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Genomic Resources Note

Sequencing and de novo transcriptome assembly of Brachypodium sylvaticum (Poaceae)\(^1\)

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Premise of the study: We report the de novo assembly and characterization of the transcriptomes of Brachypodium sylvaticum (slender false-brome) accessions from native populations of Spain and Greece, and an invasive population west of Corvallis, Oregon, USA.

Methods and Results: More than 350 million sequence reads from the mRNA libraries prepared from three B. sylvaticum genotypes were assembled into 120,091 (Corvallis), 104,950 (Spain), and 177,682 (Greece) transcript contigs. In comparison with the B. distachyon Bd21 reference genome and GenBank protein sequences, we estimate >90% exome coverage for B. sylvaticum. The transcripts were assigned Gene Ontology and InterPro annotations. Brachypodium sylvaticum sequence reads aligned against the Bd21 genome revealed 394,654 single-nucleotide polymorphisms (SNPs) and >20,000 simple sequence repeat (SSR) DNA sites.

Conclusions: To our knowledge, this is the first report of transcriptome sequencing of invasive plant species with a closely related sequenced reference genome. The sequences and identified SNP variant and SSR sites will provide tools for developing novel genetic markers for use in genotyping and characterization of invasive behavior of B. sylvaticum.

Key words: Brachypodium sylvaticum; comparative genomics; de novo transcriptome; invasive species; simple sequence repeat (SSR); single-nucleotide polymorphism (SNP).

Brachypodium sylvaticum (Huds.) P. Beauv. (slender false-brome; Poaceae), with an estimated genome size of 470 Mb and 17 chromosomes (Foote et al., 2004), is a perennial bunchgrass native to Europe, Asia, and North Africa and is closely related to the bioenergy feedstock model grass B. distachyon (L.) P. Beauv. (Wolny et al., 2011), which has a sequenced genome of 272 Mb and five chromosomes. In its native range, B. sylvaticum occurs in habitats ranging from forest understory to open meadows and tolerates conditions from full shade to full sun (Holten, 1980; Long, 1989; Aarrestad, 2000; Kirby and Thomas, 2000). In the United States, B. sylvaticum is invasive and listed as a noxious weed covering the west coast of California, Oregon, and Washington (Oregon Department of Agriculture, 2009; Washington State Department of Agriculture, 2009; Lionakis Meyer and Effenberger, 2010). It is also expanding into the eastern United States where it has been reported in Missouri and Virginia (Roy, 2010). In Oregon, B. sylvaticum forms thick monocultures in open forests at elevations from nearly sea level to approximately 1200 m. It threatens the endangered Oregon Willamette Valley oak savanna ecosystem by replacing the native flora and reduces habitat for rare butterflies (Kaye and Blakeley-Smith, 2006; Severns and Warren, 2008). False brome is shade tolerant (Murchie and Horton, 1997; Holmes et al., 2010), which makes it a particularly dangerous invasive threat to undisturbed habitats (Martin et al., 2009).

False brome was first introduced into Oregon via plant introduction studies in the early part of the twentieth century, and later identified and collected from the wild in 1939 (Chambers, 1966; Kaye and Blakeley-Smith, 2006). Field trials for exotic grasses were performed by the United States Department of Agriculture (USDA) in Corvallis at the Oregon State University facilities, and B. sylvaticum was widely planted to “improve range” throughout the western United States (Hull, 1974). Oregon State University herbarium records indicate that two separate experimental gardens were established in Eugene and Corvallis, Oregon. Genetic profiling with microsatellite markers confirm these introductions were independent and that they probably consisted of the same set of multiple accessions from the native range in Europe that had been collected by the USDA Division of Plant Introduction (Rosenthal et al., 2008). Accessions in each of these two plantings have crossed, and the invasive plants that are now spreading across Oregon forests are recombinant products of hybridization. Brachypodium sylvaticum

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has thus become increasingly common in the past 15 yr (Chambers, 1966; Kaye and Blakeley-Smith, 2006; Rosenthal et al., 2008). The introductions in Corvallis and Eugene retain unique marker signatures, but Bayesian cluster analyses indicate that similar sets of native accessions from Western Europe contributed to the hybrid genotypes that are spreading from each introduction location (Rosenthal et al., 2008).

Despite the economic and environmental impact of *B. sylvaticum*, there remains a lack of adequate genomic resources for the study of the invasive populations. To thoroughly investigate genetic differences between invasive and native populations, a reference transcriptome specific for *B. sylvaticum* is needed to precisely align, map, and interrogate gene sequences. The major aim of this study was to assemble, annotate, and characterize a high-quality reference transcriptome that will enable researchers to assess gene expression levels, conduct comparative analyses, and identify putative single-nucleotide polymorphism (SNP) and simple sequence repeat (SSR) sequence sites in the genomes of *B. sylvaticum* populations for developing genetic markers to be used in future genotyping, identification, and genetic tracking studies.

Over the past several years, next-generation sequencing (NGS) has emerged as a low-cost, large-scale, fast, and accurate approach for de novo transcriptome sequencing (reviewed in Ward et al., 2012). Moreover, the tremendous depth of coverage generated by the Illumina sequencing platform in particular enables marker and gene discovery, comparative genomics, and gene expression analysis in nonmodel organisms (Wang et al., 2010; Huang et al., 2012; Nicolai et al., 2012; Varshney et al., 2012; Wang et al., 2012; Zhao et al., 2012). We conducted RNA-Seq transcriptome assemblies on *B. sylvaticum* plants originating west of Corvallis, Oregon (hereafter referred to as Corvallis, or Brasy-Cor), Spain (Brasy-Esp), and Greece (Brasy-Gre). We generated >36 Gb of *B. sylvaticum* transcriptome and assembled the sequences into 120,091, 104,095, and 177,095 transcript contigs with an average length of 1652, 1728, and 1566 bp for samples from Corvallis, Spain, and Greece, respectively. The cDNA sequence files are provided in fasta format for each population in Appendices S1 (Brasy-Cor), S2 (Brasy-Esp), and S3 (Brasy-Gre). We estimate that these transcriptomes represent >90% of the *B. sylvaticum* gene space. Furthermore, we identified SNP and SSR sequences that could be used to design genetic markers for use in future population studies. Along with the substantial genomic resources available in a close congener (*B. distachyon*; Mur et al., 2011) providing reference and comparative material, this transcriptome assembly will be useful on a broad scale as a greatly needed resource for ecologists and geneticists conducting research on native and invasive populations of *B. sylvaticum*, and on the role of climate change and adaptation toward successful invasiveness.

### METHODS AND RESULTS

**Transcriptome sequencing and de novo assembly**—Populations from Spain and Greece were selected to investigate correlations between the Oregon, USA samples and European progenitors used in the USDA field trials. Our samples were drawn from populations in Corvallis, Oregon (population OR-C1; Rosenthal et al., 2008; GPS coordinates: 44°39′25″N, 124°45′41″W), Avila, Spain (population SAV, USDA accession PI 318962; GPS coordinates: 40°39′27″N, 5°18′38″W), and Thessaloniki, Greece (population GRE, USDA accession PI 206546; GPS coordinates: 40°37′48″N, 22°57′36″E). The OR-C1 plants were selected from field-collected seed, and the GRE and SAV seed samples were collected and maintained by the USDA Plant Germplasm division in Pullman, Washington (USA). All plants were grown in a common greenhouse garden at the Portland State University campus in Portland, Oregon, under 12 hours light at 25°C and 12 hours dark at 15°C. At 60 wk, leaf tissue was collected from two individuals per population and ground in liquid nitrogen. Total cellular DNA was extracted using a modified protocol described elsewhere (Fox et al., 2009). In brief, RNA was extracted using RNA Plant Reagent (Life Technologies, Grand Island, New York, USA) and treated with RNase-Free Turbo DNase (Life Technologies). Concentration, integrity, and extent of contamination by ribosomal RNA were assessed using an ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). Samples were prepared using the TrueSeq RNA Sample Preparation Kit (v2) and sequenced on the Illumina HiSeq 2000 instrument (Illumina, San Diego, California, USA) and Bioanalyzer 2100 (Agilent Technologies).

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| Transcriptomes       | Raw sequences | Assembled contigs |
|----------------------|---------------|-------------------|
|                      | No. of reads  | Gb                 | Total no. | Longest sequence (bp) | Average length (bp) | Median length (bp) |
| Brasy-Cor            | 120,443,086   | 12.16              | 120,091   | 16,713                  | 1616                | 1380               |
| Brasy-Esp            | 109,942,266   | 11.1               | 104,950   | 21,443                  | 1696                | 1456               |
| Brasy-Gre            | 127,927,820   | 12.92              | 177,682   | 15,289                  | 1566                | 1317               |
| *B. distachyon* (Bd21) | —            | —                 | 31,029    | 14,577                  | 1280                | 1086               |

**Table 1. Statistics of data sets from the sequencing and de novo transcriptome assemblies of the *Brachypodium sylvaticum* samples from Corvallis (Brasy-Cor), Spain (Brasy-Esp), and Greece (Brasy-Gre) and their comparison with the transcriptome of *B. distachyon* Bd21 sequenced genome.**

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Fig. 1. Sequence comparisons. (A) Histogram of frequency distribution of contig lengths of *Brachypodium sylvaticum* samples from Corvallis (Brasy-Cor), Spain (Brasy-Esp), and Greece (Brasy-Gre) de novo transcriptome assemblies compared with *B. distachyon* cDNA lengths (X-axis has been truncated at 6 kb). The frequency distribution of the *B. sylvaticum* transcriptomes closely mirrors that of the *B. distachyon* transcriptome. (B) Comparisons of number of contigs and the average length of *B. sylvaticum* assembled contigs and *B. distachyon* transcripts. Although the overall number of contigs is far greater than the number of gene loci in *B. distachyon*, the average length observed in *B. sylvaticum* transcriptomes is larger than *B. distachyon*. 
gene paralogs, and mis-assemblies. As discussed below, the vast majority of assembled contigs are well-annotated, indicating that the inflated number of contigs is not due to mis-assemblies but more likely due to transcript isoforms and/or paralogous loci.

**Homology-based transcriptome annotation**—We examined the number of homologous loci identified through BLAST to approximate the diversity and coverage of the assembled gene loci (Mount, 2007). We annotated genes based on BLAST similarity (E-value threshold cutoff of $1e^{-10}$) to sequences available in the GenBank protein database and directly to transcripts identified in the sequenced monocot genomes. We used our transcript scaffolds as queries in BLASTx to search against the National Center for Biotechnology Information (NCBI) nonredundant protein database (Table 2), which resulted in >80% correlation between the contigs of each *Brachypodium sylvaticum* assembly and GenBank peptide sequences.

Similar trends were observed when we compared the *B. sylvaticum* nucleotide sequences directly against other monocot nucleotide sequences using BLASTn (E-value threshold cutoff of $1e^{-10}$ and percent identity >90%). The *B. distachyon*, *Oryza sativa* (Japonica, MSU6), and *Sorghum bicolor* cDNA databases were obtained from Gramene BioMart (Spoon et al., 2012). In the Corvallis assembly, 103,752 (86.4%) contigs hit *B. distachyon* genes, 96,375 (80.3%) contigs hit rice genes, and 93,751 (78.1%) contigs hit *S. bicolor* genes, with similar results for the Spain and Greece assemblies (Table 2). We further examined the number of homologous loci identified through direct comparisons between *B. sylvaticum* and *B. distachyon* (Fig. 2). We found that the Corvallis assembly uncovered 28,791 (92.8%) of the gene paralogs, and mis-assemblies. As discussed below, the vast majority of assembled contigs are well-annotated, indicating that the inflated number of contigs is not due to mis-assemblies but more likely due to transcript isoforms and/or paralogous loci.

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Similar trends were observed when we compared the *B. sylvaticum* nucleotide sequences directly against other monocot nucleotide sequences using BLASTn (E-value threshold cutoff of $1e^{-10}$ and percent identity >90%). The *B. distachyon* Bd21 (v1.2), *Oryza sativa* (Japonica, MSU6), and *Sorghum bicolor* v1.4 transcriptome databases were obtained from Gramene BioMart (Spoon et al., 2012). In the Corvallis assembly, 103,752 (86.4%) contigs hit *B. distachyon* genes, 96,375 (80.3%) contigs hit rice genes, and 93,751 (78.1%) contigs hit *S. bicolor* genes, with similar results for the Spain and Greece assemblies (Table 2). We further examined the number of homologous loci identified through direct comparisons between *B. sylvaticum* and *B. distachyon* (Fig. 2). We found that the Corvallis assembly uncovered 28,791 (92.8%) of the

**Table 2. BLAST comparisons of *Brachypodium sylvaticum* transcriptomes against various databases.** Shown are the number and percentage of contigs from *B. sylvaticum* samples from Corvallis (Brasy-Cor), Spain (Brasy-Esp), and Greece (Brasy-Gre) that hit a gene in the respective database. BLASTx comparisons were made to the GenBank nonredundant peptide database (nr), while BLASTn nucleotide comparisons were made against the *B. distachyon*, *Oryza sativa*, and *Sorghum bicolor* cDNA databases (E-value threshold cutoff of $1e^{-10}$).

| Database compared | Brasy-Cor (120,091) | Brasy-Esp (104,950) | Brasy-Gre (177,682) |
|-------------------|---------------------|---------------------|---------------------|
|                   | No. of hits | % hits | No. of hits | % hits | No. of hits | % hits |
| GenBank peptide (nr) | 96,140 | 80.1 | 86,791 | 82.7 | 149,178 | 84.0 |
| *B. distachyon* v1.2 | 103,752 | 86.4 | 93,291 | 88.9 | 160,309 | 90.2 |
| *O. sativa* (japonica) vMSU6 | 96,375 | 80.3 | 86,577 | 82.5 | 148,617 | 83.6 |
| *S. bicolor* v1.4 | 93,751 | 78.1 | 84,023 | 80.1 | 144,884 | 81.5 |

**Fig. 2.** Sequence comparisons between contigs from *Brachypodium sylvaticum* samples from Corvallis (Brasy-Cor), Spain (Brasy-Esp), and Greece (Brasy-Gre) and *B. distachyon* transcripts and genome. (A) *B. distachyon* genes hit by *B. sylvaticum* contigs. Greater than 87% of *B. distachyon* loci were hit by all three *B. sylvaticum* transcriptomes, and more than 96% were hit by a minimum of one contig from at least one *B. sylvaticum* transcriptome. (B) RPKM values averaged over 0.5 megabase intervals across the *B. distachyon* genome. This image shows the uniform coverage of *B. sylvaticum* reads aligned to the five *B. distachyon* chromosomes. Blue histograms indicate RPKM values averaged along the positive strand while green histograms indicate RPKM values of minus strand alignments.
31,029 B. distachyon v1.2 transcripts, while the Spain and Greece assemblies hit 91.3% and 94.1% of the B. distachyon transcripts, respectively (Fig. 2A). When we compared the B. distachyon genes hit by all three B. sylvaticum assemblies, we found that ~96.8% (30,054) of the B. distachyon genes were hit by a contig from at least one of the three B. sylvaticum transcriptomes. In addition, 27,187 (87.6%) B. distachyon genes were commonly hit by all three B. sylvaticum transcriptome assemblies.

Further, when we extended our comparisons with other sequenced grass genomes, we found that the Corvallis contigs hit 44,538 (~67%) of 66,338 rice transcripts and 24,999 (~84%) of 29,448 sorghum transcripts with similar results for the other two B. sylvaticum assemblies. Overall, ~90% of all the B. sylvaticum contigs were assigned a homology-based annotation. While the properties of any transcriptome are uniquely associated with the spatial, temporal, and environmental factors present at the precise time of tissue sampling, these results indicate that we have sequenced the great majority of B. sylvaticum gene loci and have assembled three quality reference transcriptomes for B. sylvaticum.

**Functional characterization of the transcriptome**—All the B. sylvaticum contigs were translated into peptides by querying the longest predicted open reading frame (ORF) using the ORFPredictor tool (Min et al., 2005) and were functionally characterized using InterProScan version 4.8 (Quevillon et al., 2005; Hunter et al., 2012). Nearly half of the translated ORFs were assigned InterPro and Gene Ontology (GO) annotations (Appendix S5), which is consistent with other published annotated genomes. The functional annotations were enriched by performing Blast2GO analysis by adopting a stringent BLASTx search (E-value ≤1e-20 and percent identity ≥90%) against the NCBI GenBank nonredundant protein database (Conesa and Gotz, 2008), and the resulting best hits with GO annotations were used to project GO assignments to B. sylvaticum contigs (Gotz et al., 2008; Barrell et al., 2009). Enrichment resulted in assigning GO annotations to 69,628 Corvallis, 61,015 Spain, and 107,700 Greece contig assemblies (Appendix S6).

**Gene expression analysis**—We conducted relative gene expression analyses to assess the utility of these reference transcriptomes for future differential gene expression studies. We mapped B. sylvaticum Illumina reads to B. distachyon loci and calculated the reads per kilobase per million reads (RPKM) values. We then mapped these RPKM values to B. distachyon Bd21 reference genome.

http://www.bioone.org/loi/apps
0.5 megabase intervals (Fig. 2B). When we compared the RPKM values among the three B. sylvaticum plants, we found very high Pearson’s correlation coefficients between gene expression data sets (Appendix S7). Although the three B. sylvaticum plants do not exhibit significant expression differences over our mapped intervals, we do observe a uniform distribution of contigs mapping to the B. distachyon genome (Fig. 2B; B. distachyon v1.0 genome sequence from http://mips.helmholtz-muenchen.de/plant/brachypodium/). To further demonstrate the utility of pairing the B. sylvaticum transcriptomes and their close congener to investigate questions regarding gene expression, we used the BrachyCyc pathway tool (http://pathway.granem.org/graneme/brachycyc.shtml), which contains biochemical pathways consisting of over 7000 B. distachyon genes coding for enzymes. Using the BrachyCyc pathway tool, we mapped RPKM values to B. distachyon metabolic pathways to examine gene expression profiles of homologous genes (Appendix S7). These results demonstrate the potential of the B. sylvaticum transcriptomes for use in future studies investigating differential gene expression and metabolomics, as well as for making comparisons with B. distachyon.

Genetic variation—To quantify the number of SNP sites across the three transcriptomes, we mapped the reads from each B. sylvaticum sample to the B. distachyon genome v1.0 using Bowtie version 0.12.8 (Langmead et al., 2009). We then used custom Perl scripts to identify nucleotide differences in positions with at least eight aligned reads and 75% of those aligned reads confirming the SNP (Kimbel, unpublished). Using these criteria, we identified 394,654 putative SNPs among the three B. sylvaticum genotypes (Fig. 3A; Appendix SS). Of these, 157,835 SNPs were in sites common to all three genotypes. Although the total number of SNPs was similar among the genotypes, we observed more SNPs unique to the Greek sample (66,963) when compared to Corvallis (39,936) and Spain (40,027). To address the biological relevance of these SNPs and their potential role to be studied in the future for relevance to invasive phenotypes, we predicted the potential effects of the variants and identified a diverse set of consequences (McLaren et al., 2010). Notably, we identified more than 230,000 down-stream variant sites, more than 92,000 missense variants, and 234 stop codons introduced (Tables 3, 4). We observed only slight variation when we mapped the SNP densities of each B. sylvaticum genotype onto B. distachyon chromosomes. Generally, the B. sylvaticum SNP density mirrors the B. distachyon gene density and centromeric region (Fig. 3B). We also constructed a maximum parsimony tree based on concatenated variant sites where at least five reads from each B. sylvaticum transcriptome aligned with Bowtie (Fig. 3C). Of the 157,835 SNPs, only 628 loci were polymorphic within at least one of the three B. sylvaticum samples. These 628 positions were concatenated to generate the maximum likelihood tree depicting relative relationships among the three B. sylvaticum genotypes. While this SNP analysis shows the utility of the B. sylvaticum transcriptomes for genotyping studies, much work needs to be done to fully elucidate the relationships of the various native and invasive populations.

We mined the assembled B. sylvaticum contigs for SSRs using Perl code from the Simple Sequence Repeat Identification Tool (SSRIT; Temnykh et al., 2001; http://www.granem.org/db/markers/ssrtool), looking for di-, tri-, tetra-, penta-, and hexanucleotide SSRs with a minimum of nine, six, six, five, and five repeat units, respectively (Table 5; Appendix S9). In total, we identified 23,535 SSRs in Corvallis, 20,303 in Spain, and 32,847 in Greece transcriptome sequences (McLaren et al., 2010). Notably, we predicted the potential effects of the variants and identified a diverse set of consequences for the various gene loci.

### CONCLUSIONS

The list of noxious invasive plants identified by the Oregon Department of Agriculture (2009) includes species such as kudzu, goatgrass, knapweed, and others that exact high economic and ecological costs in regions where they have been introduced. Ecologists make distinctions among species that are introduced (able to persist), naturalized (establishes self-sustaining populations, but is not a dominant component of the vegetation), and invasive (is able to dominate habitats to the exclusion of native species). False brome meets the “invasive” criteria but has not yet become notorious because its distribution is still restricted compared to other invasive plants. Our ultimate goal is to use B. sylvaticum as a model for studying adaptation and invasiveness and for the general study of grasses. Therefore, we consider it a necessary first step to establish baseline resources for B. sylvaticum by generating de novo transcriptomes from multiple genotypes, use them to study gene expression and regulation, and identify functional nucleotide polymorphisms to develop new sets of genetic markers for future population-wide applications in Plant Sciences 1(3): 1200011 Fox et al.—Brachypodium sylvaticum transcriptome

| SNP Substitution type | Brasy-Cor | Brasy-Esp | Brasy-Gre |
|-----------------------|-----------|-----------|-----------|
| A → C                 | 14,208    | 13,836    | 14,695    |
| A → G                 | 43,635    | 42,985    | 43,135    |
| A → T                 | 9,695     | 9,834     | 10,118    |
| A → C                 | 11,998    | 12,241    | 11,459    |
| A → G                 | 18,834    | 18,072    | 17,862    |
| A → T                 | 36,201    | 37,250    | 34,717    |
| G → A                 | 35,918    | 36,980    | 34,383    |
| G → C                 | 18,962    | 18,103    | 17,813    |
| G → T                 | 11,892    | 12,143    | 11,393    |
| T → A                 | 9,647     | 9,970     | 10,039    |
| T → C                 | 44,121    | 43,302    | 43,790    |
| T → G                 | 14,077    | 13,912    | 14,371    |

Table 3. SNP consequence predictions. After aligning all of the 394,654 SNPs from the Brachypodium sylvaticum samples from Corvallis (Brasy-Cor), Spain (Brasy-Esp), and Greece (Brasy-Gre) on the B. distachyon (Bd21) genome v1.0, we predicted the potential effects of SNP variant loci on the Bd21 genes. We identified a diverse set of consequences for the various gene loci.
Table 5. Summary of SSR sites identified in the transcriptomes of *Brachypodium sylvaticum* samples from Corvallis (Brasy-Cor), Spain (Brasy-Esp), and Greece (Brasy-Gre). We identified di-, tri-, tetra-, penta-, and hexanucleotide SSRs with a minimum of nine, six, five, and five repeat units, respectively. More than 20,000 SSRs were identified in the *B. sylvaticum* transcriptomes; trinucleotide repeats were the largest class of SSRs.

| Population | Total | Dimer | Trimer | Tetramer | Pentamer | Hexamer |
|------------|-------|-------|--------|----------|----------|---------|
| Brasy-Cor  | 23,535| 6011  | 16,346 | 750      | 183      | 245     |
| Brasy-Esp  | 20,303| 5449  | 14,093 | 513      | 141      | 107     |
| Brasy-Gre  | 32,847| 8194  | 23,280 | 858      | 276      | 239     |

screening. The results of our de novo assemblies produced a relatively large number of long, reconstructed transcripts, as demonstrated by average contig lengths. Overall, we were able to assign homology-based annotations to ~90% of *B. sylvaticum* contigs, and more than 50% of the translated sequences were functionally annotated by assigning InterPro and Gene Ontology annotations. More than 96% of *B. distachyon* Bd21 gene loci were associated with *B. sylvaticum* contigs, thereby demonstrating diversity and broad coverage in our transcriptomic data. When compared to *B. distachyon*, we discovered ~390,000 SNPs, of which ~158,000 SNPs were common to the three *B. sylvaticum* samples. Based on the SNP calls, we identified the number of SNPs with consequences to the gene and transcripts including those altering the potential intron splicing sites and translated protein sequences. These resources, when paired with well-established *B. distachyon* genomic data, will be useful in the future characterization of *B. sylvaticum* invasiveness.

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