution in the Coding Sequence of Five Fine Fescue Species

To identify single nucleotide polymorphisms (SNPs) we used the diploid *F. ovina* gff3 file (JX871940.1) as the template gene model to count SNPs distribution. We found more SNPs were located within intergenic regions in the *F. ovina* complex, while in the *F. rubra* complex, SNPs were located evenly between gene coding and intergenic regions. Most InDels were located in intergenic regions of the fine fescue species (**Table 3**). Between *F. ovina* and the *F. rubra* complex, *the ropC2* gene had the most SNPs (4 vs 31). *rbcL* gene also has a high level of variation (1 vs 14.3). *rpoB*, *ccsA*, NADH dehydrogenase subunit genes (*ndhH*, *ndhF*, *ndhA*), and ATPase subunit genes (*atpA*, *atpB*, *aptF*) also showed variation between *F. ovina* and *F. rubra* complexes. Less SNP and InDel variation were found within each complex (**Table 4, Table S3 and S4**).

Table 4. Distribution of SNPs and InDels for the five fine fescue taxa sequenced in this study.

Species	*F. brevipila*	*F. ovina*	*F. rubra* ssp. *rubra*	*F. rubra* ssp. *litoralis*	*F. rubra* ssp. *fallax*
Total number of SNPs	98	134	638	615	624
SNPs in the coding region	35	52	306	301	300
SNPs in intergenic region	63	82	332	314	324
Percentage of SNPs in the intergenic region	64.29	61.19	52.04	51.06	51.92
Total number of InDels	112	102	149	156	149
InDels in the coding region	22	17	27	26	27
InDels in intergenic region	90	85	122	130	122
Percentage of InDels in the intergenic region	80.36	83.33	81.88	83.33	81.88

2.6 Nucleotide Diversity and Mutation Hotspot Identification

A sliding window analysis successfully detected highly variable regions in the fine fescue chloroplast genomes (**Figure 6, Table S5**). The average nucleotide diversity (Pi) among fine fescue species was relatively low (0.00318). The IR region showed lower variability than the LSC and SSC region. There were several divergent loci having a Pi value greater than 0.01 (*psbK-psbI*, *trnfM-trnE*, *trnC-rpoB*, *psbH-petB*, *trnL-trnF*, *trnS-rps4*, *aptB-rbcL-psaI*, and *rpl32-trnL*), but mostly within intergenic regions. The *rbcL-psaI* region contained a highly variable *accD-like* region in some species, we looked at the structural variation of 10 species in the *Festuca - Lolium* complex. We found species in broad-leaved fescue and *F. rubra* complex had similar structure, while *F. ovina* (2x, 4x) and *F. brevipila* had a 276 bp deletion in the *rbcL-psaI* intergenic region (**Figure 7**).

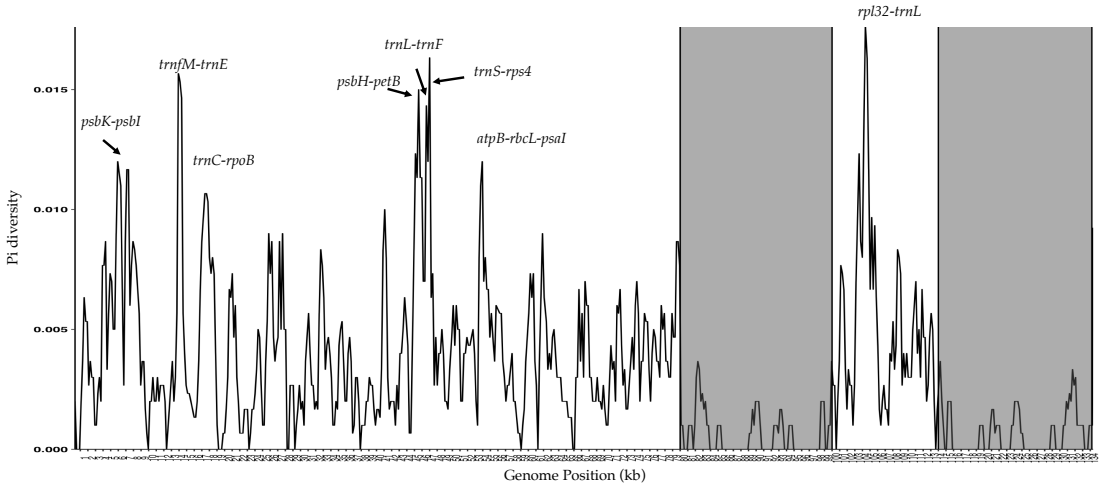

Figure 6. Sliding window analysis of fine fescue whole chloroplast genomes. Window size: 600 bp, step size: 200 bp. X-axis: the position of the midpoint of a window (kb). Y-axis: nucleotide diversity of each window. Inverted repeat regions are highlighted in grey. *rpl32-trnL*region has the most nucleotide diversity followed by *psbH- petB-trnL-trnF-trnS-rps4* region.

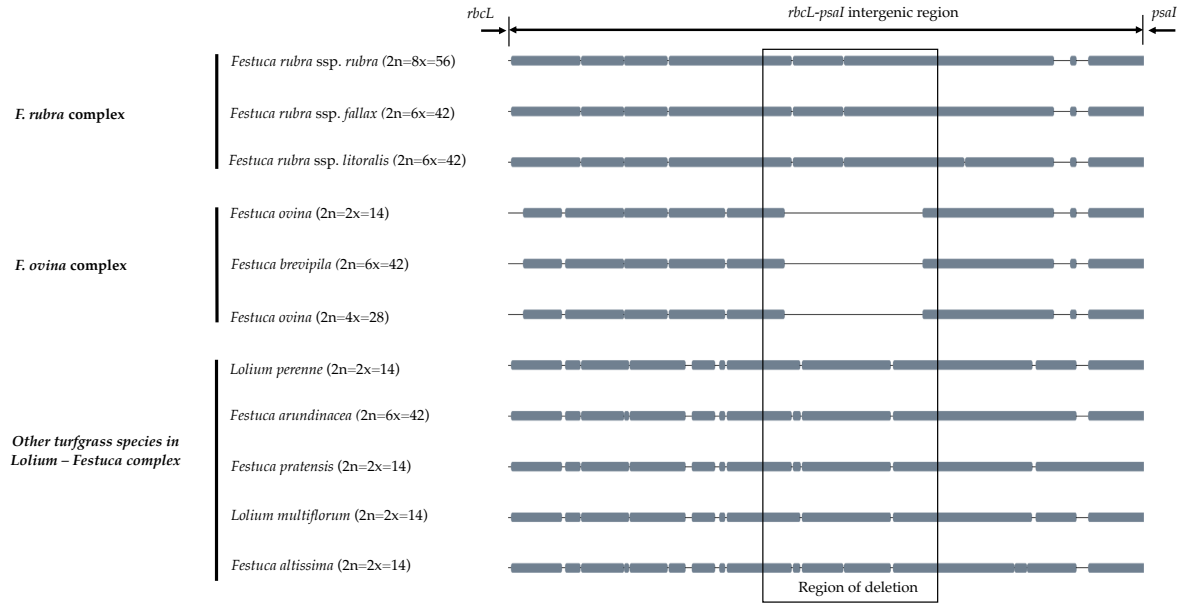

Figure 7. Alignment of *rbcL-psaI* intergenic sequence alignment shows the pseudogene *accD* is missing in both *F. ovina* (2x, 4x) and *F. brevipila* but present in the *Festuca rubra* complex and other species examined in this study. Species were ordered by complexes.

2.7 Phylogenetic Reconstruction of Fine Fescue Species

We reconstructed the phylogenetic relationships of species within the *Festuca - Lolium* complex using the chloroplast genomes sequenced in our study and eight publicly available complete chloroplast genomes including six species within the *Festuca-Lolium* complex (**Figure 8**). The dataset included 125,824 aligned characters, of which 3,923 were parsimony-informative and 91.11% characters are constant. The five fine fescue taxa were split into two clades ([ML]BS=100). In the *F. ovina* complex, two *F. ovina* accessions included in the

phylogenetic analysis, a diploid one from GenBank, and a tetraploid one newly sequenced in this study are paraphyletic to *F. brevipila* ([ML]BS=100). All three subspecies of *F. rubra* formed a strongly supported clade ([ML]BS=100). Together they are sisters to the *F. ovina* complex ([ML]BS=100).

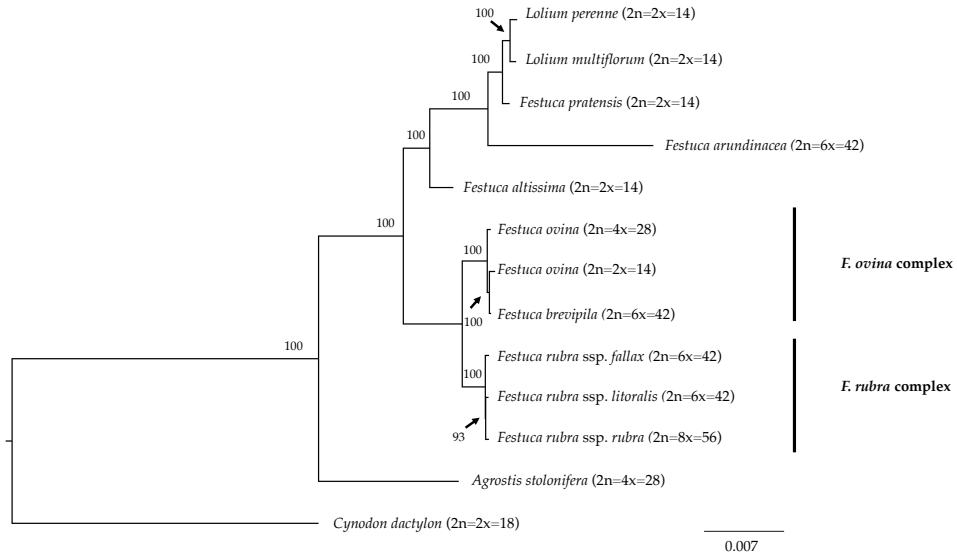

Figure 8. Maximum likelihood (ML) phylogram of the *Festuca - Lolium* complex with 1,000 bootstrap replicates. Fine fescues were grouped into previous named complexes (*F. ovina* and *F. rubra*), sister to broad leaved fescues in the *Festuca – Lolium* complex.

3. Discussion

In this study, we used flow cytometry to determine the ploidy level of five fine fescue cultivars, assembled the chloroplast genomes for each, and identified structural variation and mutation hotspots. We also identified candidate SSR loci for marker development to facilitate fine fescue species identification. Additionally, we reconstructed the phylogenetic relationships of the *Festuca-Lolium* complex using plastid genome information generated in this study along with other publicly-available plastid genomes.

Flow cytometry was able to separate *F. brevipila* cv. Beacon, *F. ovina* cv. Quatro and *F. rubra* ssp. *rubra* cv. Navigator II based on the mean PI-A value. We noticed that the average mean PI-A of the diploid *L. perenne* (63.91) was higher than the mean PI-A of diploid *F. ovina* (52.73), suggesting that *F. ovina* species has smaller genome size than *L. perenne*. The ploidy estimation in the *F. ovina* complex are fairly consistent while the estimations of genome sizes in the *F. rubra* complex are smaller than we expected, even though these two complexes are closely related. Indeed, a similar finding was reported by Huff et al [8] that *F. brevipila* has a larger genome size than *F. rubra* ssp. *litoralis* and *F. rubra* ssp. *fallax*, both of which have the same ploidy level as *F. brevipila*. The range of variation in DNA content within each complex suggest a complicated evolutionary history in addition to polyploidization [8].

While most crop plants are highly distinctive from their close relatives, *Festuca* is a species-rich genus that contains species with highly similar morphology and different ploidy level. Consequently, it is difficult for researchers to interpret species identity. In our case, it is most difficult to distinguish between *F. rubra* ssp. *litoralis* cv. Shoreline and *F. rubra*

ssp. *fallax* cv. Treazure II as they had similar PI-A values based on flow cytometry. Thus, we need different approaches to identify them. The presence and absence of rhizome formation could be taken into consideration; for example, *F. rubra* ssp. *fallax* cv. Treazure II is a bunch type turfgrass, while *F. rubra* ssp. *litoralis* cv. Shoreline forms short and slender rhizomes [20]. This method may not be effective, however, because rhizome formation can be greatly affected by environmental conditions [21, 22].

To further develop molecular tools to facilitate species identification, we carried out chloroplast genome sequencing. We assembled the complete chloroplast genomes of five low-input turfgrass fine fescues using Illumina sequencing. Overall, the chloroplast genomes had high sequence and structure similarity among all five fine fescue taxa sequenced, especially within each complex. All five chloroplast genomes share similar gene content except for the three species in the *F. rubra* complex that have a pseudogene Acetyl-coenzyme A carboxylase carboxyl transferase subunit (*accD*). The *accD* pseudogene is either partially or completely absent in some monocots. Instead, a nuclear-encoded ACC enzyme has been found to replace the plastic *accD* gene function in some angiosperm linage [23]. Indeed, even though the *accD* pseudogene is missing in the *F. brevipila* chloroplast genome, the gene transcript was identified in a transcriptome sequencing dataset (unpublished data), suggesting that this gene has been translocated to nucleus genome. Previous studies have shown that broad-leaf fescues, *L. perenne*, *O. sativa*, and *H. vulgare* all had the pseudogene *accD* gene, while it was absent in diploid *F. ovina*, *Z. mays*, *S. bicolor*, *T. aestivum*, and *B. distachyon* [16]. Broad-leaf and fine-leaf fescues diverged around 9 Mya ago [24], which raises an interesting question about the mechanisms of the relocation of *accD* among closely related taxa in the *Festuca-Lolium* complex and even within fine fescue species.

In plants, chloroplast genomes are generally considered "single copy" and lack of recombination due to maternal inheritance [25]. For this reason, chloroplast genomes are convenient for developing genetic markers for distinguishing species and subspecies. We have identified a number of repeat signatures that are unique to a single species or species complex in fine fescue. For example, complement match is only identified in *F. ovina* complex, and *F. rubra* complex has more reversed matches. We also identified two SSR repeats unique to each of the two complexes. The first one consists of AAATT/AATTT repeat units is unique to *F. rubra* ssp. *litoralis* and *F. rubra* ssp. *rubra*, and the second one consists of ACCAT/ATGGT repeat units is unique to *F. brevipila* and *F. ovina*. In cases like the identification of hexaploids *F. brevipila*, *F. rubra* ssp. *fallax*, and *Festuca rubra* ssp. *litoralis*, it is critical to have these diagnostic repeats given all three taxa share similar PI-A values from flow cytometry. Taxon-specific tandem repeat could also aid the SSR repeats for species identification. Nucleotide diversity analysis suggested that several variable genome regions exist among the five fine fescue taxa sequenced in this study. These variable regions included previously known highly variable chloroplast regions such as *trnL-trnF* and *rpl32-trnL* [13, 26]. These regions have undergone rapid nucleotide substitution and are potentially informative molecular markers for characterization of fine fescue species.

Phylogeny inferred from DNA sequence is valuable for understanding the evolutionary context of a species. The phylogenetic relationship of fine fescue using whole plastid genome sequences agrees with previous classification based on genome size estimation and morphology [8, 18]. The *F. ovina* complex includes *F. ovina* and *F. brevipila* and the *F. rubra* complex includes *F. rubra* ssp. *rubra*, *F. rubra* ssp. *litoralis* and *F. rubra* ssp. *fallax*, with the two rhizomatous subspecies (ssp. *rubra* and ssp. *literalis*) being sister to each other. Within the *Festuca – Lolium* complex, fine fescues are monophyletic and together sister to a clade consists of broad-leaved fescues and *Lolium*. In our analysis, *F. brevipila* (6x) is nested within

F. ovina and sister to the diploid *F. ovina*. It is likely that *F. brevipila* arose from the hybridization between *F. ovina* (2x) and *F. ovina* (4x). Further research using nuclear loci is needed to confirm this hypothesis.

The diversity of fine fescue provides valuable genetic diversity for breeding and cultivar development. Breeding fine fescue cultivars for better disease resistance, heat tolerance, and traffic tolerance could be achieved through screening wild accessions and by introgressing desired alleles into elite cultivars. Some work has been done using *Festuca* accessions in the USDA Germplasm Resources Information Network (GRIN) (https://www.ars-grin.gov) to breed for improved forage production in fescue species [27]. To date, there are 229 *F. ovina* and 486 *F. rubra* accessions in the USDA GRIN. To integrate this germplasm into breeding programs, plant breeders and other researchers need to confirm the ploidy level using flow cytometry and further identify them using molecular markers. Resources developed in this study could provide the tools to screen the germplasm accessions and refine the species identification so breeders can efficiently use these materials for breeding and genetics improvement of fine fescue species.

4. Materials and Methods

Plant Material

Seeds from the fine fescue cultivars seeds were obtained from the 2014 National Turfgrass Evaluation Program (www.ntep.org, USA) and planted in the Plant Growth Facility at the University of Minnesota, St. Paul campus under 16 hours daylight (25 ℃) and 8 hours dark (16 ℃) with weekly fertilization. Single genotypes of *F. brevipila* cv. Beacon, *F. rubra* ssp. *litoralis* cv. Shoreline, *F. rubra* ssp. *rubra* cv. Navigator II, *F. rubra* ssp. *fallax* cv. Treazure II, and *F. ovina* cv. Quatro were selected and used for chloroplast genome sequencing.

Flow Cytometry

To determine the ploidy level of the cultivars used for sequencing and compare them to previous work (2n=4x=28: *F. ovina*; 2n=6x=42: *F. rubra* ssp. *litoralis*, *F. rubra* ssp. *fallax*, and *F. brevipila*; 2n=8x=56: *F. rubra* ssp. *rubra*), flow cytometry was carried out using *Lolium perenne* cv. Artic Green (2n=2x=14) as the reference. Samples were prepared using CyStain PI Absolute P (Sysmex, product number 05-5022). Briefly, to prepare the staining solution for each sample, 12 µL propidium iodide (PI) was added to 12 mL of Cystain UV Precise P staining buffer with 6 µL RNase A. To prepare plant tissue, a total size of 0.5 cm x 0.5 cm leaf sample of the selected fine fescue was excised into small pieces using a razor blade in 500 µL CyStain UV Precise P extraction buffer and passed through a 50 µm size filter (Sysmex, product number 04-004-2327). The staining solution was added to the flow-through to stain nuclei in each sample. Samples were stored on ice before loading the flow cytometer. Flow cytometry was carried out using the BD LSRII H4760 (LSRII) instrument with PI laser detector using 480V with 2,000 events at the University of Minnesota Flow Cytometry Resource (UCRF). Data was visualized and analyzed on BD FACSDiva 8.0.1 software. To estimate the genome size, *L. perenne* DNA (5.66 pg/2C) was used as standard [28], USDA PI 230246 (2n=2x=14) was used as diploid fine fescue relative (unpublished data). To infer fine fescues ploidy, estimation was done using equations (1) and (2) [29].

$$\text{Sample 2C DNA Content} = \text{Standard 2C DNA Content (pg DNA)} \times \frac{(\text{Sample G1 Peak Mean})}{(\text{Standard G1 Peak Mean})} \quad (1)$$

$$\text{Sample Ploidy} = \frac{2n \times \text{Sample pg/Nucleus}}{\text{Diploid Relative pg/Nucleus}} \quad (2)$$

DNA Extraction and Sequencing

To extract DNA for chloroplast genome sequencing, 1 g of fresh leaves were collected from each genotype and DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, USA) following manufacturer instructions. DNA quality was examined on 0.8% agarose gel and quantified via PicoGreen (Thermo Fisher, Catalog number: P11496). Sequencing libraries were constructed by NovoGene, Inc. (Davis, CA) using Nextera XT DNA Library Preparation Kit (Illumina) and sequenced in 150 bp paired-end mode, using the HiSeq X Ten platform (Illumina Inc., San Diego, CA, USA) with an average of 10 million reads per sample. All reads used in this study were deposited in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA512126.

Chloroplast Genome Assembly and Annotation

Raw reads were trimmed of Illumina adaptor sequences using Trimmomatic (v. 0.32) [30]. Chloroplast genomes were *de novo* assembled using NovoPlasty v. 2.0 [31]. Briefly, *rbcL* gene sequence from diploid *F. ovina* (NCBI accession number: JX871940) was extracted and used as the seed to initiate the assembly. NovoPlasty assembler configuration was set as follows: *k-mer* size = 39; insert size = auto; insert range = 1.8; and insert range strict 1.3. Reads with quality score above 25 were used to complete the guided assembly using *F. ovina* (NCBI accession number: JX871940) as the reference. Assembled plastid genomes for each taxon were manually corrected by inspecting the alignments of reads used in the assembly. The assembled chloroplast genomes were deposited under BioProject PRJNA512126.

The assembled chloroplast genomes were annotated using the GeSeq pipeline [32] and corrected using DOGMA online interface (https://dogma.ccbb.utexas.edu) [33]. BLAT protein, tRNA, rRNA, and DNA search identity threshold was set at 80% in the GeSeq pipeline using the default reference database with the generate codon-based alignments option turned on. tRNAs were also predicted via tRNAscan-SE v2.0 and ARAGORN v 1.2.38 using the bacterial/plant chloroplast genetic code [34, 35]. The final annotation was manually inspected and corrected using results from both pipelines. The circular chloroplast map was drawn by the OrganellarGenomeDRAW tool (OGDRAW) [36].

Nucleotide Polymorphism of Fine Fescue Species

To identify genes with the most single nucleotide polymorphism, quality trimmed sequencing reads of the five fine fescues were mapped to the diploid *Festuca ovina* chloroplast genome (NCBI accession number: JX871940) using BWA v.0.7.17 [37]. SNPs and short indels were identified using bcftools v.1.9 with the setting "mpileup -Ou" and called via bcftools using the -mv function [38]. Raw SNPs were filtered using bcftools filter -s option to filter out SNPs with low quality (Phred score cutoff 20, coverage cutoff 20). The subsequent number of SNPs per gene and InDel number per gene was calculated using a custom perl script SNP_vcf_from_gene_gff.pl (https://github.com/qiuxx221/fine-fescue-).

To identify simple sequence repeat (SSR) markers for plant identification, MIcroSAtellite identification tool (MISA v 1.0) was used with a threshold of 10, 5, 4, 3, 3, 3 repeat units for mono-, di-, tri-, tetra-, penta-, and hexanucleotide SSRs, respectively [39]. The identification of repetitive sequences and structure of whole chloroplast genome was done via PEPuter program online server (https://bibiserv.cebitec.uni-bielefeld.de/reputer)

[40]. Program configuration was set with minimal repeat size set as 20 bp and with sequence identify above 90%. Data was visualized using ggplot2 in R (v 3.5.3). Finally, the sliding window analysis was performed using DnaSP (v 5) with a window size of 600 bp, step size 200 bp to detected highly variable regions in the fine fescue chloroplast genome [41].

Comparative Chloroplast Genomics Analysis

To compare fine fescue species chloroplast genome sequence variations, the five complete chloroplast genomes were aligned and visualized using mVISTA, an online suite of computation tools with LAGAN mode [42, 43]. The diploid *Festuca ovina* (NCBI accession number: JX871940) chloroplast genome and annotation were used as the template for the alignment.

Phylogenetic Analysis of Fine Fescues and Related Festuca species

To construct the phylogenetic tree of the fine fescues using the whole chloroplast genome sequence, chloroplast genomes of 8 species were downloaded from GenBank. Of the 8 downloaded genomes, perennial ryegrass (*Lolium perenne*, AM777385), Italian ryegrass (*Lolium multiflorum*, JX871942), diploid *Festuca ovina* (JX871940), tall fescue (*Festuca arundiancea*, FJ466687), meadow fescue (*Festuca pratensis*, JX871941), and wood fescue (*Festuca altissima*, JX871939) were within the *Festuca- Lolium* complex. Turfgrass species outside of *Festuca-Lolium* complex including creeping bentgrass (*Agrostis stolonifera* L., EF115543) and *Cynodon dactylon* (KY024482.1) were used as an outgroup. All chloroplast genomes were aligned using the MAFFT program (v 7) [44]; alignments were inspected and manually adjusted. Maximum likelihood (ML) analyses was performed using the RAxML program (v 8.2.12) under GTR+G model with 1,000 bootstrap [45]. The phylogenetic tree was visualized using FigTree (v 1.4.3) (https://github.com/rambaut/figtree) [46].

5. Conclusions

Five newly-sequenced complete chloroplast genomes of fine fescue taxa were reported in this study. Chloroplast genome structure and gene contents are both conserved, with the presence and absence of *accD* pseudogene being the biggest structural variation between the *F. ovina* and the *F. rubra* complexes. We identified SSR repeats and long sequence repeats of fine fescues and discovered several unique repeats for marker development. The phylogenetic constructions of fine fescue species in the *Festuca - Lolium* complex suggested a robust and consistent relationship compared to the previous identification using flow cytometry. This information provided a reference for future fine fescue taxa identification.

Supplementary Materials:

1. **Figure S1**: Flow cytometry nuclei population distribution of *L. perenne*, fine fescues, and diploid USDA PI accession. G1 populations were gated in red, G2 population was only gated in *L. perenne* in green.
2. **Table S1**: SSR loci types and number distributions of fine fescue species predicted using MISA program.
3. **Table S2**: Tandem repeat loci and repeat types predicted using PEPuter program in fine fescue species.
4. **Table S3**: SNPs number per gene distribution for fine fescue species. *rpoC2* gene has the most SNPs (31) in *F. rubra* complex comparing to *F. ovina* species.
5. **Table S4**: InDel number per gene distribution for fine fescue species. *ndhA* gene had the most InDels *F. rubra* species. *atpI* gene had the most InDesl in *F. ovina* species.
6. **Table S5**: Sliding window analysis using DnaSP at window size of 600 bp, step size 200 bp to detect highly variable regions in the fine fescue chloroplast genome

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