

Transcriptome Sequencing, Characterization, and Polymorphism Detection in

Subspecies of Big Sagebrush (*Artemisa tridentata*)

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A thesis submitted to the faculty of  
Brigham Young University  
in partial fulfillment of the requirements for the degree of

Master of Science

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August 2011

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## ABSTRACT

### Transcriptome Sequencing, Characterization, and Polymorphism Detection in Subspecies of Big Sagebrush (*Artemisia tridentata*)

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Big sagebrush (*Artemisia tridentata*) is one of the ecologically most important shrub species in western North America. The species serves as a major source of food and habitat for the near-threatened sage grouse and various other fauna. Habitat loss due to a combination of disturbances followed by establishment of invasive plant species is considered as a serious threat to sustainability of big sagebrush ecosystem. Because of its importance, restoration of this species is very crucial to those dependent on big sagebrush community. However, restoration of big sagebrush carried out by using diverse seed source can lead to imbalance and degradation in the native ecosystem. Therefore, restoration works aided by understanding of adaptive traits of big sagebrush using molecular markers will likely lead to successful restoration. The major objective of this research was to create substantial amount of nuclear sequence data and identify markers that can be used in future studies in big sagebrush. We report the development and annotation of the first expressed sequence tag (EST) collection for big sagebrush based on 454 sequencing of leaf tissue. Expressed genes of subspecies *tridentata* and *vaseyana* were sequenced using the 454 GS-FLX titanium platform, which produced 823,392 reads with an average read length of 404 bp and 702,001 reads with an average read length of 333 bp for sspp.

*tridentata* and *vaseyana*, respectively. Assembly of the reads resulted in 212,102 consensus sequences in ssp. *tridentata* and 199,439 in ssp. *vaseyana*. A combined assembly of both subspecies sequences generated 29,541 contigs with an average length of 796 bp and 275,866 singletons with an average length of 370 bp. A BLASTx search against the non-redundant (NR) protein database using the contigs obtained from a combined assembly resulted in 21,436 sequences with significant blast alignments ( $\leq 1e^{-15}$ ). Gene Ontology (GO) IDs were assigned to 18,397 sequences. A total of 20,952 SNPs were detected between the two subspecies and 1,182 SNPs were confirmed in tetraploid ssp. *wyomingensis*. In addition, 1,003 and 507 SSRs were detected in ssp. *tridentata* contigs and ssp. *vaseyana* contigs, respectively.

Keywords: *Artemisia tridentata*, EST assembly, 454 sequencing, SNPs, SSRs,  $K_a$ ,  $K_s$

## ACKNOWLEDGEMENTS

I would like to express my sincere appreciation towards everyone who assisted me in this project. I especially thank my advisor Dr Josh Udall for his guidance throughout the project, and for his constant care and concern towards my progress and intellectual development. I thank Dr Bryce Richardson for teaching me all he could about big sagebrush, and for providing excellent opportunities to showcase my research in front of veteran shrub scientists. Thanks to Dr Leigh Johnson, whose academic knowledge and research expertise were of great resources to me. I also thank Scott Yourstone and Dave Elzinga for being great friends, and for helping me in developing programming skills. I thank Brian Knaus, Durant McArthur, Felix Jimenez, Jared Price, Nancy Shaw, Rich Cronn, Robert Byers, and Stuart Sanderson who helped in very many ways during the span of the project. I also express my humble thanks and gratitude towards my parents, who while living half the circumference of the earth away, always believed in me and supported my endeavors. The funding for this work was provided by USDA Forest Service Western Forest Transcriptome Survey, National Fire Plan (research joint venture 09-JV-11221632-200) and the Great Basin Native Plant Selection and Increase Project (GBNPSIP).

## TABLE OF CONTENTS

|                                                                                                                                         |      |
|-----------------------------------------------------------------------------------------------------------------------------------------|------|
| Title Page                                                                                                                              | i    |
| Abstract                                                                                                                                | ii   |
| Acknowledgements                                                                                                                        | iv   |
| Signature Page                                                                                                                          | v    |
| List of Tables                                                                                                                          | vii  |
| List of Figures                                                                                                                         | viii |
| List of Supplemental Data                                                                                                               | ix   |
| <br>                                                                                                                                    |      |
| Transcriptome Sequencing, Characterization and Polymorphism Detection in<br>Subspecies of Big Sagebrush ( <i>Artemisia tridentata</i> ) | 1    |
| Introduction                                                                                                                            | 2    |
| Materials and Methods                                                                                                                   | 5    |
| Results                                                                                                                                 | 12   |
| Discussion                                                                                                                              | 23   |
| Conclusions                                                                                                                             | 29   |
| References                                                                                                                              | 30   |

## LIST OF TABLES

|                                                                                                                                                                        |    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Table 1: Summary report of individual and combined <i>de novo</i> assembly of the subspecies sequences generated from 454-pyrosequencing                               | 37 |
| Table 2: Frequency of SNPs between ssp. <i>tridentata</i> and ssp. <i>vaseyana</i> by mutation types                                                                   | 38 |
| Table 3: SSR frequencies of repeat type with repeat numbers in <i>A. tridentata</i> ssp. <i>tridentata</i> ( <i>A.t.t.</i> ) and ssp. <i>vaseyana</i> ( <i>A.t.v</i> ) | 39 |

## LIST OF FIGURES

|                                                                                                                                                                                           |    |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1 – Histogram of number of EST members in a contig                                                                                                                                 | 40 |
| Figure 2 – Distribution of consensus sequences from combined assembly of <i>Artemisia tridentata</i> ssp. <i>tridentata</i> and ssp. <i>vaseyana</i> in three main GO categories          | 41 |
| Figure 3 – Distribution of the number of SNPs by read coverage depth                                                                                                                      | 42 |
| Figure 4 – Distribution of contigs by number of SNPs in a combined assembly of <i>A. t.</i> ssp. <i>tridentata</i> and ssp. <i>vaseyana</i>                                               | 43 |
| Figure 5 – Frequency and distribution of SSRs in <i>A. t.</i> ssp. <i>tridentata</i> and <i>A. t.</i> ssp. <i>vaseyana</i>                                                                | 44 |
| Figure 6 – A histogram illustrating the distribution of the ratio of non-synonymous mutations to non-synonymous sites compared to synonymous mutations per synonymous sites ( $K_a/K_s$ ) | 45 |

## LIST OF SUPPLEMENTAL DATA

|                                                                                                                                                       |    |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Supplemental Table 1 – List of individuals used in the study                                                                                          | 46 |
| Supplemental Table 2 – The distribution and sequences of putative sagebrush homologs of enzymes involved in terpenoid and coumarin synthesis pathways | 47 |
| Supplemental Table 3 – Additional details of SSRs including frequencies of di- and tri-nucleotide repeats                                             | 48 |
| Supplemental Table 4 – Details of SNP primers used for SNP validation                                                                                 | 49 |
| Supplemental Table 5 – Details of SSR primers used for SSR validation                                                                                 | 50 |
| Supplemental Figure 1 – Distribution of protein domain vs number of contigs                                                                           | 51 |
| Supplemental File 1 – A list of contigs containing discriminatory SNPs between <i>ssp. tridentata</i> and <i>ssp. vaseyana</i>                        | 52 |
| Supplemental File 2 – Results for SNP validation during sequence capture                                                                              | 53 |

TRANSCRIPTOME SEQUENCING, CHARACTERIZATION, AND POLYMORPHISM  
DETECTION IN SUBSPECIES OF BIG SAGEBRUSH (*ARTEMISIA TRIDENTATA*)

## Introduction

*Artemisia* (Asteraceae) is a widely distributed plant genus that is predominantly found in temperate regions of the northern hemisphere. Some species within this genus are valued in medicine, cooking, and forage for livestock [1, 2]. Big sagebrush (*Artemisia tridentata*) is recognized for its importance to ecosystem function. Big sagebrush is one of the most abundant, widespread, and ecologically important woody shrub species in the intermountain regions of western North America. This species contains three widespread subspecies (*A. t. ssp. tridentata*, *A. t. ssp. vaseyana*, and *A. t. ssp. wyomingensis*) that occupy distinct habitats and two less common subspecies (*A. t. ssp. spiciformis* and *A. t. ssp. xericensis*) [3, 4]. Prior to the Anglo-American settlement, big sagebrush was estimated to occupy up to 100 million ha of the western United States, while contemporary estimates have shown that the area has been reduced to approximately 43 million ha [5]. Changes in land use and disturbance regimes (*e.g.*, conversion to agriculture, overgrazing and wildfire frequencies) are major factors in the degradation of these ecosystems. Such disturbances can lead to invasions by cheat grass (*Bromus tectorum*) and other weeds that fundamentally change the wildfire frequency and severely reduce the frequency of sagebrush in ecosystems where it historically dominated [6, 7]. Restoration of these ecosystems not only requires replanting of big sagebrush, but the replanting should be carried out with a basis of scientific knowledge. Early efforts toward this goal have been made by Mahalovich and McArthur [8], where the authors outline the importance of seed plantation by geographical distribution of the subspecies. Restoration of sustainable populations necessitates understanding of the local and landscape level genetic structure of natural big sagebrush populations.

Polyploidy and intra- and interspecific hybridization are likely the important factors in big sagebrush adaptation and landscape dominance. Big sagebrush subspecies occupy specific ecological niches: ssp. *tridentata* grows in alluvial flats at elevation typically lower than 1800 m, ssp. *vaseyana* is found in higher altitude uplands at elevations above 1660 m up to timberline, and ssp. *wyomingensis* occupies drier sites with shallow soils [9]. Subspecies *wyomingensis* is universally tetraploid, whereas sspp. *tridentata* and *vaseyana* are typically diploid; although both sspp. *tridentata* and *vaseyana* also include tetraploid populations [4]. Hybridization between ssp. *tridentata* and ssp. *vaseyana* is common under the appropriate ecological conditions. Hybridization among big sagebrush subspecies has been studied using reciprocal transplants, showing that natural selection tends to limit the hybrids of sspp. *tridentata* and *vaseyana* to a zone between the parental subspecies habitat [9, 10]. McArthur and Sanderson [4] suggest that hybrid zones could be repositories of genetic variation and gene exchange, and can influence the evolution of big sagebrush.

Though widely acknowledged as an important shrub of the intermountain ecosystem in western North America, limited DNA sequence data has been collected on big sagebrush. A search for *A. tridentata* nucleotide sequences in the NCBI database yielded less than 50 nucleotide sequences. As a genus, *Artemisia* has approximately 3.8 million sequences (~823 Mbp) of which 3.7 million reads are archived in the Sequence Read Archive (SRA), from *A. annua* EST projects [2, 11], and an ongoing *A. annua* genome project [11]. *A. annua* is a medicinal herb native to temperate Asia and is not found in the western hemisphere. Sequences of *A. tridentata* are needed to conduct research studies involving phylogenetics, population genetics, and ecological genetics in North American big sagebrush populations. Transcriptome

sequencing and annotation, and marker detection within big sagebrush EST sequences will provide a rapid means to satisfy these information needs and set the stage for future studies.

In this study, we characterized the leaf transcriptome of two big sagebrush subspecies, *A.t. ssp. tridentata* and *A.t. ssp. vaseyana* and compared the resulting ESTs. We also sequenced *A. t. ssp. wyomingensis* ESTs to assess SNP distribution in this subspecies compared to *sspp. tridentata* and *vaseyana*. Our objectives were to 1) identify and characterize a large number of expressed genes in *A. tridentata*, 2) detect sequence differences within and between *sspp. tridentata* and *vaseyana* that could be used as markers to further our understanding of evolutionary changes that contribute to the adaptive and ecological niche of this species, and 3) gain inference into the origins of the tetraploid *A. t. ssp. wyomingensis*.

## Materials and Methods

### *Plant materials and RNA extraction*

Young leaves from two subspecies of big sagebrush, *A. tridentata* ssp. *tridentata* and *A. tridentata* ssp. *vaseyana*, were harvested from plants growing in USDA Shrub Lab greenhouse in Provo, UT for 454-pyrosequencing. The plants were grown from seeds collected in their natural habitat near Park Valley, UT. The leaves were flash frozen in liquid N<sub>2</sub> and stored in -80°C until further use. RNA extraction was performed using approximately 0.1 g of frozen leaf tissue, following a modified hot borate procedure [12]. The extracted RNA was analyzed for quality and quantified using Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA) before using for cDNA synthesis.

### *cDNA library preparation for 454-pyrosequencing*

cDNA was created using 1 µg of total RNA using the SMART cDNA synthesis kit (Clontech Laboratories, Inc., Mountain View, CA), but the cDNA synthesis primer for first strand synthesis was replaced by a modified oligo-dT primer (5'-AAGCAGTGGTATCAACGCAGAGTCGCAGTCGGTACTTTTTTCTTTTTTV-3') [13]. The poly-T stretch in the primer is broken by inserting a Cytosine to minimize the potential sequencing problems due to the presence of a long poly-A homopolymer stretch. The cDNA library was normalized using the Trimmer Kit (Evrogen, Moscow, Russia) to limit redundant sequencing of highly expressed genes. We did not directly test normalization values since so few transcripts were known for big sagebrush prior to this report. The normalization control included

with the Trimmer Kit was reduced in copy number as expected. Because this control was normalized as expected, we assumed that a similar normalization of highly expressed genes also occurred in our two sagebrush samples. Adaptors ligation and single strand selection were done as described in the GS FLX Titanium General Library Preparation Kit (Roche, Branford, CT) with modifications. One half-plate was sequenced for each subspecies at the Brigham Young University DNA sequencing center, Provo, UT.

#### *Illumina sequencing of *A. t. ssp. wyomingensis* and SNP mapping*

Leaves were harvested from two young *A. t. ssp. wyomingensis* plants growing in USDA Shrub Lab greenhouse in Provo, UT (Supplemental Table 1). The plants were grown from seeds collected in their natural habitat in two different states – Montana and Utah. Geographic information on sampled individuals is provided in Supplemental Table 1. Tetraploid confirmation was conducted on a Partec PAII flow cytometer. Leaves from each plant along with a known *A. tridentata* ssp. *tridentata* diploid standard were finely chopped in a buffer and then nuclei were stained with DAPI solution (CyStain UV Precise P, Partec). Total RNA was harvested and quantified in the same manner as mentioned above. The RNA was processed for sequencing following directions in the Illumina mRNA Sequencing Sample Prep Guide (part #1004898 rev. D., Illumina, Inc., San Diego, CA), with the addition of custom barcoded adapters designed for the paired-end sequencing process [14]. The quality of the libraries was validated using the Agilent 2100 Bioanalyzer. The prepared libraries of the ssp. *wyomingensis* individuals were multiplexed in approximately equal concentrations and sequenced in two separate runs (one single end 80 bp run, and a second paired end 80 bp run) on the Illumina Genome Analyzer at

the Oregon State University Center for Gene Research and Biocomputing, Corvallis, OR. Pooled libraries were loaded onto one lane of an Illumina Genome Analyzer II at 5 pM concentration. Cluster generation and sequencing used Illumina version 3.0 reagents, and image acquisition and base calling used the Illumina pipeline version 1.5. These Illumina sequences were used only to verify in *ssp. wyomingensis* the SNP loci detected on the combined assembly of *sspp. tridentata* and *vaseyana* obtained from 454 sequences.

Bowtie [15] was used to sort and align the Illumina reads to the ‘reference’ combined assembly, with no gaps and allowing a single base mismatch. The mismatch alignment results were compared to the SNPs obtained from the combined assembly of two subspecies, and the output was parsed so that the SNPs were covered by 1 or more *ssp. wyomingensis* reads. For confident SNP calling, we required only the SNPs covered by coverage of 20x or more be counted; and 10% of all the reads overlapping a SNP were required to be of a particular variant in order to avoid SNP detection that could have resulted from sequencing errors.

#### *EST sequence de novo assembly and annotation*

A combined *de novo* assembly of the sequences from both subspecies was performed using CLC Genomics Workbench Version 3.7.1 (CLC bio, Cambridge, MA). The sequence ends were trimmed to remove the barcodes added during library preparation, and any sequence shorter than 50 bp was not included in building the assembly. The mismatch cost for the nucleotides was set at 2 while both the insertion cost and deletion cost for nucleotides in the reads were set at 3. The length fraction and the similarity of the sequences were set at 0.5 and 0.9, respectively. Any conflicts among the individual bases in the reads were resolved by voting for the base with

maximum number of repetitions. A minimum read length of 200 bp was set for an assembled sequence to be counted as a contig. Identical parameters were also used to create individual assemblies from both subspecies. Homologies of the contigs and singletons were identified by comparing against the NCBI NR protein database using BLASTx with cut-off e-value of  $1e^{-15}$ . The blast results were imported into Blast2GO Version 2.4.2 for mapping the consensus sequences into GO terms. To summarize the distribution of the sequences into GO terms of three main categories – biological processes, cellular components and molecular functions, GO annotations were formatted for input into the GOSlim program [16]. The consensus sequences from combined assembly of both subspecies were also searched against the Pfam-A database using the HMMER software Version 3.0 [17]. Protein sequences generated by ESTScan Version 2-2.1, using the *Arabidopsis thaliana* gene sequences as the reference matrix, were used for this purpose.

### *Polymorphism detection*

SNPs were identified between the subspecies using the Perl script used by Maughan *et al.* [18]. For the nucleotides to be counted as a SNP, the following parameters were required: 1) the coverage depth of the read at the SNP was  $\geq 8$ ; 2) the minimum frequency of the minor allele was 20%; and 3) within each possible nucleotide at that SNP position,  $\geq 90\%$  of its bases at the SNP position are from a single subspecies (*i.e.* ‘heterozygosity’ at 10%). For example, a G $\leftrightarrow$ A SNP would be included in the list of SNPs at coverage of 100x, if, out of 100 aligned sequences, 80 sequences came from one subspecies with at least 72 sequences calling for a G, and 20

sequences came from another subspecies with at least 18 sequences calling for an A at the SNP position. Primers for SNP validation were designed using Primer3 [19].

Perl script MISA (MicroSATellite identification tool, [20]) was also used to identify SSRs in the assembled consensus sequences. Motif repeats between di and deca-units were searched for in the assembly of each subspecies. For repetitive nucleotide stretches to be counted as an SSR unit, di-nucleotide repeats had to be present in frequency of 7 units, tri-nucleotide to octa-nucleotide repeats in frequency of 5 units and nona- and deca-nucleotide in frequency of 4 repeats. The interruption distance among any type of compound SSR units was set at 100 bp. Using the same parameters used by MISA to detect SSRs in each subspecies dataset, the program SSR locator [21] was used to detect SSRs and design primers in the combined assembly of sequences of both subspecies. An additional, custom Perl script was written to bioinformatically distinguish polymorphic SSR loci between the two subspecies ('true' SSRs). An SSR would be counted as a polymorphic SSR if an indel of the whole repeat motif and/or few bases in the motif was detected in the consensus sequences of each subspecies.

### *Polymorphism Validation*

Sanger re-sequencing of both subspecies cDNA validated consensus sequences containing SNPs and the SSRs. Fifteen putative SNP loci and SSR loci were randomly selected for PCR amplification. We selected 11 SNPs with transition mutation and 4 with transversion mutations for PCR amplification followed by SNP validation by re-sequencing. For validation purpose, we selected equal number of transitions and transversions (3 each, 6 total). The SSR primers were designed to amplify 4 di-, 5 tri-, and 4 tetra-, 1 penta- and 1 hexanucleotide SSRs of varying

lengths. Two tetraploid ssp. *tridentata* individuals, two diploid and one tetraploid ssp. *vaseyana* individuals and two tetraploid ssp. *wyomingensis* individuals from geographically divergent sources were used for SNP and SSR marker evaluation in genomic DNA. Geographic information on these individuals is provided in Additional File 5. The following settings were followed for PCR amplification of both SNP and SSR loci: 2 mM MgCl<sub>2</sub>, 1x PCR buffer, 0.2 mM dNTPs, 0.6 μM of each primer, 1 U *Taq* polymerase and dH<sub>2</sub>O to a final reaction volume of 10 μl ran in the following thermal profile: 94°C 3 min, 35x (94°C 30s, T<sub>m</sub> depending upon the primers used 45s, 72°C 1 min) and final extension of 72°C 10 min. PCR reactions of both SNP loci and SSR loci were cleaned using Qiaquick PCR Purification kit (Qiagen, Valencia, CA), and then mixed with appropriate primers prior to sequencing. Fragments were sequenced with an ABI 3730xl DNA analyzer at the University of Wisconsin Biotechnology Center, Madison, WI. The primers used for SNP and SSR validation are listed in Additional File 5.

SNPs and SSRs were validated by sequencing 369 genes containing 572 SNPs in sspp. *tridentata* and *vaseyana* using sequence capture. DNA extractions were made of NVT-2 and UTT-2 (sspp. *tridentata* collected from 39°29'22" 117°85'17" and 38°30'60" 109°38'76", respectively) and UTV-1 and UTV-3 (sspp. *vaseyana* collected from 39°34'13" 111°52'21" and 38°34'13" 109°21'73", respectively) RNA baits (120-mer) of the target genes were synthesized after the contigs obtained from the combined assembly. The DNA libraries for bait hybridization were prepared using GS FLX Titanium Rapid Library Preparation Kit (Roche, Branford, CT, USA). The baits were then hybridized to the library and the capture sequences were prepared for 454-sequencing following the MYselect protocol (MYcroarray, Ann Arbor, MI, USA). The captured DNA libraries were pooled and sequenced at the Brigham Young University sequencing center, Provo, UT. Using identical parameters for SNP and SSR calling as before, the

obtained reads were mapped to the reference contigs in order to place individual sequencing reads at previously called SNPs and SSRs using Genomics Workbench 4 (CLCBio, Aarhus, Denmark). Custom Perl scripts were used to assess SSR and SNP coverage and validate the presence of SNP bases in distinct DNA samples of sspp. *tridentata* and *vaseyana*.

#### *Analysis of synonymous and non-synonymous mutations*

To find the changes in amino acid sequences due to single nucleotide mutations, we aligned sspp. *tridentata* and *vaseyana* contigs that had their coding frame predicted using ESTScan. The proportion of non-synonymous to synonymous mutations ( $K_a/K_s$ ) was calculated for each contig, following the Jukes-Cantor corrected model of substitution using Bioperl modules [22]. We however modified the Jukes-Cantor equation by adding one unit to both non-synonymous and synonymous substitutions in order to obtain a valid  $K_a/K_s$  estimation in instances where either type of substitution was absent. Without this modification, we would have obtained  $K_a/K_s$  value equal to zero for genes with no observed non-synonymous substitutions, regardless of their  $K_s$  values. Similarly, genes without any synonymous substitutions would have undefined  $K_a/K_s$ .

## Results

### *EST sequencing, assembly, and characterization*

We created two normalized cDNA libraries from leaf tissues of two subspecies of *A. tridentata*: ssp. *tridentata* and *vaseyana*. Independent sequencing of these cDNA libraries generated 823,392 sequence reads (median length of 696 bp) containing 332,578,737 bases of sequence from ssp. *tridentata* and 702,001 sequence reads (median length of 529 bp) containing 233,854,535 bases of sequence from ssp. *vaseyana* (Table 1). Assuming a limited amount of sequence divergence between the two subspecies' coding sequence, both cDNA sets were combined and the sequences were assembled *de novo* to build a single combined assembly (Table 1). This assembly resulted in 29,541 contigs and 275,866 singleton sequences. Of the contigs, ESTScan [23] predicted that 25,998 (88%) contained protein coding sequences, of which 25,089 (96%) had open reading frames  $\geq 200$  bp. Of the singletons, ESTScan predicted that 136,305 (49.4%) of the singleton sequences contained protein coding sequences, of which 112,028 (82.2%) sequences had open reading frames  $\geq 200$  bp. Of the 29,541 contigs, 2,381 contigs were comprised of only ssp. *tridentata* reads and 3,137 contigs were comprised of only ssp. *vaseyana* reads (Figure 1). Also, EST read coverage ranged from 2 reads to 3,161 reads in a contig, with a median of 23 EST reads per contigs. Unless stated otherwise, the combined assembly sequences were used for subsequent bioinformatic analyses.

Protein homologies between these novel big sagebrush ESTs and the NR protein database were identified using BLASTx. Results showed that out of 29,541 assembled contig sequences, 21,436 sequences (72.6%) had significant alignments ( $\leq 1e^{-15}$ ) and out of 275,866 singleton sequences, 70,836 sequences (25.7%) had significant alignments ( $\leq 1e^{-15}$ ). Of the contigs with

hits, 9,696 (45.2%) sequences had their best blast alignment to *Vitis vinifera* and 215 (7.3%) and 80 (0.27%) to *Helianthus annuus* and *Lactuca sativa* respectively, both of which are in the Asteraceae. Only fifty-four (0.2%) contigs had their best matches to *Artemisia annua*, owing to the limited number of Artemisia proteins (167) in the NR protein database. The BLAST results of the NR database were utilized by Blast2GO [24] to annotate the EST sequences with GO terms. One or more GO IDs were assigned to 18,397 (62.3%) contigs with a maximum of 21 GO IDs assigned to a single sequence. The distributions of contigs in three, non-mutually exclusive GO categories: biological process (BP), cellular component (CC), and molecular function (MF) were well represented by a diverse set of putative biological functions (Figure 2). In BP category, the most abundant GO term was metabolic process (29.9%), followed by cellular process (21.8%), and unknown biological process (8.4%). In CC category, unknown cellular component was the most abundant (32.5%), followed by cell component (32.0%) and intracellular component (25.3%). Similarly in the MF category, binding was the most abundant category (32.1%), followed by catalytic activity (19.7%), and transferase activity (16.8%). The three groups (BP, CC and MF) are non-exclusive, and therefore, contigs annotated to one category can also be annotated in other categories.

Comparison of the 29,541 contig sequences against the Pfam-A domain database with an e-value cutoff at  $1e^{-5}$  resulted in 15,812 contigs (53.5%) matching at least one protein domain model. The distribution of the domains ranged from a maximum of 13 domains assigned to the same contig to a minimum of one domain per contig (Supplemental Figure 1). The three most common domains were the Protein kinase domain (Pkinase, Pfam ID: PF00069.18), followed by the Protein tyrosine kinase domain (Pkinase\_Tyr, Pfam ID: PF07714.10), and the RNA recognition motif domain (RRF\_1, Pfam ID: PF00076.15).

### *Genes associated with secondary metabolites synthesis in A. tridentata*

Big sagebrush leaves are known to synthesize and store large quantities of terpenoids on the epidermal surfaces of glandular leaf trichomes [25]. Therefore, a search was conducted among the annotated contigs to identify putative genes that code for enzymes involved in terpenoid synthesis via the Mevalonic acid (MVA) and 2-C-Methyl-D-Erythritol-4-Phosphate (MEP) biosynthetic pathways [2]. Most of the enzymes involved in these pathways were detected in our annotated contig sequences, and are presented in the additional materials (Supplemental Table 2). We also searched the annotated contigs for enzymes involved in coumarin biosynthesis, as coumarin derivatives exhibit differential fluorescence among big sagebrush subspecies, which is considered as a tool for subspecies identification [26, 27]. Coumarins in plants are derived via the phenylpropanoid pathway from *p*-coumaroyl CoA [28, 29]. At the top of the phenylpropanoid pathway, phenylalanine lyase acts on the substrate L-phenylalanine, and converts it to cinnamate (often, *trans*-cinnamate) which is then oxidized to *p*-coumarate by the enzyme cinnamate 4-hydroxylase. The enzyme 4-hydroxycinnamoyl CoA ligase then ligates an S-CoA group to *p*-coumarate, forming *p*-coumaroyl CoA. In our annotated contig dataset, we were able to detect these three main enzymes involved in coumarin synthesis: phenylalanine lyase, cinnamate 4-hydroxylase, and 4-hydroxycinnamoyl Co A ligase. Individual tests of enzymatic substrates and products are needed to confirm their roles in coumarin synthesis within big sagebrush.

### *Discovery and frequency of SNPs in ESTs*

A large number of resident SNPs were discovered within the accessions examined in this study. A search for SNPs yielded 59,093 ssp. *tridentata* (1 SNP / 246 bp) SNPs with an average base

coverage of 18x and 61,028 ssp. *vaseyana* SNPs (1 SNP / 207 bp) with an average base coverage of 15x. These putative SNPs may represent a high level of heterozygosity that exists in big sagebrush through its large effective population size and its wind-pollination reproductive strategy. Future segregation analysis would be needed to ascertain if these putative SNPs are truly two alleles at a single locus or an artifact of the sequencing and/or assembly process. These individual assemblies contained many fewer SNPs than detected in the combined assembly (135,310) prior to filtering those SNPs that were not discriminatory between sspp. *tridentata* and *vaseyana* (*i.e.* nearly homogeneous within a single DNA source, but different between the two DNA sources within a contig).

When only SNPs that were near homogenous within the two accessions were considered, a total of 20,952 SNPs were detected between the sspp. *tridentata* and *vaseyana* with base coverage at SNP positions ranging from 8x (2,352 SNPs) to 321x (1 SNP) with an average of 20x (Figure 3). We chose to exclude SNPs that were less than 90% homogeneous within either accession 1) to provide a reduced set SNPs that could discriminate between the two subspecies and 2) to allow for some degree of sequencing error at the SNP position when there was low sequence coverage. To test if SNP heterogeneity within a DNA source had significantly inflated the number of discriminatory SNPs between the two subspecies, the threshold of homogeneity for SNPs was raised to 99%. This resulted in the detection of 18,173 SNPs indicating that 13% of the SNPs originally detected either ssp. *tridentata* or ssp. *vaseyana* had more than one base at that position albeit at a low frequency. These multiple bases could be the result of heterozygosity, differential expression of alleles, assembly error, or sequencing error. The SNP density in the EST sequences between these subspecies was 1 SNP / 1123 bp. The analysis showed that 8,598 (29%) of the total consensus sequences contained at least one SNP and SNPs

per contig ranged from 4,037 contigs with a single SNP to a single contig with 39 SNPs (Figure 4). Some of the contigs with high number of SNPs (>13 SNPs) are likely artifacts of applying a single set of assembly parameters to a heterogeneous set of genes – each with their own evolutionary history. Collectively, contigs with an abundance of SNPs (>13) are best avoided in future population genetic studies, though individually each contig with a multitude of SNPs might provide an interesting case study of gene or gene family evolution. Contigs with high number of SNPs have been documented and highlighted in Supplemental File 1. Of 20,952 SNPs, 16,317 SNPs were distributed in the putative coding sequence and 4,365 SNPs were in the 5' or 3' untranslated regions. Forty-two percent of the identified SNPs fit within the 20 to 30% range for minor allele frequency, 30% within the 30 to 40% and the remaining 28% within the 40 to 50% range. As expected, the transition mutations (A/G or C/T) were the most abundant, outnumbering the transversion mutations (A/T, C/A, G/C, G/T) by 3.4x margin (Table 2). All SNP information of the combined assembly and the sequences with SNPs have been deposited in dbSNP in Genbank. The SNPs are submitted under the handle UDALL\_LAB (Genbank: ss252842630 to ss252863866; build B133). Full contig sequences are available upon request.

#### *Discovery and frequency of SSRs in ESTs*

The individual and combined assemblies of ESTs were used for the SSR analysis. The individual assembly analysis resulted in a total of 908 contigs containing 1,003 SSRs and 466 contigs containing 507 SSRs in *ssp. tridentata* and *ssp. vaseyana*, respectively. Homopolymer SSRs that are reported by default in MISA were not reported because of known limitations of 454 sequencing chemistry. The occurrence and frequency of different SSR motif repeats in EST sequences of the two subspecies were explored (Table 3). Although both subspecies have a

similar number of reads, the frequency of each type of SSR motif was almost doubled in ssp. *tridentata* compared to ssp. *vaseyana* (Figure 5). As might be expected from data containing open reading frames, the most common type of repeat was a trinucleotide motif (74.7% in ssp. *tridentata*, 88% in ssp. *vaseyana*), followed by a dinucleotide motif (18.5 in ssp. *tridentata*, 5.3% in ssp. *vaseyana*) and a hexanucleotide motif (3% in ssp. *tridentata*, 4% in ssp. *vaseyana*; Table 3). Repeat motifs unique to each subspecies were also detected. Excluding the counts of SSRs in compound formation, subspecies *tridentata* had 143 unique SSRs and ssp. *vaseyana* had 51 unique SSRs, relative to each other. The most dominant repeat motif overall is AC/GT with a frequency of 15.15% (152 of 1,003) in ssp. *tridentata*, whereas the most dominant repeat motif in both subspecies is ACC/GGT with a frequency of 13.4% (134 of 1003 in ssp. *tridentata*) and 20.7% (105 of 507 in ssp. *vaseyana*). We were unable to detect any CG/GC motif in either subspecies' EST sequences. Additional details about di- and trinucleotide repeat motifs in both subspecies are listed in Supplemental Table 3.

In addition to MISA-detected SSRs, a custom Perl script was used to identify putative polymorphic SSRs between ssp. *tridentata* and ssp. *vaseyana* in the combined assembly. Within an assembled contig, the polymorphic SSRs were identified by counting differences in the numbers of repeat motifs during informatic comparison of ssp. *tridentata* sequences to ssp. *vaseyana* sequences. This resulted in detection of 119 polymorphic SSRs in 117 contigs between the two subspecies. Comparing these 119 SSR motif structures to the SSR motif structures identified in individual assemblies, we observed that 111 (93%) SSRs in the combined assembly were found to be identical to those in the individual assemblies and 8 (7%) had a different number of repeats than detected in the individual assemblies.

### *SNP and SSR validation*

SNPs between *A. tridentata* subspecies were directly validated using two different experimental approaches: PCR amplification and subsequent Sanger re-sequencing of cDNA amplicons (SNP primers are listed in Supplemental Table 4 and SSR primers are listed in Supplemental Table 5) and by re-sequencing targeted loci by sequence capture. Analysis of fragment sizes on agarose gel confirmed amplification of all (15 loci) targeted with primers in both subspecies cDNA. Of these loci, 6 were chosen for Sanger re-sequencing. SNP base coverage among the chosen loci ranged from 9 to 27X and from 20% to 46% in their minor allele frequency. Three SNPs were transitions (1 C↔T; 1 G↔A; 1 T↔C) and three were transversions (2 T↔G; 1 C↔G). The bases of each SNP were validated in cDNA from both subspecies, confirming their respective identification within the combined assembly.

In a separate experiment, SNPs were also validated using sequence capture in two distinct collections of ssp. *tridentata* and two distinct collections of ssp. *vaseyana* (result for each SNP are listed Supplemental File 2). 369 ESTs containing 572 discriminating SNPs between the two subspecies were targeted for re-sequencing by sequence capture. These targets were selected based on putative EST function (terpenoid biosynthetic pathway) rather than SNP density. Reads obtained from sequence capture were mapped to their respective reference targets (consensus sequences of contigs from the combined assembly) resulting in reads that overlapped 473 SNPs in 152 contigs. Both SNP bases were detected at 60% of the SNP positions and 47% of the SNP bases were discriminatory between these four independent collections of sspp.

*tridentata* and *vaseyana*. 8% of the SNP positions had an additional or third base at the SNP position.

To validate SSRs, 15 loci were selected from combined sequence assembly. Ten of the 15 primer pairs amplified loci in leaf cDNA from both subspecies. Of these 10 loci, 5 loci were selected for Sanger re-sequencing. Re-sequencing of the selected PCR-amplified cDNA sequences confirmed the MISA-detected SSRs (Supplemental Table 4). Variation in repeat length of the re-sequenced loci was verified between subspecies in 3 of the 5 loci. Of these three SSRs, (CTT)<sub>6</sub> and (GCA)<sub>7</sub> were bioinformatically detected *a priori* as polymorphic, based on the Perl script parameters, whereas (ATA)<sub>5</sub> was not, suggesting that the number of bioinformatically identified polymorphic SSRs was an underestimate of the number of truly polymorphic SSRs. We expect that more SSR loci likely exist but were overlooked due to the stringent parameters used in our bioinformatic analysis. The sequence capture experiment also validated a number of SSRs in contig consensus sequences of the combined assembly. Capture targets included 17 putative SSRs, of which 14 had overlapping reads from sequence capture. In every instance, the presence of an SSR was confirmed. Five SSRs were polymorphic in repeat number, four SSRs contained SNP polymorphisms in one or more repeats, and five SSRs did not have any polymorphisms detected in the sequence capture reads.

#### *Marker evaluation in genomic DNA*

Because of our interest in marker utilization for population genetic studies in genomic DNA (as opposed to ESTs), 15 SSR and 15 SNP primer pairs were evaluated in big sagebrush genomic DNA. Genomic SSR loci were also amplified from the same individuals using the same primers

used for SSR validation in cDNA. Fourteen (93%) SSR loci out of 15 SSR loci amplified in both sspp. *tridentata* and *vaseyana* and 11 (73%) SSR loci out of 15 SSR loci amplified in ssp. *wyomingensis*. These 11 primers pairs produced fragments of expected sizes in all three subspecies, but the sizes were not screened for length polymorphism. Re-sequencing of genomic DNA amplicons for SSR validation was not performed, but we expect that the amplified genomic DNA fragments also contain the targeted SSRs.

Of the 15 SNP primer pairs, 11 (73%) amplified targeted loci in all three subspecies including the five loci used for cDNA SNP validation. The genomic fragments of these five loci were sequenced in two ssp. *tridentata* individual collections, three ssp. *vaseyana* individual collections and two ssp. *wyomingensis* individual collections. For two loci, we observed that both sspp. *tridentata* and *vaseyana* were homozygous at each SNP allele (as expected from the combined assembly) while ssp. *wyomingensis* was dimorphic (i.e. contained both bases). In two different loci, ssp. *wyomingensis* sequences contained a single variant matching either sspp. *tridentata* or sspp. *vaseyana* variant. The remaining SNP remained unconfirmed due poor Sanger sequencing results. Additional SNP validation of individual SNP loci would have been an overly laborious process since other sequencing methods exist for validating larger numbers of SNPs (sequence capture and Illumina re-sequencing). Instead of individually genotyping SNP additional loci, genotypic assessment of ssp. *wyomingensis* at putative SNPs loci was determined *en masse* using Illumina sequencing (see below).

#### *Detection of allelic SNP variants in ssp. wyomingensis*

Approximately 2.5 million and 10.5 million Illumina reads were obtained from the Montana and Utah *ssp. wyomingensis* samples, respectively. After trimming the 5' ends of the sequences to remove barcodes, the sequences were aligned to the combined EST assembly (obtained from 454 sequencing of normalized cDNA libraries) as a sequence reference. This resulted in 695 SNPs mapped at a depth of  $\geq 20x$  with 10% of the reads containing at least one variant in the Montana sample of which 251 SNPs matched with both allelic variants of the other two subspecies (*ssp. tridentata* and *vaseyana*) while 138 SNPs matched to *ssp. tridentata* variant and 306 SNPs matched to *ssp. vaseyana* variant. In the Utah sample, 1,039 SNPs were detected at a depth of  $\geq 20x$  with 10% of the reads containing at least one variant, 458 of which matched with both allelic variants of the other two subspecies while 157 matched to *ssp. tridentata* variant and 424 SNPs matched to *ssp. vaseyana* variant. Reads from the Montana sample verified SNPs among 484 contigs of the combined assembly and reads from the Utah sample verified SNPs were distributed among 767 contigs. This variability of SNP detection is not surprising as the leaf tissue samples were collected in different environments under different conditions and the cDNA fragments were not normalized or modified in any way to alter the association between gene expression levels and number of reads per gene.

#### *Directional selection in subspecies genes*

To detect functional sequence diversity and diversifying selection, putative exon sequences were compared between *ssp. tridentata* and *vaseyana*. Comparison of synonymous base substitutions ( $K_s$ ) and non-synonymous base substitutions ( $K_a$ ) between the two subspecies would suggest whether these sequences were influenced by stabilizing or diversifying selection. The

distribution of the calculated  $K_a/K_s$  ratios for the contigs was found to be ranging from 0.0132 to 6.4000; however any  $K_a/K_s$  value greater than 2.5000 was discarded during the analysis assuming that such high values likely resulted from alignment errors. The resultant distribution had a median of 0.2959 (standard deviation = 0.2627). A bootstrap analysis with 146,744 data points at 95% confidence level ( $\alpha = 0.025$ ) was also performed on the  $K_a/K_s$  distribution, which resulted in 0.0791 as the lower limit and 1.0880 as the upper limit of the confidence interval bracket. A histogram of the  $K_a/K_s$  distribution is shown (Figure 6). Following a method similar to Novaes *et al.* [30], we further classified genes with  $K_a/K_s < 0.15$  to be under stabilizing selection, and  $K_a/K_s$  between 0.50 and 2.50 for diversifying selection, and compared the  $K_a/K_s$  distribution with the gene annotation results. This approach gave us 978 annotated contigs in stabilizing category and 923 annotated contigs in diversifying category. In both categories, the distribution of GO IDs by contigs was the highest for the GO class ‘molecular function’ with 103 unique GO IDs distributed among 508 (51.9%) contigs in stabilizing category and 103 GO IDs distributed among 448 (48.5%) contigs in diversifying category.

## Discussion

Previous reports of ESTs from non-model, yet ecologically and economically important organisms have been successfully sequenced and annotated using next generation sequencing [30-32]. Here, we have used 454 next-generation sequencing generate the first transcriptome sequence data for big sagebrush, a key ecological species of the western North America. Similar to reports of other efforts, the assembled ESTs of big sagebrush were further analyzed to generate a functional characterization of the transcriptome and discover putative molecular markers (SSRs and SNPs). A surprisingly high level of nucleotide diversity was also found within individual assemblies of ESTs from big sagebrush accessions.

To generate a functional characterization of the big sagebrush transcriptome, we compared the contigs and singletons obtained from the combined assembly to peptides within the non-redundant protein database using BLASTx. The low number of matches (54 contigs) to *Artemisia annua* sequences is probably due to fewer number of *A. annua* sequences available in the NR database compared to species such as *Vitis vinifera*. We expect that the numbers of hits will substantially increase with the eventual publication and annotation of an *A. annua* and other *Artemisia* and Asteraceae genome sequences. A majority (69.8%) of the assembled sequences did not align with any peptide in the NR database, possibly indicating the presence of substantial number of novel genes in *A. tridentata* transcriptome and related taxa. Genes of unknown function are not unexpected, as the discovery of novel genes has been demonstrated in other EST sequencing projects within non-agricultural plant families [2, 33].

Many of the contigs and singleton ESTs identified in this study are expected to have ecological and adaptive relevance. Previous studies relating sagebrush biochemistry to mule deer feeding preference suggest strong correlation between the composition and concentration of secondary metabolites, especially terpenoids, and mule deer preference of sagebrush [34, 35]. We were able to identify many, but not all, of the genes coding enzymes involved in MVA, MEP, and phenylterpenoid pathways. The failure to detect all genes from these pathways could be explained by a lack of transcriptome coverage and/or by a lack of pathway documentation of these specific genes [36]. The detection of major enzymes involved in phenylpropanoid pathway in big sagebrush and variation within these pathways may aid in elucidating herbivore preferences and trade-offs between defense responses.

#### *Polymorphisms in A. tridentata ESTs*

A large number of SNP and SSR markers between the two subspecies were discovered and different subsets of SNPs were validated using cDNA, genomic DNA, Illumina cDNA sequencing of ssp. *wyomingensis*, and sequence capture. The SNPs presented here represent differences between the subspecies as well as differences between individual big sagebrush plants since our EST sequencing pool was comprised of only a single sample of two *A. tridentata* leaf transcriptomes. The high numbers of SNPs within accessions, apparent in the individual assemblies (of two individuals), in the validation using ssp. *wyomingensis*, and in the sequence capture assemblies (of four individuals), suggest significant amounts of nucleotide diversity between individual genomes of *Artemisia*. This evidence is supported by three findings. 1) When discriminating SNPs between ssp. *tridentata* and ssp. *vaseyana* were re-identified at a higher stringency than 90% (at 99%), 13% of the SNPs were not detected because of a single

parameter requiring a degree of homogeneity among residues originating from a single DNA source. 2) Using Illumina sequencing, only 36% and 44% of the SNP positions had both alleles detected in the *ssp. wyomingensis* samples respectively, where nearly all of the SNP positions were at least represented by one or the other allele. 3) Using sequence capture, only 47% of the 473 SNP positions were validated as discriminatory between *ssp. tridentata* and *ssp. vaseyana*, but 60% of the SNP positions had both bases detected. Thus, 13% of the sequence capture validated SNP positions also appeared to be heterogeneous (two nucleotides) within the collected individuals used for sequence capture. Much of this nucleotide diversity at initially identified SNP loci could be due to heterozygous loci, though we are careful not to describe it as such until allelism between nucleotide residues is more fully established through segregation analysis. Recall that these EST sequences contain both coding and non-coding sequence (particularly the 3' UTR as the poly-A tail was used for priming the cDNA synthesis). A high level of nucleotide diversity in these coding and non-coding sequences is certainly plausible considering the very large effective population size of sagebrush and wind-pollination strategy [37].

Given the high level of heterozygosity due to the out-crossing nature of big sagebrush populations [37], we expect that a large number of inter-subspecies SNPs and intra-subspecies SNPs could be used in conducting subspecies level association genetics studies. To date, little or no sequence of big sagebrush has been made publically available, thus the SNPs reported here represent a starting point for such future population genetic studies of sagebrush. While they may form the basis of future molecular studies, precaution is needed because informative SNP comparisons will likely depend on the specific individuals selected for genetic analysis. Alternatively, our study suggests that a sequenced based approach to population genetics such as a population-wide genome reduction strategy [18] or amplicon analysis should be considered.

Such an approach would avoid extrapolation of SNPs specific to these individuals to a larger population of individuals (*e.g.* subspecies' specific SNPs that were likely due to genetic variation between individuals, 13%) by generating accession-specific data for each newly sequenced accession. Implementation of such study among spatially distributed big sagebrush populations would 1) enlighten our understanding of natural selection on genes and gene complexes controlling adaptive traits, and the evolution of these trait-linked loci and 2) provide relatedness metrics between natural populations of these subspecies and their hybrid zones. However, these questions are out of the scope of this particular study which aims to primarily characterize EST sequences of big sagebrush and provide insight regarding the origins of *ssp. wyomingensis*.

Regarding the discovered SSRs, we were surprised to find that all SSR repeat motif types detected were much more abundant in *ssp. tridentata* compared to *ssp. vaseyana*. The reduced levels of SSR in *ssp. vaseyana* ESTs compared to *ssp. tridentata* could be due to differential gene expression since different loci were sampled with our non-replicated experimental design. While leaves from both plants were harvested at the same time in common garden, phenological differences between the subspecies might have caused differences in expression levels and thus, changes in the number and types of detected SSRs. While gene expression could explain some of the differences, many such EST-SSRs have been found to be reliable molecular markers in other species [33, 38-41] and they represent hypothetical (*i.e.* testable) genetic divergences between the subspecies.

*K<sub>a</sub>/K<sub>s</sub> and gene evolution in big sagebrush*

The ratio of synonymous and non-synonymous mutations between sspp. *tridentata* and *vaseyana* suggest possible selection pressure resulting in the maintenance of subspecies divergence, as similar trends have been observed in various organisms [42-45]. Since natural selection shapes phenotypes and genotypes in favor of adapted traits, the  $K_a/K_s$  ratio of less than 1 for a large number of contigs could be a result of either stabilizing or diversifying selection within both subspecies, depending upon the magnitude of the ratio. Or if divergence times are very recent, it could also be the hallmark of purifying selection on the adapted common ancestor of these two subspecies. For example, Contig\_29840 ( $K_a/K_s = 0.106$ ) was annotated for ‘aquaporin’ protein. Considering that big sagebrush grows in variety of soils and arid plains, valleys and foothills of mountains, the importance of aquaporin proteins in water balance is critical and the genes coding for aquaporin proteins could have been under stabilizing selection. A formal investigation of molecular evolution within these species (with a proper outgroup) would place selection pressure relative to species divergence.

#### *Exploring the inter-subspecies hybridization hypothesis*

Hybridization can be of great importance to the ecological adaptation and subsequent evolution of offspring because of the novel genetic recombination and spatial selection [46-48]. Generally, allopolyploid formation is considered to have arisen through hybridization between unreduced gametes [49-51]. Several studies have been conducted on polyploid hybrid populations formed from *A. t. ssp. tridentata* and *A. t. ssp. vaseyana* to investigate hybridization events. Generally, these allotetraploid hybrid populations are formed in a narrow zone between the two ecotypes [37, 52-55]. In this study, we did not select a tetraploid ssp. *wyomingensis* along with diploid representatives of its two neighboring ssp. *tridentata* and ssp. *vaseyana* populations. Instead,

selected *ssp. tridentata* and *ssp. vaseyana* accessions were chosen for EST sequencing based on penetrance of specific, subspecies morphological markers (*i.e.* trueness to type). Thus, variation at SNP loci for the diploid-tetraploid comparison is a mixture of individual variation, variation within inter-mating populations, and variation between subspecies in this study. Based on the number of Illumina reads that actually did map to discriminating SNPs between *ssp. tridentata* and *vaseyana*, the tetraploid *ssp. wyomingensis* samples appeared to contain both alleles for a large number of loci (251/695 Montana; 458/1,039 Utah). The presence of both alleles at approximately one-third of the loci suggests that *ssp. wyomingensis* either originated as an allotetraploid from a hybridization event of  $2n$  gametes between *ssp. tridentata* and *vaseyana* or formed as a autopolyploid from both diploid subspecies with subsequent hybridization. Since allopolyploids have been reported between diploids and tetraploids of *ssp. tridentata* and *ssp. vaseyana* [9, 37, 54, 56], a similar scenario is plausible for the origin of *ssp. wyomingensis*. A focused genetic study within and between putative hybrid zones of big sagebrush is needed to further elucidate the origins and reproducibility of hybridization processes involved in *ssp. wyomingensis* formation. If tetraploid recurrence is a common feature of *ssp. wyomingensis*, perhaps only populations of *ssp. tridentata* and *ssp. vaseyana* need active management during environmental conservation of wildlands because a tetraploid hybrid between the two locally adapted accessions could be expected to form and populate geographic zones between the diploid subspecies.

## Conclusions

This study is the first of its kind to perform transcriptome sequencing of big sagebrush subspecies, generating large selections of genetic resources (EST sequences, SNP markers and microsatellites) for this ecologically important group of range and forest plants. The EST sequences were annotated to identify putative gene functions, and select genes involved in putative terpenoid and coumarin synthesis were bioinformatically identified. The distribution of SNPs among *A. tridentata* subspecies and the estimation of depth and divergence of mutations provide insights about the magnitude of neutral divergence and natural selection between these subspecies, and a foundation of sequence references for future population genomic and functional genetic studies. The cost-effective, rapid and reliable way of obtaining nuclear sequences through transcriptome sequencing also provided insights on gene divergence and marker development in big sagebrush. Future studies integrating common garden, provenance and reciprocal transplantation of defined genetic stocks with this genomic information will immeasurably add to our understanding patterns of genes and their roles in adaptive traits among big sagebrush populations.

## REFERENCES

1. Pareto G: **Artemisie. Ricerca ed applicazione.**: Quaderni Agricoli Suppl; 1985.
2. Wang W, Wang Y, Zhang Q, Qi Y, Guo D: **Global characterization of *Artemisia annua* glandular trichome transcriptome using 454 pyrosequencing.** *BMC Genomics* 2009, **10**(1):465.
3. McArthur ED, Stevens R: **Composite shrubs.** In: *Restoring western ranges and wildlands: 2004; Fort Collins, CO, USA.*: USDA Forest Service, Rocky Mountain Research Station; 2004: 493-437.
4. McArthur ED, Sanderson SC: **Cytogeography and chromosome evolution of subgenus *Tridentatae* of *Artemisia* (Asteraceae).** *American Journal of Botany* 1999, **86**:1754–1775.
5. West NE: **Synecology and disturbance regimes of sagebrush steppe ecosystems.** In: *Proceedings of the sagebrush steppe ecosystems symposium: 2000; Boise, ID, USA.*: USDI Bureau of Land Management; 2000: 15–26.
6. Pyke DA: **Invasive exotic plants in sagebrush ecosystems of the intermountain west.** In: *Proceedings: sagebrush steppe ecosystems symposium: 2000; Boise, ID, USA.*: USDI Bureau of Land Management; 2000: 43-54.
7. Rowland MM, Wisdom MJ, Suring LH, Meinke CW: **Greater sage-grouse as an umbrella species for sagebrush-associated vertebrates.** *Biological Conservation* 2006, **129**:323-335.
8. Mahalovich MF, McArthur ED: **Sagebrush (*Artemisia* spp.) seed and plant transfer guidelines.** *Native Plant Journal* 2004, **5**:141-147

9. Wang H, McArthur ED, Sanderson SC, Graham JH, Freeman DC: **Narrow hybrid zone between two subspecies of big sagebrush (*Artemisia tridentata*: Asteraceae). IV. Reciprocal transplant experiments.** *Evolution* 1997, **4**:155-205.
10. Miglia KJ, McArthur ED, Moore W, Wang H, Graham JH, Freeman DC: **Nine-year reciprocal transplant experiment in the gardens of the basin and mountain big sagebrush (*Artemisia tridentata*: Asteraceae) hybrid zone of Salt Creek Canyon: the importance of multiple-year tracking of fitness.** *Biological Journal of the Linnean Society* 2004, **86**:213-225.
11. Atta T, Robelen G: **Cytogenetic relationship within cultivated Brassica analyzed in amphihaploids from the three diploid ancestors.** *Can J Genet Cytol*, **28**:323-329.
12. Wan CY, Wilkins TA: **A modified hot borate method significantly enhances the yield of high-quality RNA from Cotton (*Gossypium hirsutum* L.).** *Analytical Biochemistry* 1994, **223**(1):7-12.
13. Meyer E, Aglyamova G, Wang S, Buchanan-Carter J, Abrego D, Colbourne J, Willis B, Matz M: **Sequencing and de novo analysis of a coral larval transcriptome using 454 GSFlx.** *BMC Genomics* 2009, **10**(1):219.
14. Cronn R, Liston A, Parks M, Gernandt DS, Shen R, Mockler T: **Multiplex sequencing of plant chloroplast genomes using Solexa sequencing-by-synthesis technology.** *Nucleic Acids Research* 2008, **36**(19):e122.
15. Langmead B, Trapnell C, Pop M, Salzberg S: **Ultrafast and memory-efficient alignment of short DNA sequences to the human genome.** *Genome Biology* 2009, **10**(3):R25.

16. McCarthy F, Wang N, Magee GB, Nanduri B, Lawrence M, Camon E, Barrell D, Hill D, Dolan M, Williams WP *et al*: **AgBase: a functional genomics resource for agriculture**. *BMC Genomics* 2006, **7**(1):229.
17. Eddy SR: **Profile hidden Markov models**. *Bioinformatics* 1998, **14**(9):755-763.
18. Maughan PJ, Yourstone SM, Jellen EN, Udall JA: **SNP discovery via genomic reduction, barcoding, and 454-pyrosequencing in amaranth**. *The Plant Genome* 2009, **2**:260–270.
19. Rozen S, Skaletsky H: **Primer3 on the WWW for general users and for biologist programmers**. In., vol. 132; 1999: 365-386.
20. Thiel T, Michalek W, Varshney RK, Graner A: **Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.)**. *Theoretical and Applied Genetics* 2003, **106**:411-422.
21. da Maia LC, Palmieri DA, de Souza VQ, Kopp MM, de Carvalho FI, Costa de Oliveira A: **SSR Locator: Tool for simple sequence repeat discovery integrated with primer design and PCR simulation**. *International Journal of Plant Genomics* 2008, **2008**.
22. **BioPerl** [[www.bioperl.org](http://www.bioperl.org)]
23. Iseli C, Jongeneel CV, Bucher P: **ESTScan: a program for detecting, evaluating, and reconstructing potential coding regions in EST sequences**. *Proceedings International Conference on Intelligent Systems for Molecular Biology* 1999:138-148.
24. Götz S, García-Gómez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, Robles M, Talón M, Dopazo J, Conesa A: **High-throughput functional annotation and data mining with the Blast2GO suite**. *Nucleic Acids Research* 2008, **36**(10):3420-3435.

25. Kelsey RG: **Foliage biomass and crude terpenoid productivity of big sagebrush (*Artemisia tridentata*)**. In: *Proceedings: Symposium on the Biology of Artemisia and Chrysothamnus: 1986; Ogden, UT*: USDA Forest Service; 1986: 375-388.
26. Stevens R, McArthur ED: **A simple field technique for identification of some sagebrush taxa**. *Journal of Range Management* 1974(27):325-326.
27. Shumar ML, Anderson JE, Reynolds TD: **Identification of subspecies of big sagebrush by ultraviolet spectrophotometry**. *Journal of Range Management* 1982, **35**(1):60-62.
28. Kai K, Shimizu B-i, Mizutani M, Watanabe K, Sakata K: **Accumulation of coumarins in *Arabidopsis thaliana***. *Phytochemistry* 2006, **67**(4):379-386.
29. Fritig B, Hirth L, Ourisson G: **Biosynthesis of the coumarins: Scopoletin formation in tobacco tissue cultures**. *Phytochemistry* 1970, **9**(9):1963-1975.
30. Novaes E, Drost D, Farmerie W, Pappas G, Grattapaglia D, Sederoff R, Kirst M: **High-throughput gene and SNP discovery in *Eucalyptus grandis*, an uncharacterized genome**. *BMC Genomics* 2008, **9**(1):312.
31. Parchman T, Geist K, Grahnen J, Benkman C, Buerkle CA: **Transcriptome sequencing in an ecologically important tree species: assembly, annotation, and marker discovery**. *BMC Genomics* 2010, **11**(1):180.
32. Vera JC, Wheat CW, Fescemyer HW, Frilander MJ, Crawford DL, Hanski I, Marden JH: **Rapid transcriptome characterization for a nonmodel organism using 454 pyrosequencing**. *Molecular Ecology* 2008, **17**(7):1636-1647.
33. Zeng S, Xiao G, Guo J, Fei Z, Xu Y, Roe B, Wang Y: **Development of a EST dataset and characterization of EST-SSRs in a traditional Chinese medicinal plant, *Epimedium sagittatum* (Sieb. Et Zucc.) Maxim.** *BMC Genomics* 2010, **11**(1):94.

34. Bray RO, Wambolt CL, Kelsey RG: **Influence of sagebrush terpenoids on mule deer preference.** *Journal of Chemical Ecology* 1991, **17**(11):2053-2062.
35. Personius TL, Wambolt CL, Stephens JR, Kelsey RG: **Crude terpenoid influence on mule deer preference for sagebrush.** *Journal of Range Management* 1987, **40**(1):84-88.
36. Bourgaud F, Hehn A, Larbat R, Doerper S, Gontier E, Kellner S, Matern U: **Biosynthesis of coumarins in plants: a major pathway still to be unravelled for cytochrome P450 enzymes.** *Phytochemistry Reviews* 2006, **5**(2):293-308.
37. McArthur ED, Welch BL, Sanderson SC: **Natural and artificial hybridization between big sagebrush (*Artemisia tridentata*) subspecies.** *Journal of Heredity* 1988, **79**:268-276.
38. Han Z, Wang C, Song X, Guo W, Gou J, Li C, Chen X, Zhang T: **Characteristics, development and mapping of *Gossypium hirsutum* derived EST-SSRs in allotetraploid cotton.** *Theor Appl Genet* 2006, **112**:430 - 439.
39. Zhang Y, Lin Z, Li W, Tu L, Nie Y, Zhang X: **Studies of new EST-SSRs derived from *Gossypium barbadense*.** *Chin Sci Bull* 2007, **52**:2522 - 2531.
40. Yu Y, Wang Z, Feng C, Zhang Y, Lin Z, Zhang X: **Genetic evaluation of EST-SSRs derived from *Gossypium herbaceum*.** *Acta Agronomica Sinica* 2008, **34**:2085 - 2091.
41. Yu Y, Yuan D, Liang S, Li X, Wang X, Lin Z, Zhang X: **Genome structure of cotton revealed by a genome-wide SSR genetic map constructed from a BC1 population between *Gossypium hirsutum* and *G. barbadense*.** *BMC Genomics* 2011, **12**(1):15.
42. Arunyawat U, Stephan W, Stadler T: **Using multilocus sequence data to assess population structure, natural selection, and linkage disequilibrium in wild tomatoes.** *Molecular Biology and Evolution* 2007, **24**(10):2310-2322.

43. Bamshad M, Wooding SP: **Signatures of natural selection in the human genome.** *Nature Reviews Genetics* 2003, **4**(2):99-111.
44. Ford MJ: **Applications of selective neutrality tests to molecular ecology.** *Molecular Ecology* 2002, **11**(8):1245-1262.
45. Tian D, Araki H, Stahl E, Bergelson J, Kreitman M: **Signature of balancing selection in *Arabidopsis*.** *Proceedings of the National Academy of Sciences of the United States of America* 2002, **99**(17):11525-11530.
46. Arnold ML: **Natural hybridization and evolution.** Oxford: Oxford University Press 1997.
47. Durrett R, Buttel L, Harrison R: **Spatial models for hybrid zones.** *Heredity* 2004, **84**:9–19.
48. Rieseberg LH: **Hybrid origins of plant species.** *Ecology and Systematics* 1997, **28**:359–389.
49. McArthur ED: **Breeding systems in shrubs.** San Diego, CA: Academic Press, Inc.; 1989.
50. Otto SP, Whitton J: **Polyploid incidence and evolution.** *Annual Review of Genetics* 2000, **34**(1):401-437.
51. Husband BC, Schemske DW: **Cytotype distribution at a diploid–tetraploid contact zone in *Chamerion (Epilobium) angustifolium* (Onagraceae).** *Am J Bot* 1998, **85**(12):1688-1694.
52. Freeman DC, Turner WA, McArthur ED, Graham JH: **Characterization of a narrow hybrid zone between two subspecies of big sagebrush (*Artemisia tridentata*: Asteraceae).** *American Journal of Botany* 1991, **78**:805-815.

53. Graham JH, Freeman DC, McArthur ED: **Narrow hybrid zone between two subspecies of big sagebrush (*Artemisia tridentata*: Asteraceae). II. Selection gradients and hybrid fitness.** *American Journal of Botany* 1995, **82**:709-716.
54. McArthur ED, Mudge J, Buren RV, Anderson WR, Sanderson SC, Babbel DG: **Randomly amplified polymorphic DNA analysis (RAPD) of *Artemisia* subgenus *Tridentatae* species and hybrids.** *Great Basin Naturalist* 1998, **58**:12-27.
55. McArthur ED, Blauer AC, Plummer AP, Stevens R: **Characteristics and hybridization of important intermountain shrubs. III. Sunflower family.** *Res Pap* 1979, INT-**220**:82.
56. Weber DJ, Gang DR, Halls SC, Smith BN, McArthur ED: **Inheritance of hydrocarbons in subspecific big sagebrush (*Artemisia tridentata*) hybrids.** *Biochemical Systematics and Ecology* 1994, **22**(7):689-697.

## TABLES

Table 1: Summary of individual and combined *de novo* assembly of the subspecies sequences generated from 454-pyrosequencing

| Assembly                  |            | Count     | Average length | N50 (bp) | Total bases |
|---------------------------|------------|-----------|----------------|----------|-------------|
| ssp.<br><i>tridentata</i> | Reads      | 823,392   | 403.91         |          | 332,578,737 |
|                           | Singletons | 191,745   | 403.62         |          | 77,391,754  |
|                           | Contigs    | 20,357    | 716            | 869      | 14,587,705  |
| ssp.<br><i>vaseyana</i>   | Reads      | 702,001   | 333.13         |          | 233,854,535 |
|                           | Singletons | 179,189   | 331.51         |          | 59,402,844  |
|                           | Contigs    | 20,250    | 624            | 797      | 12,641,189  |
| ssp.<br>combined          | Reads      | 1,525,393 | 371.34         |          | 566,433,272 |
|                           | Singletons | 275,866   | 370.18         |          | 102,121,262 |
|                           | Contigs    | 29,541    | 796            | 1.003    | 23,521,465  |

Table 2: Frequency of SNPs between *ssp. tridentata* and *ssp. vaseyana* by mutation types

|               | SNP type | Count | % Count | % Total |
|---------------|----------|-------|---------|---------|
| Transitions   | C/T      | 6456  | 30.81   | 62.24   |
|               | A/G      | 6586  | 31.43   |         |
| Transversions | A/T      | 2352  | 11.23   | 37.7    |
|               | C/G      | 1533  | 7.32    |         |
|               | A/C      | 2043  | 9.75    |         |
|               | G/T      | 1970  | 9.40    |         |
|               | Total    | 20940 | 99.94   | 99.94   |

Table 3: SSR frequencies of repeat type with repeat numbers in *A. tridentata* ssp. *tridentata* (*A.t.t.*) and ssp. *vaseyana* (*A.t.v.*)

| Motif<br>length | Repeat number |               |               |               |               |               |               |               |               |               |               |               | Total         |               |
|-----------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
|                 | <i>A.t.t.</i> | <i>A.t.v.</i> | <i>A.t.t.</i> | <i>A.t.v.</i> | <i>A.t.t.</i> | <i>A.t.v.</i> | <i>A.t.t.</i> | <i>A.t.v.</i> | <i>A.t.t.</i> | <i>A.t.v.</i> | <i>A.t.t.</i> | <i>A.t.v.</i> | <i>A.t.t.</i> | <i>A.t.v.</i> |
|                 | <b>5</b>      |               | <b>6</b>      |               | <b>7</b>      |               | <b>8</b>      |               | <b>9</b>      |               | <b>≥10</b>    |               |               |               |
| <b>Di</b>       | -             | -             | -             | -             | 104           | 15            | 34            | 2             | 17            | 1             | 31            | 9             | 186           | 27            |
| <b>Tri</b>      | 431           | 250           | 186           | 127           | 75            | 35            | 30            | 16            | 10            | 10            | 17            | 8             | 749           | 446           |
| <b>Tetra</b>    | 23            | 10            | 5             | 2             | 3             | 0             | 1             | 0             | 1             | 0             | 0             | 0             | 33            | 12            |
| <b>Penta</b>    | 2             | 0             | 2             | 1             | 0             | 1             | 0             | 0             | 1             | 0             | 0             | 0             | 5             | 2             |
| <b>Hexa</b>     | 22            | 16            | 3             | 0             | 2             | 3             | 1             | 1             | 1             | 0             | 1             | 0             | 30            | 20            |
| <b>≥Hepta</b>   | 0             | 0             | 0             | 0             | 0             | 0             | 0             | 0             | 0             | 0             | 0             | 0             | 0             | 0             |
| <b>Total</b>    | 478           | 276           | 196           | 130           | 184           | 51            | 66            | 19            | 30            | 11            | 49            | 17            | 1003          | 507           |
| <b>%</b>        | 47.6          | 54.5          | 19.5          | 25.6          | 18.3          | 10.1          | 6.6           | 3.7           | 3.0           | 2.2           | 4.9           | 3.8           |               |               |

## FIGURES

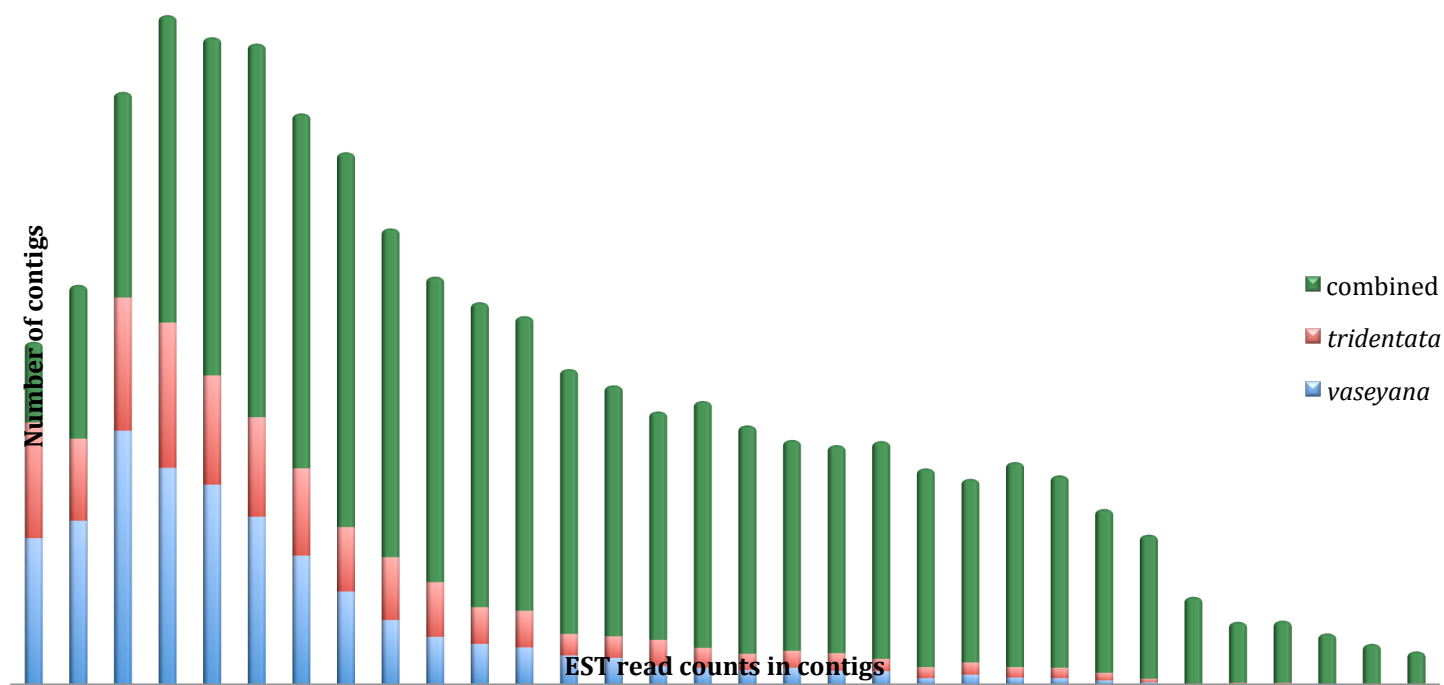


Figure 1: Histogram of number of EST members in a contig.

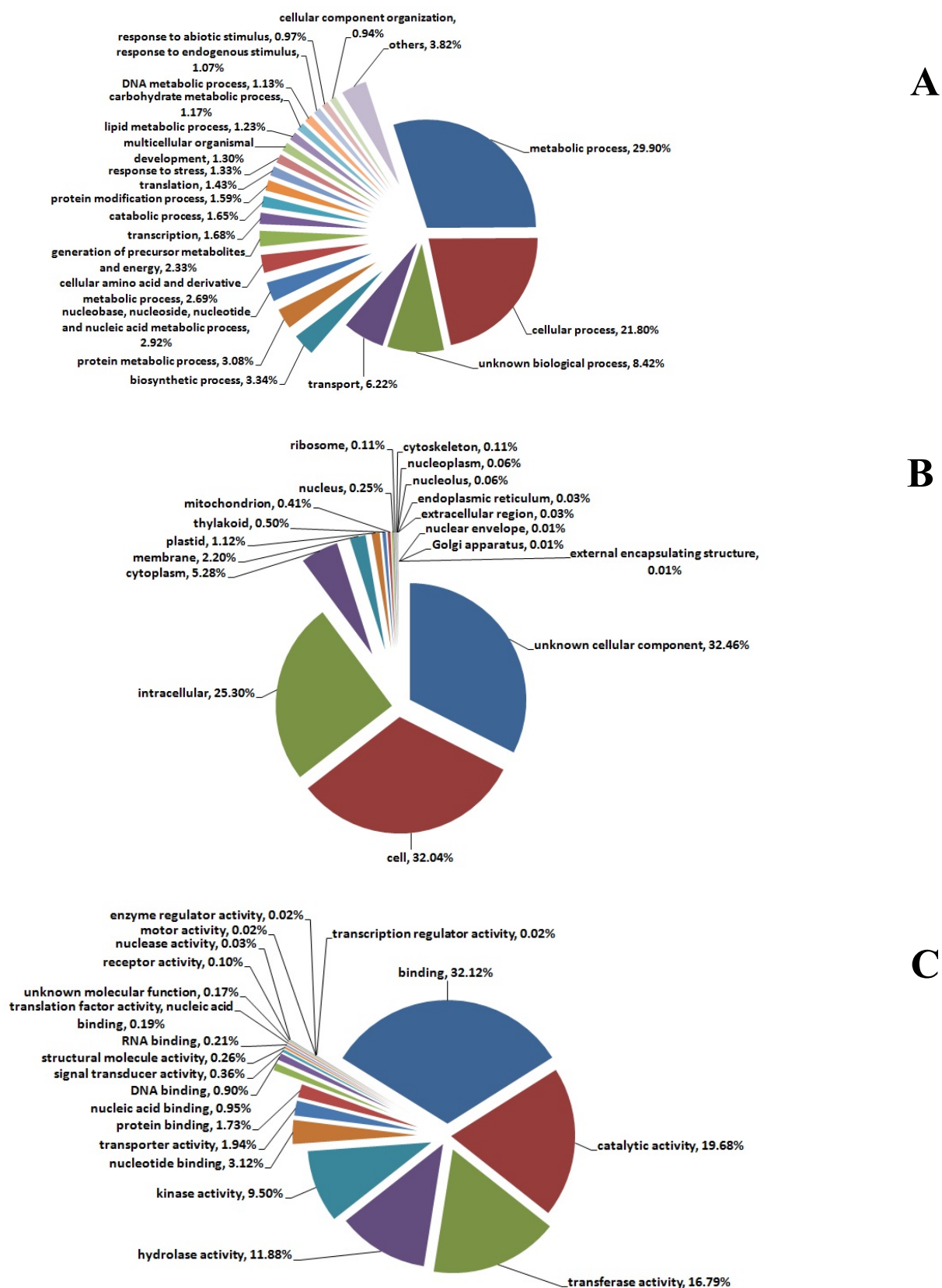


Figure 2: Distribution of consensus sequences from combined assembly of *Artemisia tridentata* ssp. *tridentata* and ssp. *vaseyana* in three main GO categories.

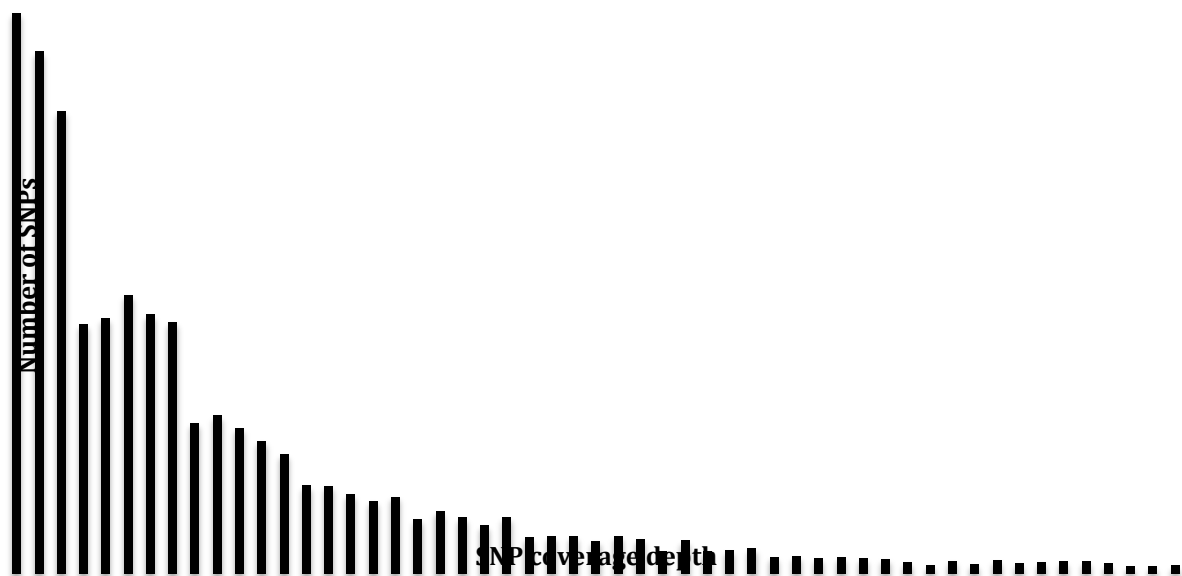


Figure 3: Distribution of the number of SNPs by read coverage depth.

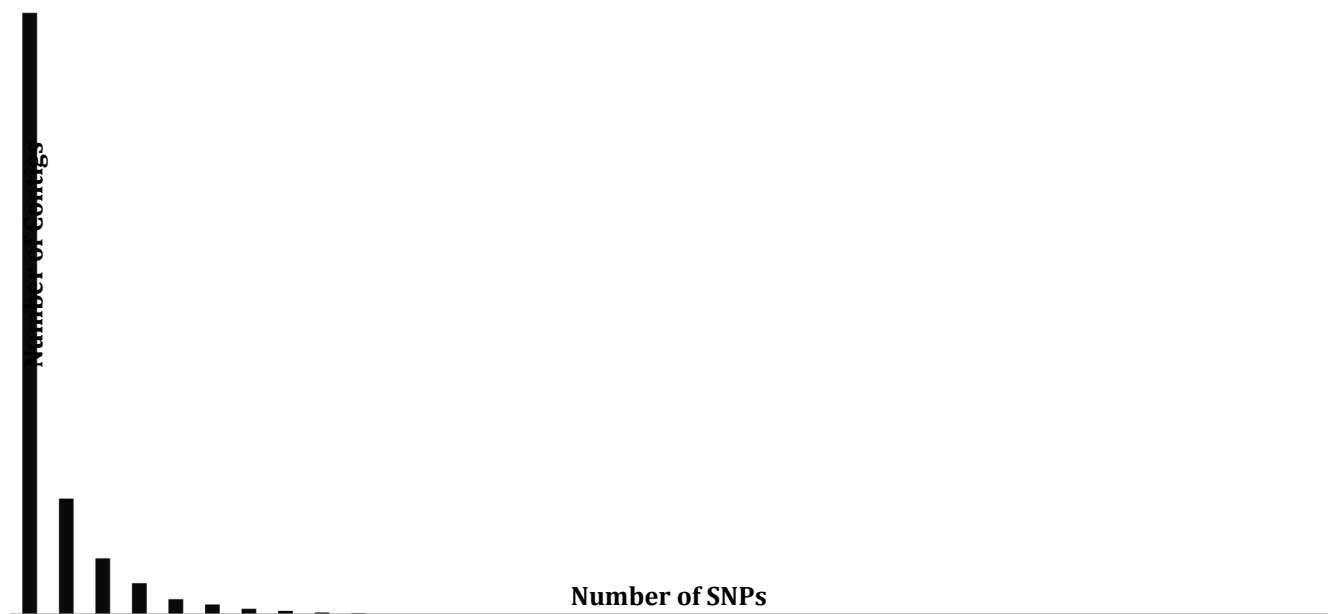


Figure 4: Distribution of contigs by number of SNPs in a combined assembly of *A. t.* ssp. *tridentata* and ssp. *vaseyana*.

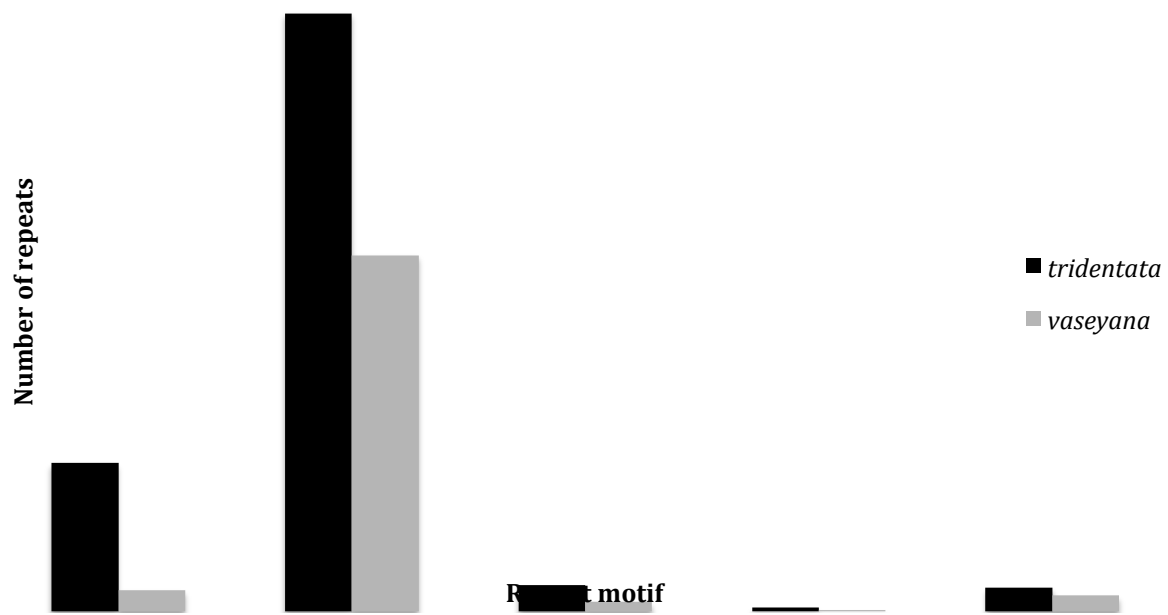


Figure 5: Frequency and distribution of SSRs in *A. t. ssp. tridentata* and *A. t. ssp. vaseyana*

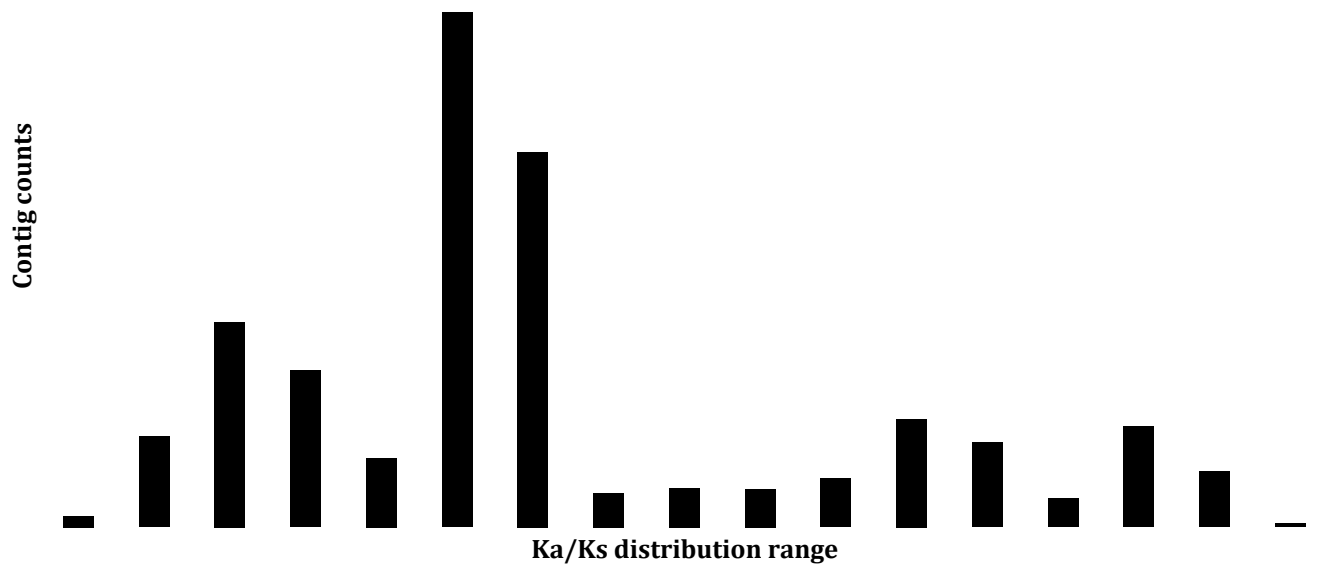


Figure 6: A histogram illustrating the distribution of the ratio of non-synonymous mutations to non-synonymous sites compared to synonymous mutations per synonymous sites ( $K_a/K_s$ ).

## SUPPLEMENTAL DATA

Supplemental Table 1: List of individuals used in the study

### Samples used for 454 sequencing

| Site                 | Latitude    | Longitude     | Elevation (m) | Plant ID | Putative race     | Ploidy |
|----------------------|-------------|---------------|---------------|----------|-------------------|--------|
| near Park Valley, UT | 41°49'1.2"N | 113° 17' 42"W | 1680          | N/A      | <i>tridentata</i> | 2      |
| near Park Valley, UT | 41°49'1.2"N | 113° 17' 42"W | 1680          | N/A      | <i>vaseyana</i>   | 2      |

### Samples used for Illumina sequencing

| Site            | Latitude      | Longitude      | Elevation (m) | Plant ID | Putative race       | Ploidy |
|-----------------|---------------|----------------|---------------|----------|---------------------|--------|
| Powderville, MT | 45°39'16.31"N | 105° 9'16.12"W | 972           | MTW1     | <i>wyomingensis</i> | 4      |
| Scooby, UT      | 41°51'23.97"N | 113° 3'0.20"W  | 1438          | UTW2     | <i>wyomingensis</i> | 4      |

### Samples used for marker evaluation

| Site               | Latitude      | Longitude      | Elevation (m) | Plant ID | Putative race       | Ploidy |
|--------------------|---------------|----------------|---------------|----------|---------------------|--------|
| Desert View, AZ    | 35°55'2.57"N  | 111°46'22.33"W | 1898          | AZT1     | <i>tridentata</i>   | 4      |
| Crab Creek, WA     | 46°56'55.68"N | 119°14'13.56"W | 312           | WAT2     | <i>tridentata</i>   | 4      |
| Benton, CA         | 37°43'24.30"N | 118°35'33.35"W | 2330          | CAV2     | <i>vaseyana</i>     | 2      |
| Currant, NV        | 38°49'5.99"N  | 115°17'0.66"W  | 2132          | NVV2     | <i>vaseyana</i>     | 2      |
| Robber's Roost, NV | 36°18'10.31"N | 115°36'37.42"W | 2410          | NVV3     | <i>vaseyana</i>     | 4      |
| Meeker, CO         | 40°10'52.63"N | 108°27'19.72"W | 1788          | COW2     | <i>wyomingensis</i> | 4      |
| La Sal, UT         | 38°19'28.60"N | 109°25'46.58"W | 1797          | UTW1     | <i>wyomingensis</i> | 4      |

Supplemental Table 2: Contig sequences in the EST collection annotated as enzymes involved in terpenoid and coumarin synthesis pathways

| Pathway            | Enzymes                                                                                          | No. of hits<br>(ssp.<br><i>tridentata</i> ) | No. of hits<br>(ssp.<br><i>vaseyana</i> ) | No. of hits<br>(ssp.<br>combined) |
|--------------------|--------------------------------------------------------------------------------------------------|---------------------------------------------|-------------------------------------------|-----------------------------------|
| MEP                | 1-deoxy-D-xylulose 5-phosphate synthase (DXS)                                                    | 51                                          | 100                                       | 111                               |
|                    | 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXP)                                            | 83                                          | 118                                       | 64                                |
|                    | 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCT)                                      | 22                                          | 22                                        | 22                                |
|                    | 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK)                                          | 63                                          | 126                                       | 168                               |
|                    | 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS)                                      | 22                                          | 22                                        | 16                                |
|                    | 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase (HDS)                                    | 0                                           | 0                                         | 0                                 |
|                    | 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR)                                   | 20                                          | 12                                        | 24                                |
|                    | isopentenyl diphosphate/ dimethylallyl diphosphate isomerase (IDI, also involved in MVA pathway) | 36                                          | 20                                        | 36                                |
|                    | isopentenyl diphosphate/ dimethylallyl diphosphate synthase (IDS)                                | 0                                           | 0                                         | 0                                 |
| MVA                | acetoacetyl-coenzyme A thiolase (AACT)                                                           | 39                                          | 21                                        | 27                                |
|                    | 3-hydroxy-methylglutaryl coenzyme A synthase (HMGS)                                              | 0                                           | 0                                         | 0                                 |
|                    | 3-hydroxy-methylglutaryl coenzyme A reductase (HMGR)                                             | 0                                           | 0                                         | 0                                 |
|                    | Mevalonate kinase (MK)                                                                           | 0                                           | 50                                        | 10                                |
|                    | phosphomevalonate kinase (PMK)                                                                   | 0                                           | 0                                         | 15                                |
|                    | mevalonate disphosphate decarboxylase (MDC)                                                      | 20                                          | 0                                         | 20                                |
| Coumarin synthesis | phenylalanine ammonia lyase                                                                      | 29                                          | 45                                        | 48                                |
|                    | cinnamate 4-hydroxylase                                                                          | 28                                          | 70                                        | 260                               |
|                    | 4-coumarate CoA ligase                                                                           | 322                                         | 215                                       | 255                               |

Supplemental Table 3: Additional details of SSRs including frequencies of di- and tri-nucleotide repeats

| Repeat<br>Motif | Repeat Number |               |               |               |               |               |               |               |               |               |               |               | Total         |               |
|-----------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
|                 | <i>A.t.t.</i> | <i>A.t.v.</i> | <i>A.t.t.</i> | <i>A.t.v.</i> | <i>A.t.t.</i> | <i>A.t.v.</i> | <i>A.t.t.</i> | <i>A.t.v.</i> | <i>A.t.t.</i> | <i>A.t.v.</i> | <i>A.t.t.</i> | <i>A.t.v.</i> | <i>A.t.t.</i> | <i>A.t.v.</i> |
|                 | 5             |               | 6             |               | 7             |               | 8             |               | 9             |               | ≥10           |               |               |               |
| AC/GT           | -             | -             | -             | -             | 89            | 11            | 27            | 1             | 11            | 1             | 25            | 6             | 152           | 19            |
| AG/CT           | -             | -             | -             | -             | 14            | 0             | 6             | 1             | 6             | 0             | 6             | 3             | 32            | 4             |
| AT/AT           | -             | -             | -             | -             | 1             | 4             | 1             | 0             | 0             | 0             | 0             | 0             | 2             | 4             |
| AAC/GTT         | 69            | 26            | 21            | 10            | 16            | 5             | 4             | 3             | 1             | 0             | 4             | 1             | 115           | 45            |
| AAG/CTT         | 68            | 37            | 37            | 16            | 12            | 3             | 3             | 3             | 2             | 3             | 5             | 2             | 127           | 64            |
| AAT/ATT         | 21            | 9             | 15            | 8             | 5             | 4             | 1             | 0             | 0             | 0             | 0             | 1             | 42            | 22            |
| ACC/GGT         | 82            | 58            | 42            | 33            | 7             | 9             | 3             | 4             | 0             | 1             | 0             | 0             | 134           | 105           |
| ACG/CTG         | 20            | 7             | 8             | 5             | 4             | 1             | 2             | 0             | 0             | 1             | 0             | 0             | 34            | 14            |
| ACT/ATG         | 50            | 41            | 22            | 16            | 12            | 2             | 8             | 3             | 4             | 2             | 4             | 0             | 100           | 64            |
| AGC/CGT         | 22            | 19            | 4             | 12            | 4             | 3             | 1             | 1             | 2             | 1             | 0             | 1             | 33            | 37            |
| AGG/CCT         | 21            | 12            | 9             | 6             | 4             | 3             | 1             | 0             | 0             | 0             | 0             | 0             | 35            | 21            |
| AGT/ATC         | 68            | 39            | 28            | 21            | 11            | 5             | 7             | 2             | 1             | 2             | 4             | 3             | 119           | 72            |
| CCG/CGG         | 10            | 2             | 0             | 0             | 0             | 0             | 0             | 0             | 0             | 0             | 0             | 0             | 10            | 2             |

Supplemental Table 4: Primers used for SNP validation

| SNP type | Primer Sequence                                      | T <sub>m</sub> (°C) | Amplified? | Polymorphic? |
|----------|------------------------------------------------------|---------------------|------------|--------------|
| C/T      | 5'-GGATGGGTTATGCTACCGAA                              | 54.4                | Yes        | Yes          |
|          | 5'-ATGTGCATGGGCATGAAGTA                              | 54.6                |            |              |
| C/G      | 5'-AGATATCGCCCGAAACATTG                              | 52.8                | Yes        | Yes          |
|          | 5'-AATGTCTGCTCCCATTGTCA                              | 54.3                |            |              |
| T/G      | 5'-AACAAAGATACAGTCGCTAACCA                           | 54.5                | Yes        | Yes          |
|          | 5'-TGTCTGGTAGCTGAATTTCCAA                            | 54.2                |            |              |
| C/T      | 5'-CGTGTTCGGTATCTCAAGGAT                             | 54.3                | Yes        | Not tested   |
|          | 5'-GTGGAACACGTGCTTTGAAC                              | 54.8                |            |              |
| G/A      | 5'-CACCAGATTGACCCGAGATT                              | 54.5                | No         | N/A          |
|          | 5'-GTGGAACACGTGCTTTGAAC                              | 56.9                |            |              |
| G/A      | 5'-AAGGGTTTGCAGAAGAGAGTGT                            | 56.5                | Yes        | Not tested   |
|          | 5'-TGAGTTGGCATATGTTGATTAC                            | 53.5                |            |              |
| T/G      | 5'-CCCATGCTGCAATAGACAAC                              | 54.1                | Yes        | Yes          |
|          | 5'-GGGTTATAATTATCATGATGTGCTGA                        | 53.1                |            |              |
| G/A      | 5'-TGAGCTATAACCTGAGAAAGTTACAAA                       | 54.2                | Yes        | Yes          |
|          | 5'-CCGTGATCTAGGAATGCCA                               | 54.2                |            |              |
| T/C      | 5'-TCAAGTTCATGAGGCAGCAA                              | 54.7                | Yes        | Yes          |
|          | 5'-GAATGGAGCAAGTCCCTTCA                              | 54.8                |            |              |
| T/C      | 5'-CCAGCTCGTTCACACAAACA                              | 55.8                | Yes        | Not tested   |
|          | 5'-ATGGCACTTGGACCTTGAAC                              | 55.6                |            |              |
| A/G      | 5'-ACACTGACGACATGGTTCTACACTTGTACAAACCCAAACCC         | 67                  | Yes        | Not tested   |
|          | 5'-TACGGTAGCAGAGACTTGGTCTTGCGAACTTCAAGGTCGGTA        | 67.6                |            |              |
| A/G      | 5'-ACACTGACGACATGGTTCTACAAATCCATCTATGATCTTACAGTCAAGG | 65                  | No         | N/A          |
|          | 5'-TACGGTAGCAGAGACTTGGTCTAGGTGAGGTTGTAGGGTAATAACG    | 66.4                |            |              |
| T/G      | 5'-ACACTGACGACATGGTTCTACAAGGATATTCGTGGAACGAGG        | 66.1                | No         | N/A          |
|          | 5'-TACGGTAGCAGAGACTTGGTCTCAATCAAGTGATACACCAACCA      | 66.4                |            |              |
| G/A      | 5'-ACACTGACGACATGGTTCTACATGATGGGACTGTTGTTGCTG        | 66.8                | No         | N/A          |
|          | 5'-TACGGTAGCAGAGACTTGGTCTATCTCTCCAGGTGCTTTGGA        | 67.4                |            |              |
| G/A      | 5'-ACACTGACGACATGGTTCTACAAAAGCCTCTCGTGTGTTGTCC       | 66.8                | Yes        | Not tested   |
|          | 5'-TACGGTAGCAGAGACTTGGTCTGGCTCCTTCTGATGAAGCTG        | 68                  |            |              |

'Not tested' means the loci were not sequenced.

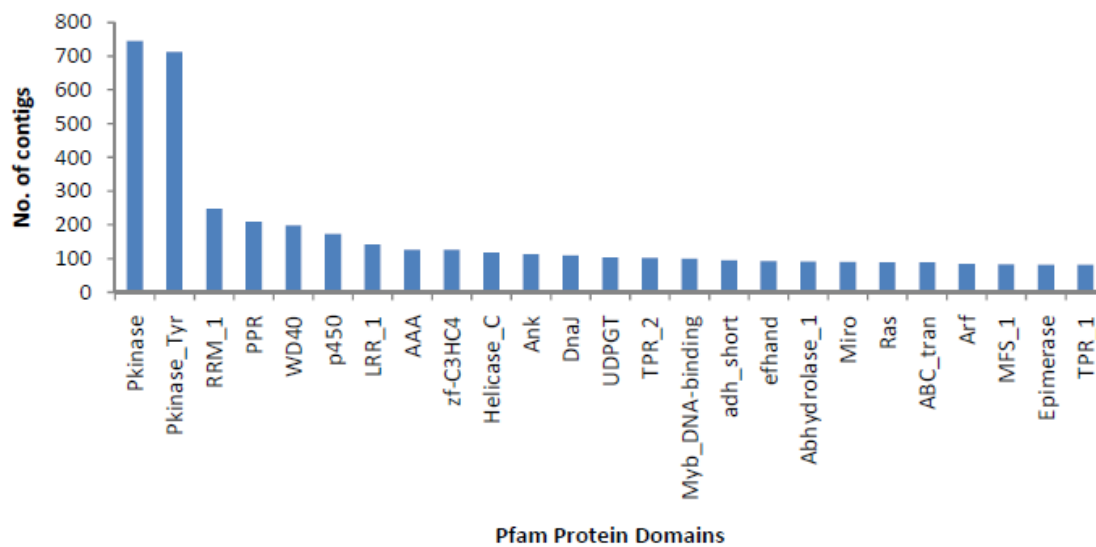
Supplemental Table 5: Primers used for SSR validation

| SSR type  | Primer Sequence          | Tm (°C) | Amplified? | Polymorphic? |
|-----------|--------------------------|---------|------------|--------------|
| (CA)11    | 5'-GCAGTGGTATCAACGCAGAG  | 59.47   | No         | N/A          |
|           | 5'-TATTGCATCAACAGCCAACC  | 59.55   |            |              |
| (ATA)5    | 5'-GGCAAATTAAGGTGCAAGGA  | 60.07   | Yes        | No           |
|           | 5'-TATAAAACCCACGCCGTCTC  | 59.96   |            |              |
| (TCA)9    | 5'-GGTGGGATAAGTGTGTCGCT  | 59.99   | Yes        | No           |
|           | 5'-CCTTCTGCGTCTCAAGTTCC  | 59.98   |            |              |
| (GCA)7    | 5'-AGGACCCTTGCAAGCAGTAA  | 59.88   | Yes        | Yes          |
|           | 5'-TTAACCCCTGTGGAGGAGGTG | 59.96   |            |              |
| (TG)7     | 5'-ACCCCGTCGCTTTAGTTCTT  | 60.13   | Yes        | Not tested   |
|           | 5'-TTGCTCAAACCTGAATCACGC | 59.99   |            |              |
| (TAGGGT)5 | 5'-TGCAAACCCATCATACGAAA  | 59.93   | No         | N/A          |
|           | 5'-TGAAATCATTGCCTGACCAA  | 60.05   |            |              |
| (CAAAC)6  | 5'-CTGCCTCCTTTCCTCACTTG  | 59.98   | No         | N/A          |
|           | 5'-TGTGTTGGTTGTTTGCTGGT  | 60.05   |            |              |
| (GAT)8    | 5'-TTCGTCTTTCCTGTTCGCTT  | 59.99   | Yes        | Not tested   |
|           | 5'-GCCCCAACCTCCTCTTCATCT | 60.59   |            |              |
| (CTT)6    | 5'-ATCCTTCAACAACGGAAACG  | 59.97   | Yes        | Yes          |
|           | 5'-TGACAACAACAGCAACAGCA  | 60.07   |            |              |
| (GA)9     | 5'-CCGAAAAGCAAGAACCTCTG  | 59.99   | No         | N/A          |
|           | 5'-AACAGTCCCCGCCTTATTCT  | 59.96   |            |              |
| (TC)10    | 5'-GCAGTGGTATCAACGCAGAG  | 59.47   | Yes        | Not tested   |
|           | 5'-ATTTCTGTGGGGTCAGTTGC  | 59.97   |            |              |
| (TTAG)6   | 5'-ACCGCATCGCTGATAATCTC  | 60.21   | No         | N/A          |
|           | 5'-TTCCAACAATCAACCCCAT   | 60.03   |            |              |
| (GGAT)5   | 5'-CATCATTTTGTGACCCACTT  | 55.85   | Yes        | No           |
|           | 5'-ACCCTAACACGCTCACCATC  | 59.99   |            |              |
| (CTTT)7   | 5'-ATCGCCAGAAAAGCTTGAAA  | 59.96   | Yes        | Not tested   |
|           | 5'-TGTTAGTGAACGGGTGTCCA  | 60      |            |              |
| (AAGG)10  | 5'-AACCCCTGCACACCAGTAGG  | 60.03   | Yes        | Not tested   |
|           | 5'-GCTCTGTGCCTTTCCTTTTG  | 59.99   |            |              |

'Not tested' means the loci were not sequenced.

Supplemental Figure 1: Distribution of protein domain vs number of contigs

The number of contigs on Y-axis represents total number of contigs that had a match against a protein domain. Only the top 25 most common domains (of 3065 domains found) are illustrated in the figure.



Supplemental File 1: A list of contigs containing discriminatory SNPs between ssp. tridentata and ssp. vaseyana including contig name, SNP position, base for each subspecies, read count per base, flagged contigs with >13 SNPs, and SNPs that were found to be heterogeneous when the parameter of homogeneity was raised to 99%

Because of its large size, the file has been stored in the following link:

[https://lswiki.byu.edu/index.php/Thesis\\_materials\\_only](https://lswiki.byu.edu/index.php/Thesis_materials_only)  
(direct link: [https://lswiki.byu.edu/images/f/f0/Thesis\\_-\\_Additional\\_File\\_1\\_-\\_SNP\\_info.xlsx](https://lswiki.byu.edu/images/f/f0/Thesis_-_Additional_File_1_-_SNP_info.xlsx))

## Supplemental File 2: Results for SNP validation during sequence capture

Because of its large size, the file has been stored in the following link:

[https://lswiki.byu.edu/index.php/Thesis\\_materials\\_only](https://lswiki.byu.edu/index.php/Thesis_materials_only)

(direct link: [https://lswiki.byu.edu/images/d/d3/Thesis\\_-\\_Additional\\_File\\_2\\_-\\_SNP\\_validation.xlsx](https://lswiki.byu.edu/images/d/d3/Thesis_-_Additional_File_2_-_SNP_validation.xlsx))

