**Title:** Building a Reference Transcriptome for the Hexaploid Hard Fescue Turfgrass (*Festuca brevipila*) Using a Combination of PacBio Isoseq and Illumina Sequencing

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**Abstract**

Hard fescue (*Festuca brevipila* Tracey, 2n=6x=42) is a cool-season turfgrass with a fine leaf texture that performs well under low-input management. Breeding and genetics studies of *F. brevipila* have been limited due to the complexity of its hexaploid genome. To advance our knowledge of *F. brevipila* genomics, we used PacBio isoform sequencing to develop a reference transcriptome for this species. Here, we report the *F. brevipila* reference transcriptome generated from root, crown, leaf, and seed head tissues. We obtained 59,510 full-length transcripts, of which 38,595 were non-redundant full-length transcripts. The longest and shortest transcripts were 11,487 and 58 bp, respectively. To test the polyploid origin of *F. brevipila,* we sequenced three additional transcriptomes using closely related species on an Illumina platform. The results of our phylotranscriptomic analysis supported the allopolyploid origin of *F. brevipila*. Overall, the *F. brevipila* Pacbio Isoseq reference transcriptome provided the foundation for transcriptome studies and allowed breeders for gene discovery in this important turfgrass species.

**Keywords:** hard fescue, Pacbio Isoseq, RNA sequencing, phylogenetics, turfgrass

## Introduction

Festuca (*Festuca* L., Poaceae) is a large and diverse genus that includes more than 450 species (Clayton and Renvoize, 1986). Several species in this genus have been used as ground cover turfgrass: (1) broader-leaf fescues that includes tall fescue (*Festuca arundinacea*) and meadow fescue (*Festuca pratensis*) and (2) fine-leaf fescues (Wilkinson and Stace, 1991). Five fine-leaved fescue taxa are of more interest to turfgrass breeders because of their performance in shade and drought tolerance, and adaption to low fertility and acidic soils (Carroll, 1943;Hanson and Juska, 1969;Reiter et al., 2017). These five fine fescue taxa include hard fescue (*Festuca brevipila* Tracey, 2n=6x=42), sheep fescue (*Festuca ovina*, 2n=4x=28), both of which belong to *Festuca ovina* complex; and *Festuca rubra* complex taxa: strong creeping red fescue (*Festuca rubra* ssp. *rubra* 2n=8x=56), slender creeping red fescue (*Festuca rubra* ssp. *littoralis* 2n=6x=42), and Chewings fescue (*Festuca rubra* ssp. *fallax* 2n=6x=42) (Huff and Palazzo, 1998). Previous phylogenetic study using plastid genomes have resolved the relationships among these five taxa (Qiu et al., 2019). Of the five fescue taxa, *F. brevipila* has a particularly attractive target for breeding and genetics improvement due to its excellent performance in low input environments (Ruemmele et al., 1995).

Modern breeding programs in model crop species rely heavily on the use of genomic and molecular biology tools, such as marker-assisted breeding and CRISPR-Cas 9 based genome editing to improve consumer and field production traits (Varshney et al., 2005; Gupta et al., 2010; Shan et al., 2013; Phing Lau et al., 2016). To apply these tools in turfgrass breeding, there is a need to build genomic and transcriptome resources. However, adapting molecular breeding techniques and genomic studies into *F. brevipila* has been challenging because of the lack of genetic information. As of September 2019, there were 174 nucleotide sequences for *F. brevipila*

on National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov), while perennial ryegrass (*Lolium perenne*), the turfgrass species for which the most genomics work has been done, has a reference genome available, 45,149 Sequence Read Archive (SRA) accessions, and more than 600,000 nucleotide sequences on GenBank.

RNA sequencing is a powerful tool to generate genomic data for marker development and enable researchers to study the dynamics of gene expression of plants under stress. As the cost of Illumina sequencing continues to drop, more and more researchers are using this approach for gene discovery and gene identification. Without a reference genome, researchers use *de novo* assembled short reads to generate a reference to conduct gene expression study (Grabherr et al., 2011). However, this approach is error-prone in polyploids with highly similar subgenomes (Chen et al., 2019), making it difficult to obtain full-length transcripts or identify alternative splicing (Alkan et al., 2011; Ozsolak and Milos, 2011).

PacBio sequencing, also known as long-read sequencing, has average read length over 10 kb (Rhoads and Au, 2015) and is particularly suitable for generating reference transcriptomes in polyploid species. With the capability to obtain full-length RNA sequences (Isoseq), PacBio Isoseq has been used for the discovery of new genes, isoforms, and alternative splicing events; this technology is improving genome annotation in crop species such as maize (*Zea mays*), rice (*Oryza sativa*), sugarcane (*Saccharum officinarum*), and sorghum (*Sorghum bicolor*) (Wang et al., 2016; Hoang et al., 2017; McCormick et al., 2018; Zhang et al., 2019). In turfgrass and ground cover related genetics research, PacBio Isoseq has been used to study the transcriptome and phylogeny of bermudagrass (*Cynodon dactylon*)and red clover (*Trifolium pratense*) (Chao et al., 2018; Zhang et al., 2018). Due to the highly outcrossing and polyploid nature of *F. brevipila*,

PacBio isoform sequencing is essential for generating a reference transcriptome useful for plant breeding and genetics research.

For plant breeding and germplasm improvement, USDA PI accession have proven to be a valuable resources (Pastor-Corrales, 2003; Chang and Hartman, 2017). Our previous work showed out of 127 USDA accessions, only 17.3% were confirmed to be hexaploid, while 33.1% were diploid and 26.8% were tetraploid (Qiu et al., 2020). For this reason, it is important to understand the subgenomes of the hexaploid so breeders could potentially create synthetic hexaploid to introgress traits from lower ploidy taxa. Previous GC content, average chromosome size, and genome size based evidence suggested the presence of allo/auto-polyploid in *Festuca* ssp. (Šmarda et al., 2008). However, so far there lacks nuclear sequence-based evidence to test the polyploid origin of *F. brevipila*. Previous study using the amplified fragment length polymorphism (AFLP) maker followed by neighbor-joining analysis suggested that *F. valesiaca* (2n=2x=14) is closely related to *F. brevipila* (Ma et al., 2014). Through sequencing the chloroplast genomes our previous work suggested *F. ovina* subsp *ovina* (2n=2x=14) and *F. ovina* (2n=4x=28) were both closely related to *F. brevipila* (Qiu et al., 2019). Transcriptome sequencing followed by phylogenetic analyses has been previously used to resolve allopolyploid origin of strawberry (Edger et al., 2019). Therefore here we adopt a similar phylotranscriptomic approach to test the allopolyploid origin of *F. brevipila* and investigate the relationship among its close relatives.

In this study, we used the Pacbio Sequel II platform to conduct isoform sequencing to develop a reference transcriptome for the hexaploid *F. brevipila*. We then evaluated and annotated the reference transcriptome using publicly available protein databases. We also used Illumina HiSeq 4000 RNA sequencing on leaf samples of *F. brevipila*, and closely related taxa

*F. ovina* subsp *ovina* (two accessions, one diploid and one tetraploid) and *F. valesiaca* to test the allopolyploid origin of *F. brevipila*.

## Material and Methods

### ***Plant Materials***

*Festuca brevipila* breeding material SPHD15-3 from the University of Minnesota breeding program was used for this study (plant materials available upon request). A clonal population was vegetatively propagated and grown using the BRK Promix soil (Premier Tech, USA) in the Plant Growth Facility at the University of Minnesota, St. Paul campus under 14 hours daylight and 8 hours darkness with bi-daily irrigation and weekly fertilization (906 grams of Ammonium sulfate, 950 grams of Peat-lite 20-10-20 and 38 grams of Sprint 330 mixed into 5 gallons of water with a Dositron fertilizer injector set at a 1:100 ratio).

To capture a broad representation of the *F. brevipila* transcriptome using PacBio Isoform sequencing, we collected root, crown, leaf, and inflorescence tissues. Plants were vernalized in a 4°C cold room for 3 months with 8 hours daylight and 16 hours darkness The plants were then moved back to greenhouse conditions as described above with day time temperature of 25°C to recover for one month and allow inflorescence development. The root, crown, leaf, and

inflorescence tissues were harvested and flash frozen for RNA extraction (**Figure 1**).

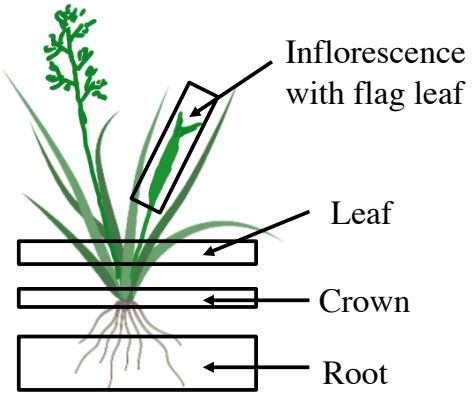


**Figure 1**. Plant tissues used for PacBio Isoform and RNA sequencing.

To perform phylogenetic study on *F. brevipila* and closely related taxa, leaf samples of *F. ovina* subsp *ovina* (PI 676177), *F. valesiaca* (PI 422463), *F. ovina* cv. Quatro, and *F. brevipila* were grown in the greenhouse with the same condition described above and leaf samples were harvested and flash frozen for RNA extraction.

***RNA Extraction and Sequencing***

To maintain RNA integrity, RNA extractions for Isoseq were done using a Quick-RNA Miniprep Kit (ZYMO Research, Catalog number R1055) in a cold room following the manufacturer's instructions. RNA extraction for Illumina sequencing was performed at room temperature using the same method. A Qubit Fluorometer (ThermoFisher Scientific) was used for initial RNA quantification, before an Agilent 2100 Bioanalyzer (Agilent) was used to assess the RNA integrity. Only samples with a RIN >8.0 were used for sequencing library construction.

For PacBio Isoform sequencing, four sequencing libraries (one for each tissue type) were constructed using the SMARTer PCR cDNA Synthesis Kit (ClonTech, Takara Bio Inc., Shiga,

Japan) by tissue type with no size selection by NovoGene, China. Sequencing was performed on a PacBio Sequel II (PacBio, CA) instrument. Illumina sequencing libraries were constructed using a TruSeq® Stranded mRNA Library Prep kit (Illumina) and sequenced on an Illumina HiSeq 4000 instrument in 150 bp paired-end sequencing mode. All sequencing data generated from this study have been deposited in NCBI SRA under bioProject PRJNA598357.

***Illumina Sequencing Data Processing and de novo Assembly***

Illumina adaptor sequences were trimmed using Trimmomatic with the default settings (v 0.32) (Bolger et al., 2014). Quality trimming was performed using the seqtk tool with -q 0.01 (https://github.com/lh3/seqtk). Trinity (v 2.4.0) was used to assemble transcriptomes of *F. ovina* subsp *ovina*, *F. valeciaca*, *F. ovina* cv. Quatro, and *F. brevipila* (Grabherr et al., 2011) with max memory 62G, 24 CPUs, bflyCalculateCPU, and minimum contig size of 200 bp.

***PacBio isoform sequencing (Iso-Seq) Data Processing***

The software SMRTlink (v 2.3.0, PacBio) was used to filter and process original sequencing files, with minLength 0, minReadScore 0.8 to produce the subreads file. To identify full-length transcripts, the Isoseq 3 (v 3.1.0, PacificBiosciences) pipeline was installed locally using conda (Anaconda-2.3.0) under bioconda following instruction by PacBio (https://github.com/PacificBiosciences/IsoSeq_SA3nUP). Circular consensus sequences (ccs) bam files were generated using ccs command with --noPolish, --maxPoaCoverage 10, and 1 minimum passes options. To classify full-length (FL) reads, we identified and trimmed the 5' and 3' cDNA primers (Primer sequences can be found in **Table S1)** in the ccs using lima with --dump-clips, --no-pbi, --peak-guess option. Poly(A) tails in FL reads that were at least 20 bp long were removed with --require-polya. Bam files produced from the previous steps for four tissues types were merged into one dataset, the source files were also merged into a subreadset.xml file.

Clustered reads were polished using the isoseq3 polish function to produce high-quality FL transcripts (expected accuracy ≥ 99% or QV ≥ 30).

To correct the PacBio Isoseq FL transcripts, high quality Illumina paired-end reads from *F. brevipila* leaf tissue were used in the LoRDEC -correct function in LoRDEC program (v 0.6) with configuration *k-mer* size = 19 and 21, solid-*k-mer* = 3, error rate 0.4, and maximum branch time 200 (Salmela and Rivals, 2014).

***Removing Sequence Contamination and Collapsing Redundant Sequences***

To remove microbial sequence contamination in both Isoseq and Illumina *de novo* transcriptomes, bacterial and virus genomes were downloaded from NCBI ftp://ftp.ncbi.nih.gov/genomes/archive/old_refseq/Bacteria/all.fna.tar.gz, ftp://ftp.ncbi.nih.gov/genomes/Viruses/all.fna.tar.gz, a mapping index was built using bwa64 (v 0.5.9-r16) included in the deconseq program (v 0.4.3) (Schmieder and Edwards, 2011). Contaminant transcripts were removed using the deconseq.pl script in the deconseq program before performing downstream analysis. To merge and remove redundant transcripts in each transcriptome 'cd-hit-est' from the CD-HIT (v 4.8.1) package was used with the following parameters: -c 0.99 -G 0 -aL 0.00 -aS 0.99 -AS 30 -M 0 -d 0 -p 1 (Isoseq transcriptome); -c 0.98 -p 1 -T 10 (*de novo* assembly) (Li and Godzik, 2006).

***Transcriptome Completeness Analysis***

To evaluate the completeness of the assembled *F. brevipila* transcriptomes, we used 1335 core embryophyte genes (embryophyta_odb10) from Benchmarking Universal Single-Copy Orthologs (BUSCO v3) (Simão et al., 2015). Besides individual evaluation of Isoseq and *de novo* transcriptomes, we also combined non-redundant full length (NR-FL) transcripts and non-redundant (NR) *de novo* transcriptomes and used cd-hit-est to cluster redundant sequences with

95% sequence identity (Li and Godzik, 2006). The completeness of the combined assembly was also evaluated using the same BUSCO method described above. To evaluate how combined assembly could improve short read mapping, we mapped RNA sequencing reads used for *de novo* assembly to the two individual and the combined assembly using bowtie2 program (Langmead and Salzberg, 2012). Mapping statistics was summarized using the samstat tool (v 1.5.1) (Lassmann et al., 2010).

***Coding Genome Reconstruction***

To reconstruct the coding genome of *F. brevipila*, non-redundant full length transcripts were processed using the COding GENome reconstruction Tool (Cogent, v 3.3, https://github.com/Magdoll/Cogent) with minimap (v 2.17-r941, https://github.com/lh3/minimap2), Mash (v 1.0.2, https://github.com/marbl/Mash/releases), and BioPython (v. 1.71). Briefly, k-mer similarity was used to partition the non-redundant Pacbio Isoseq transcriptome into gene families. Undirected weight graphs were constructed based on the percentage of matching k-mer sketches. Python scripts run_preCluster.py, generate_batch_cmd_for_Cogent_family_finding.py (https://github.com/Magdoll/Cogent) with k-mer size 30, and reconstruct_contig.py were used to reconstruct the coding genome.

***Transcriptome Functional Annotation***

The NR-FL transcriptome was searched against NCBI NR protein and SwisProt, UniProt protein database using diamond BLASTx (v 0.9.13) with e-value < 1e-5.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the KEGG Automatic Annotation Server (KASS, https://www.genome.jp/kegg/kaas/). *Oryza sativa*, *Brassica napus*, *Zea mays*, and *Arabidopsis thaliana* were set as references with a single-

directional best hit model. Gene Ontology (GO) terms were produced by the interproscan program (Quevillon et al., 2005).

***Identification of lncRNAs and miRNA from PacBio Sequences***

The prediction of long non-coding RNAs (lncRNAs) was performed using an improved *k-mer* scheme (PLEK) tool (v 1.2) with a minimum sequence length of 200 bp (Li et al., 2014). Putative miRNA precursors were identified by BLASTn of NR-FL sequences against the plant miRNAs database (http://www.mirbase.org) that includes both hairpin and mature miRNA with e-value ≤ $10^{-5}$ (Kozomara and Griffiths-Jones, 2013). For each sequence the BLASTn hit with the highest bit score was kept.

***Phylotranscriptomic Analyses of Festuca-Lolium Taxa***

To test the allopolyploid origin of *F. brevipila*, we also generated the transcriptome for three additional taxa (*F. ovina* subsp. *ovina*, *F. valeciaca*, *F. ovina*) in this study using *de novo* assembly method described above. In addition, we included four publicly available transcriptomes from closely related species selected according to previous phylogenetic analyses based on chloroplast sequences (**Table 1**)(Hand et al., 2013; Qiu et al., 2019). Protein and coding sequences of all *Festuca – Lolium* taxa were predicted using the TransDecoder program (https://github.com/TransDecoder) (Wu and Watanabe, 2005). *Brachypodium distachyon* was included as the outgroup.

**Table 1.** Sources of transcriptome and chloroplast genome sequences used in this study. Sequences newly generated in this study were in bold.

| Taxon | Transcriptome Sources | Chloroplast Genome Sequence |
|---|---|---|
| ***Festuca ovina* subsp. *ovina*** (2n=2x=14) | Illumina (SRR10997225) | *de novo contigs* |

| | | |
|---|---|---|
| ***Festuca ovina*** (2n=4x=28) | Illumina (SRR10997223) | MN309824.1 (same genotype for used for Illumina sequencing) |
| ***Festuca valesiaca*** (2n=2x=14) | Illumina (SRR10997224) | *de novo contigs* |
| ***Festuca brevipila*** (2n=6x=42) | Pacbio Isoseq with Illumine short reads correction (http://hdl.handle.net/11299/211413) | *de novo contigs* |
| *Lolium perenne* | Perennial Ryegrass Genome Sequencing Project | AM777385.2 |
| *Lolium multiflorum* | ftp.ebi.ac.uk GBXX01 | JX871942.1 |
| *Festuca pratensis* | ftp.ebi.ac.uk GBXZ01 | JX871941.1 |
| *Festuca arundinacea* | ftp.ebi.ac.uk GCTP01 | KM974751.1 |
| *Brachypodium distachyon* | https://plants.ensembl.org (v 3.0) | LT558603.1 |

Orthology inference was carried out using protein sequences of all nine taxa in OrthoFinder 2 (Emms and Kelly, 2019). Single-copy protein sequences were aligned using the MUSCLE program (v 3.8.31) with the parameters -maxiters 16 -diags -sv (Edgar, 2004). To improve the phylogenetic resolution among closely related taxa, we used the PAL2NAL program (v 14) to align coding sequences according to the protein alignment (Suyama et al., 2006). The resulting coding sequence alignments were trimmed using TrimAl with the parameters -gt 0.9 -cons 60, which removes all positions in the alignment with gaps in 10% or more of the sequences and keeps at least 60% of the sequence alignment (Capella-Gutiérrez et al., 2009).

To trace the maternal relationships of the focal taxa, chloroplast gene sequences for *F. ovina* subsp. *ovina*, *F. valeciaca*, and *F. brevipila* were extracted from *de novo* assembled transcriptomes by aligning assembled contigs to the *F. ovina* reference chloroplast genome (MN309824) using Burrows-Wheeler Aligner (Li and Durbin, 2009). Sequences for the other five taxa were obtained from NCBI by BLASTn using *F. ovina* reference chloroplast genome sequences as the query. *Brachypodium* chloroplast sequence was used as the outgroup (**Table 1**).

Three fragments of chloroplast genome sequence that covered *rps2-atpA*, *rpl32 -ccsA*, and *clpP – psbH* region had the highest completeness and taxon representation and were aligned using MAFFT (v 7) and concatenated (Katoh and Standley, 2013).

Phylogenetic trees were constructed using the RAxML program (v 8.2.12) for both the concatenated nuclear and chloroplast alignments under the GTR+GAMMA model with 1,000 bootstrap (Stamatakis, 2006). The phylogenetic trees were visualized using FigTree v 1.4.3 (Rambaut, 2012). Because the chloroplast sequences and transcriptomes for *L. perenne, L. multiflorum, F. pratensis, F. arundinacea, B. distachyon* were downloaded from NCBI/EBI and they might not come from the same genotypes, for these taxa, we only present the nuclear and chloroplast phylogenies without carrying out conflict analyses.

Next, we further explored phylogenetic conflict between chloroplast and nuclear gene trees conflicts among *F. ovina* subsp. *ovina*, *F. ovina,* and *F. valeciaca* using transcriptomes generated in this study and the reference transcriptome for *F. brevipila*. *Lolium perenne* and *B. distachyon* were included as outgroups. We carried out a new OrthoFinder run with a reduced set of taxa and we used single-copy genes predicted from the new run. The coding sequence alignment for each single-copy gene was generated using the methods described above. Gene trees were estimated using the RAxML program (v 8.2.12) under GTR+GAMMA model with 1000 bootstrap. A species tree was estimated using ASTRAL v 5.6.3 using gene tress from previous step (Mirarab et al., 2014). The concordance and conflicts among gene trees and were visualized using Phypart (Smith et al., 2015) by mapping single-copy gene trees rooted by *Brachypodium distachyon* to the rooted species tree from ASTRAL. Tree concordance and conflicts were plotted using the python script phypartspiecharts.py available at

https://github.com/mossmatters/phyloscripts/tree/ master/phypartspiecharts (last accessed on Feb 2, 2020)

## Results

### ***Festuca brevipila Transcriptome Assembly***

Using Pacbio Isoseq, a total of 76.3 GB of sequence was generated from four SMRT cells, 73.91 Gb (48,788,662) of which were subreads. The Isoseq 3 pipeline identified 60,719 putative high-quality full length (FL) transcripts and 487 low-quality FL transcripts. After removing microbial contamination and plastid genome sequences, 59,510 FL transcripts were retained (98.01%) with a N50 of 2,668 bp, a total length of 130,657,908 bp. For Illumina sequencing on *F. brevipila* leaf tissue, a total of 37,100,522 paired-end reads were generated (SRR10995913). The reads were used for PacBio reads error correction. Finally, we generated the *F. brevipila* transcriptome which included 59,510 FL transcripts with a N50 of 2,667 bp and a total length of 130,631,252 bp. After removing redundant sequences, we obtained 38,556 (64.78%) unique transcripts with a total length of 82,782,352 bp and a N50 of 2,584 bp. The longest and shortest transcripts were 11,487 and 58 bp, respectively. The GC% content of the transcriptome was 51.5%, with the majority of transcripts size above 1,000 bp (83.3%) **Figure 2**.

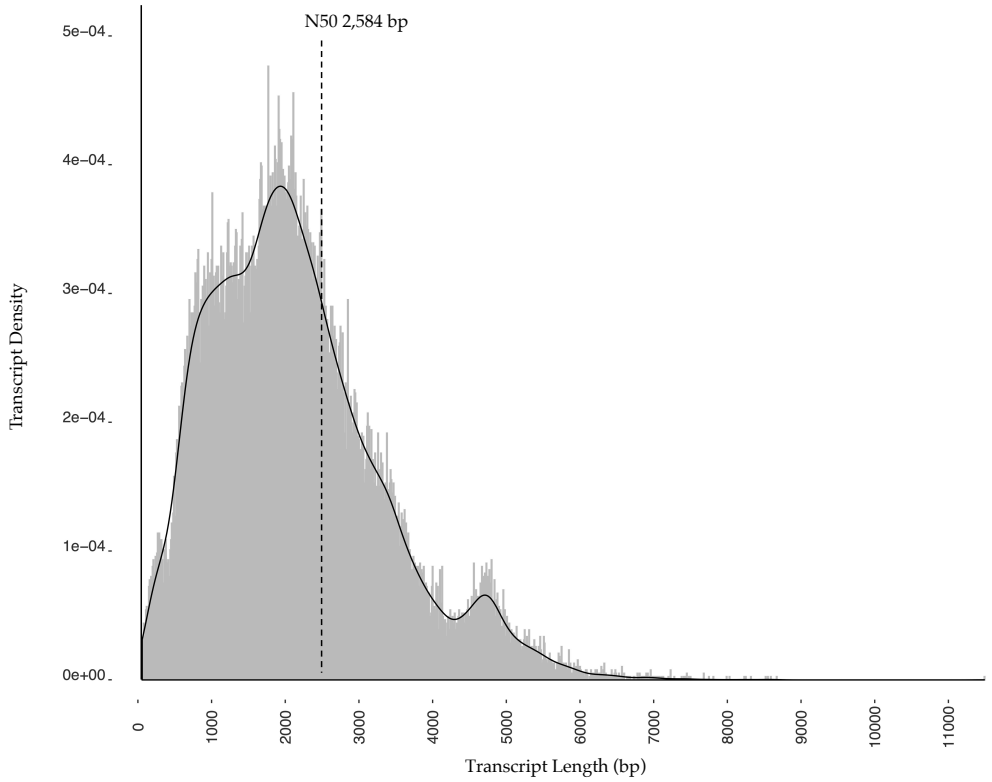


**Figure 2**. Transcript length distribution after redundant sequence removal.

*De novo* assembly of llumina reads recovered 325,781 assembled contigs. After contamination and redundancy removal, 302,471 contigs were retained with a N50 of 1,157 bp. Because of the complexity of the hexaploid genome, a short and long reads combined assembly was generated that included 248,318 transcripts and an N50 of 1,405 bp. A total of 20,063 transcripts were included from the Isoseq transcriptome by cd-hit-est (**Table 2**).

**Table 2.** Statistics from *Festuca brevipila* PacBio Isoseq, *de novo* transcriptome, and combined assemblies. No decontamination was carried out for the combined assembly.

| **Assembly** | **Total Contigs** | **Contigs After Contaminants Removal** | **Contigs After Redundancy Removal** | **N50 (bp)** |
|---|---|---|---|---|
| *de novo* | 325,781 | 322,110 | 302,471 (92.84%) | 1,157 |
| PacBio Isoseq | 60,719 | 59,510 | 38,556 (64.79%) | 2,584 |
| Combined Transcritpome (cd-hit-est) | 341,027 | NA | 248,314 (72.81%) | 1,405 |

When evaluating the completeness of the assemblies, the Illumina *de novo* assembly showed better coverage (94.6%, fragmented BUSCO included) than the PacBio Isoseq transcriptome (71.85%, fragmented BUSCO included). The combined assembly outperformed the individual assemblies for better coverage (96.07%, fragmented BUSCO included) and completeness (89.24%) (**Figure 3**).

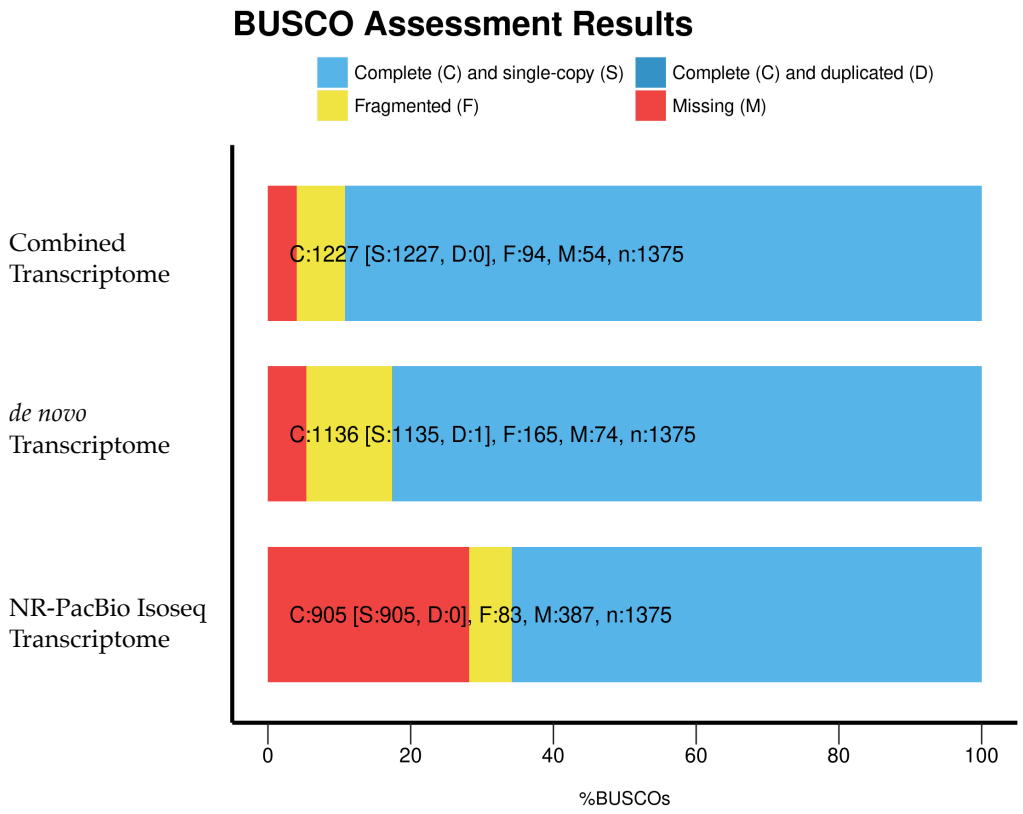


**Figure 3**. BUSCO assessment of the three assemblies. The combined assembly had the most complete BUSCO genes and the least missing genes.

Around 60% of the fragmented and 35% of the missing BUSCO genes from the Illumina-only assembly were complete in the hybrid assembly (**Figure 4A**). Similarly, 54% of the fragmented and 78% of the missing BUSCO genes from the PacBio assembly were marked as

completed in the hybrid assembly (**Figure 4B**).

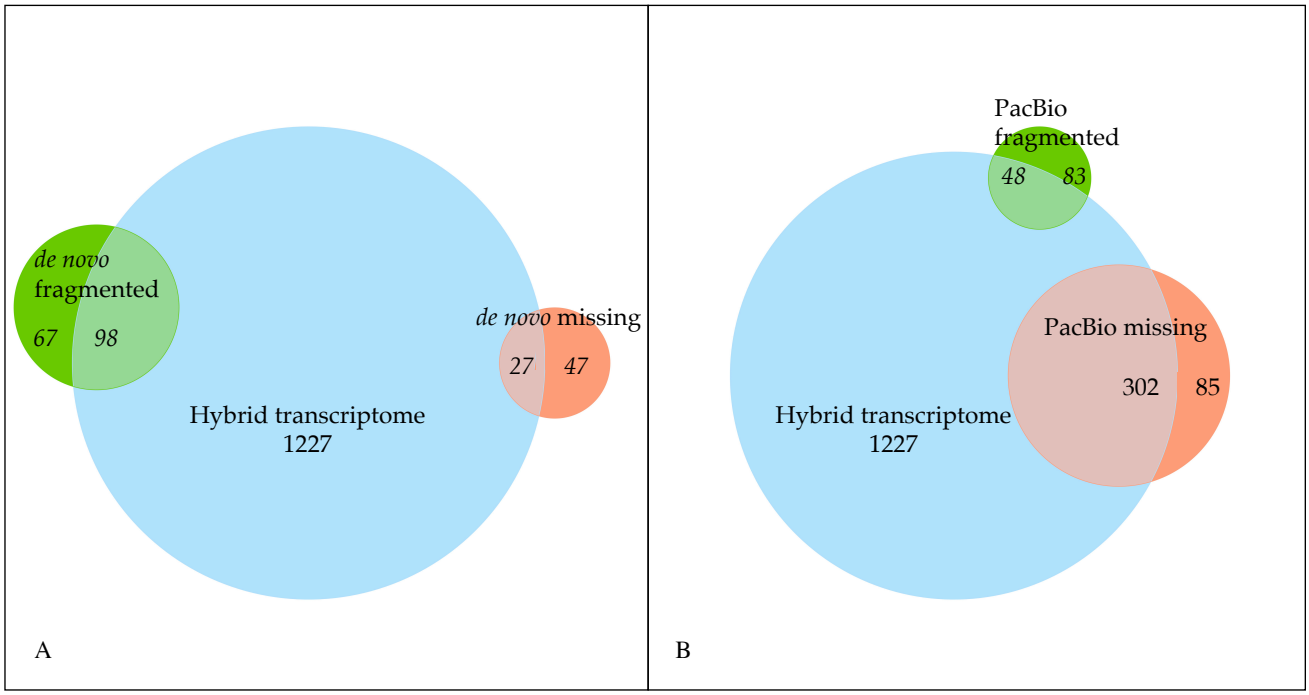


**Figure 4**. Comparison of BUSCO completeness. Fragmented or missing BUSCO in the Illumina assembly (A) or the PacBio assembly (B) but were complete (1,227) in the hybrid assembly.

When mapping Illumina reads from the *F. brevipila* leaf tissue back to the three assemblies, the percent of unmapped reads for PacBio Isoseq, Illumina, and combined assembly was 34.5%, 38.2%, and 28.5%, respectively.

***Coding Genome Reconstruction***

In the absence of a reference genome, we reconstructed the coding genome for detecting potential splicing events in future studies. After k-mer based clustering and minimizing edges of graphs assuming each gene family had the similar k-mer pattern, the Cogent program reconstructed 6,043 gene families (**Figure 5**). Of them, 4,384 gene families had one path, 1,285 had 2 paths, and one had the most paths of 19. A total of 153 transcripts were absent from the final coding genome.

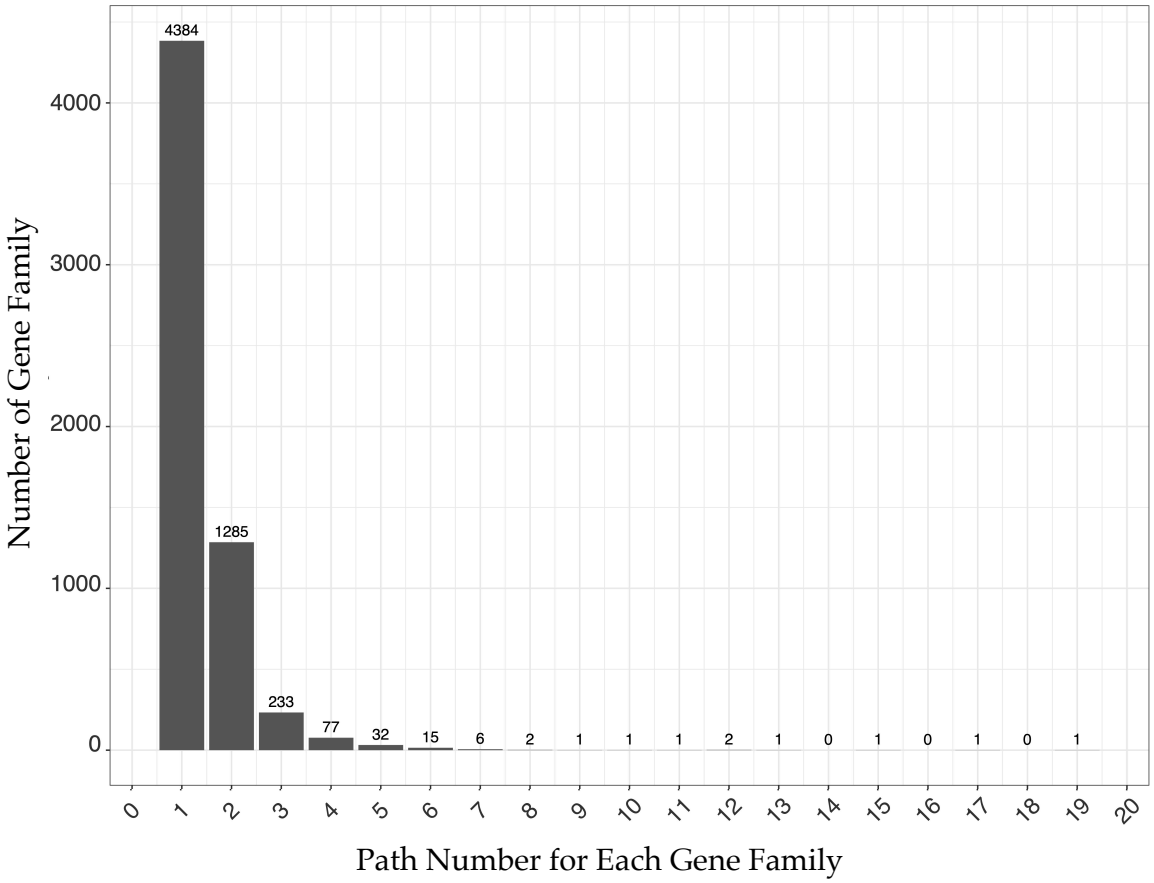


**Figure 5**. Distribution of number of paths for constructed gene families from coding genome reconstruction. The majority of gene families had one path. A total of eight gene families had more than ten paths in the reconstructed coding genome.

***<u>Transcript Functional Annotation</u>***

We carried out functional annotation of the PacBio Isoseq transcriptome using multiple databases including NCBI NR protein, UniRef90, SwisProt, KEGG, and Pfam (GO terms) (**Figure 6**). Around 25% transcripts had annotation in all five databases and 36,067(93.54%) transcripts had annotation from more than one database.

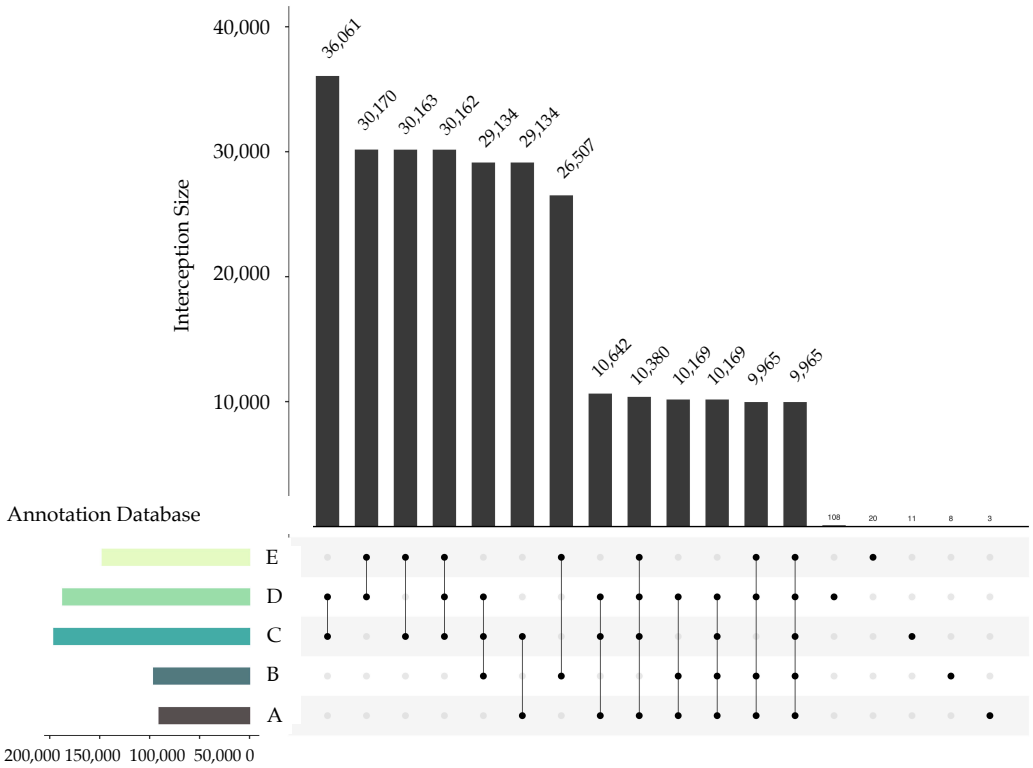


**Figure 6**. Comparison of *Festuca brevipila* PacBio Isoseq transcriptome annotation using different databases. A: Kyoto Encyclopedia of Genes and Genomes; B: Gene Ontology; C: NCBI NR Protein; D: UniRef90; E: SwisProt.

***Identification of lncRNAs and miRNA from PacBio Sequences***

In the NR PacBio Isoseq transcriptome, we identified 7,868 transcripts that were long non-coding RNA. A total of 145 transcripts were identified as hairpin miRNA, and non-plant hits were removed from further analysis. A total of 39.04% of the miRNA was annotated in *Hordeum vulgare*, 19.18% was annotated in *O. sativa*, and 17.8% was annotated in *F. arundinacea* (**Table S2**). The most copies found was hvu-MIR6179 with 16 copies, followed by far-MIR1119 and osa-MIR2927, both of which had 11 copies (**Figure 7**)

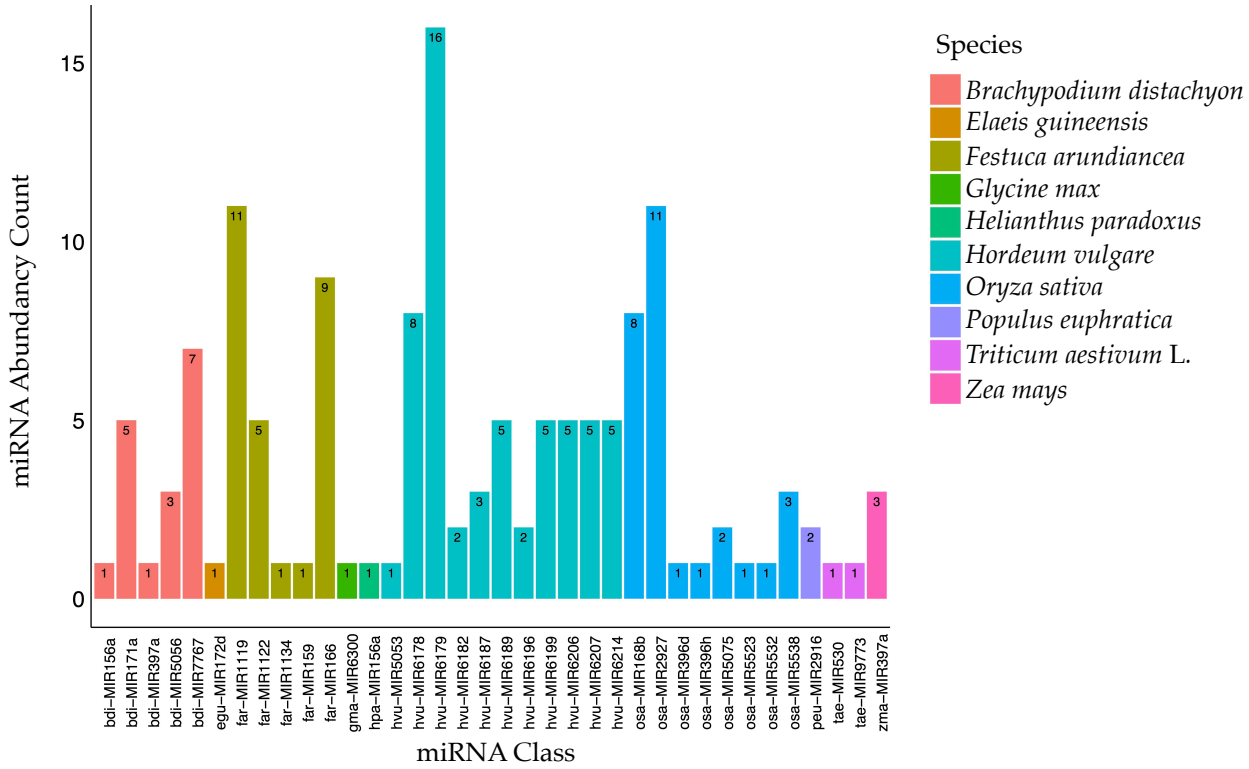


**Figure 7**. miRNA identified in the PacBio Isoseq reference transcriptome. Most of the homologous miRNAs were identified in barley (*Hordeum vulgare*) and tall fescue (*Festuca arundiancea*). With MIR6179, MIR1119, and MIR2927 had the most abundance.

***<u>Phylotranscriptomic Analyses of the Festuca-Lolium Complex</u>***

A total of 86,445,109 paired end reads were generated from the Illumina sequencing for *F. ovina* subsp. *ovina*, *F. valeciaca*, *F. ovina*. After removing microbes contaminates, the assembly statistics was summarized in **Table 3**.

**Table 3**. Summary statistics of Illumina sequencing and *de novo* transcriptome assembly. No decontamination was carried out for the three assemblies.

| **Taxon** | **Number of raw reads** | **Number of Contigs** | **N50 (bp)** |
|---|---|---|---|
| *F. ovina* (4x) | 26,640,768 | 242,794 | 1,230 |
| *Festuca ovina* subsp. *ovina* (2x) | 24,719,943 | 179,393 | 1,664 |
| *F. valeciaca* (2x) | 35,084,398 | 157,926 | 1,770 |

OrthoFinder identified 29 single copy nuclear genes across the 9 taxa to reconstruct the nuclear gene trees and three chloroplast genome regions that included 9,680 bp sequence which covered the *rps2-atpA*, *rpl32 -ccsA*, and *clpP – psbH* regions. They were likely to be DNA contamination in the RNA sequencing library, because these contigs spanned gene and intergenic regions. The topology between concatenated nuclear and plastome datasets were mostly congruent, except the placement of *F. brevipila* and *F. valesiaca* (**Figure 8**).

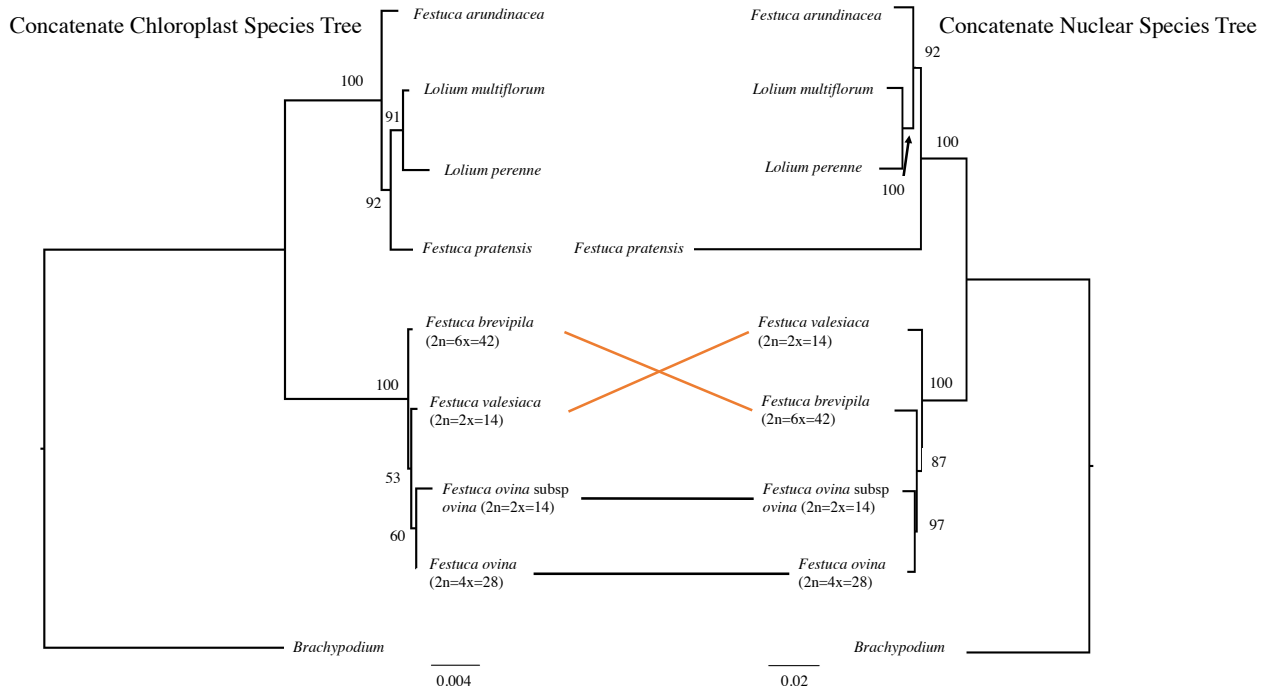


**Figure 8**. Nuclear and chloroplast species tree of the 9 taxa used in this study. In *F. ovina* complex, concordance relationship was linked in black while conflicts were linked in orange.

To explore the conflict among gene trees within the *F. ovina* complex, we assembled a second phylotranscriptomic dataset with all four taxa in the complex and two outgroups. OrthoFinder recovered 286 single copy genes. The resulting species tree topology by ASTRAL was congruent with the concatenated tree recovered from the 9-taxa nuclear dataset (**Figure 9**).

However, significant proportion of informative gene trees (bootstrap >50) supported alternative placements of *F. brevipila* (red and green in Fig. 9) being either sister to *F. valesiaca*, *F. ovina* 2x, or *F. ovina* ssp. *ovina* 4x.

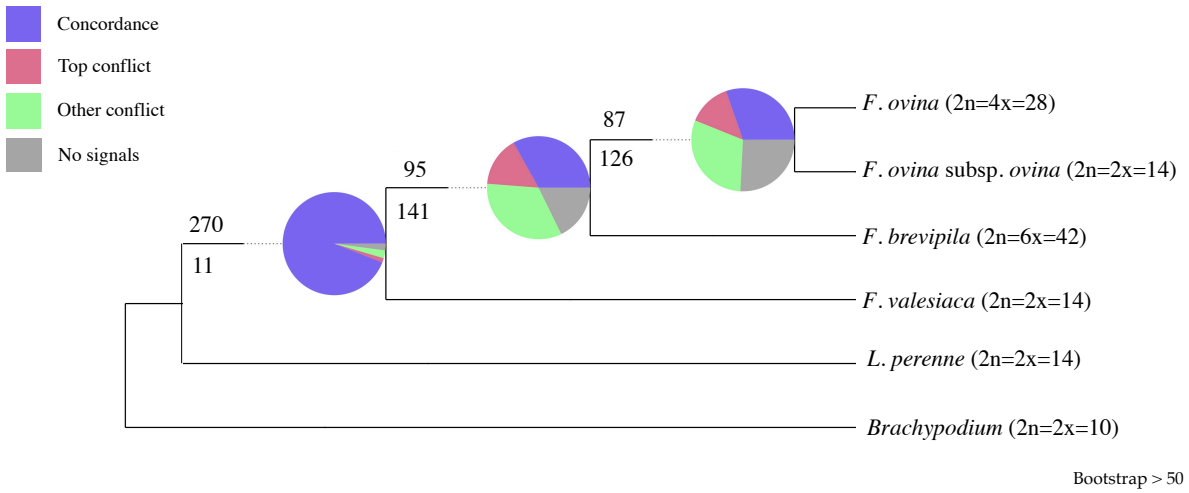


**Figure 9**. Species tree of *F. ovina* complex reconstructed by ASTRAL using 286 single-copy genes. Pie charts at nodes indicate the number of gene trees that were concordant (blue) or conflict (top conflict in red and all other conflicts in green) with the species tree topology. Total number of concordance gene trees were above the branch and the total number of conflicting gene trees were below the branch.

**Discussion**

In this study, we used the PacBio isoform sequencing platform to generate a reference transcriptome of *F. brevipila* using root, leaf, crown, and seed head tissue types. We used multiple protein and pathway databases to annotate the transcriptome and reconstruct the coding genome in the absence of a reference genome. We used Illumina sequencing data from *F. brevipila* leaf tissue to generate a *de novo* assembly and a combined assembly to improve the coverage and of the transcriptome. In addition, we carried out phylotranscriptomic analyses to test the allopolyploid origin of *F. brevipila*.

We took advantage of both short and long reads sequencing platform to develop an improved *F. brevipila* reference transcriptome. Comparing our PacBio Isoseq, Illumina *de novo*, and the combined assembly, PacBio Isoseq transcriptome had the best N50 of 2,584 bp while *de novo* assembly only had N50 of 1,157 bp. The combined contigs had the highest BUSCO completes (89.2%), reduced fragmented BUSCO (6.8%), and the lowest missing BUSCO (4%). This suggested the combined contigs improved the overall completeness of the assembly. In addition, when mapping Illumina sequencing reads to the three assemblies, the combined assembly has the least unmapped reads, followed by Pacbio Isoseq, and *de novo* assembly. The PacBio assembly has the best mapping quality (42.9% MAPQ >=30), followed by the *de novo,* and then the hybrid assembly. The combined assembly had the highest number of reads with MAPQ <3 (Mapping to multiple region of the transcriptome), which suggested that during the process to combine multiple assemblies using cd-hit-est, the sequence identity threshold played a crucial role. In our case, we used a 95% sequencing identity cut off. By using a lower cut off, we would likely increase the unique mapping (MAPQ>=30), however, we would potentially lose homolog or paralog information due to the hexaploidy nature of the taxon. Similarly, if we used a more stringent cutoff, we would be likely to maintain more sequence information but lose more unique reads. This is an inherent problem in polyploid transcriptomics and future whole genome sequencing will help researchers to overcome these limitations.

Although PacBio Isoseq has proven to improve the transcriptome quality, it has several limitations. First of all, it has a higher cost and lower sequencing coverage compared to the Illumina dataset. In our case, based on the BUSCO evaluation, 83 out of the 988 present BUSCO genes are fragmented. When comparing the BUSCO assessment result, the PacBio Isoseq assembly had 28.8% BUSCO genes missing while the short read *de novo* assembly had only

5.4% missing even though the data came from single tissue. In addition, PacBio sequencing has a higher error rate. However, all these limitation could be reduced by increasing the number of SMRT cell for sequencing, improvement of sequencing chemistry, enzyme (Hi-Fi), and a combination of using Illumina short reads sequencing and long reads (Rhoads and Au, 2015; Wenger et al., 2019).

When a genome is large and complex, obtaining a well-annotated genome is challenging. Pacbio Isoseq allowed us to reconstruct the transcribed regions of the genome (coding genome) using isoform transcripts to simplify the process. When constructing the *F. brevipila* coding genome using k-mer based gene family clustering approach, 153 transcripts failed to cluster into gene families. This is likely due to the undetected cycles in the graph. Using different k-mer lengths could potentially resolve the problem, although we also tried k-mers of 40 and 50 and still failed to reconstruction the transcripts. In our dataset, the majority of genes families had one (72.54%) or two (21.26%) paths. Having multiple paths is likely due to a single path cannot resolve the variation from paralogs, exon skipping, and sequencing artifacts. The coding genome generated in this study will be used in future *F. brevipila* genomics research to predict and visualize RNA alternative splicing events.

In addition to the reconstruction of coding genome, which enables researchers to look for RNA alternative splicing events, we also identified the potential microRNA (miRNA) precursors. When searching miRNA precursor transcripts in our dataset, we found MIR6179, MIR1119, and MIR2927 had the highest presence in the four tissue types sampled. Previous studies have shown that MIR1119 is essential in regulating plant drought tolerance in wheat and dehydration stress in barley (Kantar et al., 2010; Shi et al., 2018). MIR2927 has shown to regulate abiotic (salt) and biotic (virus) stress in rice (Sanan-Mishra et al., 2009). The function of

MIR6179 was found to associate with gametocidal action in wheat (Wang et al., 2018). It is likely that the high abundancy of those miRNAs reflect the importance of the pathways being targeted in *F. brevipila*.

Finally, to test the allopolyploid origin of *F. brevipila*, we used the transcriptome data to construct the phylogenetic relationship among closely related taxa in the *Festuca – Lolium* complex. When comparing the phylogeny from maternal (chloroplast) vs. bi-parentally inherited (nuclear) genes, we noticed a conflict at the placement of *F. brevipila* (6x) and *F. valeciaca* (2x; Fig. 8). Among nuclear gene trees (Fig. 9), 40.25% of informative gene trees supported the nuclear species tree topology, with no single dominant alternative topology. Given the short internal branch length among *F. brevipila* (6x), *F. valeciaca* and *F. ovina* (2x and 4x), hybridization, incomplete lineage sorting, and assembly artifact may contribute to the conflict among the nuclear genes. However, when we plotted the synonymous substitutions per site (*Ks*), we did not observe a clear peak in any of our ingroup species (**Figure S1**), indicating that the polyploidy event happened relatively recently. Together our data had no clear support for or against an allo- vs. autopolyploidy origin of *F. brevipila*. Previous studies suggest that *Festuca* taxa can hybridize with relatives easily (Jenkin and Jenkin, 1955). It is possible that *F. brevipila*, *F. ovina*, *F. ovina* subsp *ovina* share some subgenomes. Ideally, we could use ortholog group information to further investigate this event, however, due to the lack of a polyploid reference genome, it is challenging to tease apart homeologs in our current dataset. Future studies could be focused on whole genome sequencing and screening additional taxa that are closely related to *F. brevipila* to identify the potential subgenome of this important hexaploid turfgrass taxon.

**Conclusion**

In this study, we developed the reference transcriptome of *F. brevipila* using PacBio isoform sequencing and Illumina sequencing. We also carried out phylotranscriptomic analyses to test the allopolyploid origin of *F. brevipila*. However, the results were unable to distinguish auto- vs. allopolyploid due to the recent polyploidy events in *F. brevipila*. This dataset provided insight about a complicated hexaploid transcriptome and laid the foundation for future genomics research on this important turfgrass taxon.

**Author Contributions:** Y. Q. performed the experiments, analyzed the data, and wrote the manuscript; Y. Y. helped with phylogenetic analysis; C. D. H. helped with the genomics analysis; E. W. secured funding for this project, supervised this research, provided suggestions, and comments. All authors contributed to the revision of the manuscript and approved the final version.

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**Conflicts of Interest:** The authors declare no conflict of interest.

**Supplemental Information**

**SI Table 1.** Primer sequences used for ccs file trimming using the lima program

| **Primer ID** | **Primer Sequence** |
|---|---|
| NEB_5p | GCAATGAAGTCGCAGGGTTGGG |
| NEB_Clontech_3p | GTACTCTGCGTTGATACCACTGCTT |
| Clontech_5p | AAGCAGTGGTATCAACGCAGAGTACATGGGG |

**Table S2** . miRNA gene families identified in *F. brevipila* NR transcriptome.

| Species | miRNA family |
|---|---|
| *Brachypodium distachyon* (5) | bdi-MIR156a |
| | bdi-MIR171a |
| | bdi-MIR397a |
| | bdi-MIR5056 |
| | bdi-MIR7767 |
| *Festuca arundinacea* (5) | far-MIR1119 |
| | far-MIR1122 |
| | far-MIR1134 |
| | far-MIR159 |
| | far-MIR166 |
| *Hordeum vulgare* (12) | hvu-MIR5053 |
| | hvu-MIR6178 |
| | hvu-MIR6179 |
| | hvu-MIR6182 |
| | hvu-MIR6187 |
| | hvu-MIR6189 |
| | hvu-MIR6196 |
| | hvu-MIR6199 |
| | hvu-MIR6206 |
| | hvu-MIR6207 |
| | hvu-MIR6214 |
| *Oryza sativa* (8) | osa-MIR168b |
| | osa-MIR2927 |
| | osa-MIR396d |
| | osa-MIR396h |
| | osa-MIR5075 |
| | osa-MIR5523 |
| | osa-MIR5532 |
| | osa-MIR5538 |
| *Zea mays* (1) | zma-MIR397a |
| *Triticum aestivum* (2) | tae-MIR530 |
| | tae-MIR9773 |
| *Populus euphratica* (1) | peu-MIR2916 |
| *Glycine max* (1) | gma-MIR6300 |
| *Elaeis guineensis* (1) | hpa-MIR156a |

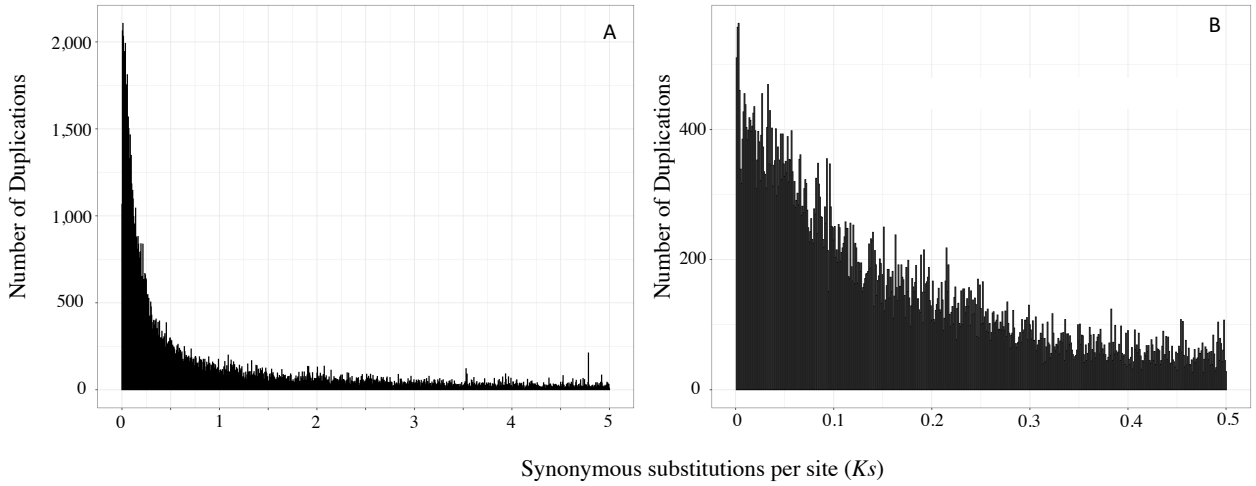


**Figure S1** The Synonymous substitutions per site (*Ks*) analysis of *F. brevipila* using *de novo* contigs that were mapped to PacBio Isoseq trnascriptome. Analysis was done using FASTKs program (https://github.com/mrmckain/FASTKs). *Ks* was subset to 5.0 in (A), and 0.5 in (B). No peaks were identified based on this analysis

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