Transcriptomes of *Saussurea* (Asteraceae) Provide Insights into High-Altitude Adaptation

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**Abstract:** Understanding how species adapt to extreme environments is an extension of the main goals of evolutionary biology. While alpine plants are an ideal system for investigating the genetic basis of high-altitude adaptation, genomic resources in these species are still limited. In the present study, we generated reference-level transcriptomic data of five *Saussurea* species through high-throughput sequencing and *de novo* assembly. Three of them are located in the highland of the Qinghai-Tibet Plateau (QTP), and the other two are close relatives distributed in the lowland. A series of comparative and evolutionary genomics analyses were conducted to explore the genetic signatures of adaptive evolution to high-altitude environments. Estimation of divergence time using single-copy orthologs revealed that *Saussurea* species diversified during the Miocene, a period with extensive tectonic movement and climatic fluctuation on the QTP. We characterized gene families specific to the alpine species, including genes involved in oxidoreductase activity, pectin catabolic process, lipid transport, and polysaccharide metabolic process, which may play important roles in defense of hypoxia and freezing temperatures of the QTP. Furthermore, in a phylogenetic context with the branch model, we identified hundreds of genes with signatures of positive selection. These genes are involved in DNA repair, membrane transport, response to UV-B and hypoxia, and reproductive processes, as well as some metabolic processes associated with nutrient intake, potentially responsible for *Saussurea* adaptation to the harsh environments of high altitude. Overall, our study provides valuable genomic resources for alpine species and gained helpful insights into the genomic basis of plants adapting to extreme environments.

**Keywords:** high-altitude adaptation; *Saussurea*; transcriptomes; positive selection; extreme environments; lineage-specific genes

1. **Introduction**

Understanding the genetic basis of species adapting to high altitude is a long-standing theme of evolutionary biology [1–5]. Species inhabiting high-altitude environments have...
to face a variety of abiotic stresses, such as reduced oxygen availability, rapid fluctuations in temperature, and high ultraviolet (UV) radiation [6–8]. These stresses present strong selective pressure and have driven striking phenotypic and genetic adaptations [9–12]. Advances in high-throughput sequencing, especially RNA sequencing (RNA-Seq), enable the collection of gene sequences for non-model species [13], opening up a new era for comparative evolutionary studies on genetic adaptations [14–16].

The Qinghai-Tibet Plateau (QTP) is the highest (average elevation above 4000 m) and the largest (ca. 2.5 million km$^2$) plateau in the world [17,18], representing an ideal natural laboratory for investigating ecological adaptation in high-altitude environments. The uplift of the QTP creates profound climatic and environmental diversity, making this area one of the world’s richest temperate floras [19–21]. While several genome-wide studies on adaptive evolution on the QTP have been conducted, including humans [2,9], plants [4–6], vertebrates [11], and bacteria [22], our knowledge of genetic adaptation to high-altitude is far from adequate, due to empirical studies showing that different organisms adapt to high altitudes via multiple genetic routes [11]. Especially in the alpine scree of the QTP (usually elevation above 4500 m), characterized by freezing temperatures, high winds, strong UV rays, and poor soil quality, plants have evolved extraordinarily diverse traits for adaptation, such as leafy bracts, woolly trichomes and cushion plants [23–25]. Despite increased understanding of the ecological benefits of these adaptive traits [26,27], the genetic mechanisms underlying high-altitude adaptation are still poorly studied.

Saussurea DC. is one of the most species-rich genera in Asteraceae [28], comprising approximately 400 species, traditionally recognized as four subgenera, distributed in Asia, Europe, and North America, with the highest diversity in the QTP [28,29]. Species in Saussurea are present in all possible terrestrial habitats, including steppes, moist forests, cold and dry alpine meadows, and scree slopes above 5000 m, demonstrating the capability to be highly adaptive [28–31]. Previous investigations indicated that Saussurea originated in the middle Miocene, and the uplift of the QTP promoted the rapid diversification of this alpine lineage [30,32]. In addition, Saussurea exhibits extraordinary morphological diversity, such as species with woolly trichomes, so-called ‘snow rabbits’, in S. subg. Eriocoryne, and species with leafy bracts, so-called ‘snow lotuses’, in S. subg. Amphilaena [28,29]. A recent study revealed that these attractive traits of Saussurea are the result of convergent adaptation to high-altitude environments [31] and are prevalent among species inhabiting alpine scree of the QTP [26], making the genetic basis behind ecological adaptation more enigmatic.

In the present study, we sampled three Saussurea species from alpine scree of the QTP (S. pachyneura Franch., S. salwinensis J. Anthony, and S. velutina W.W. Smith) as well as two lowland relatives (S. amara (L.) DC. and S. amurensis Turcz. ex DC.) to generate reference-level transcriptome sequences using RNA-seq and de novo assembly. By conducting comparative and evolutionary genomics analyses, including the detection of lineage-specific gene families and genes under positive selection on different branches, we aim to gain insights into the genetic basis of adaptive evolution to high-altitude environments at the genomic level.

2. Results

2.1. De novo Assembly and Annotation of Unigenes

We generated 80.0–92.1 million reads per species, yielding 12.0–13.8 Giga-base (Gb) of RNA-seq data per species. After quality control, 79.8–92.0 million reads per species remained, consisting of 11.7–13.6 Gb clean data per species (Supplementary Table S2). De novo assembly yielded 176,265–270,778 transcripts with 151.5–237.3 million base and 91,980–147,303 unigenes with 65.9–87.5 million base, respectively. The median length of transcripts was 417 bp–611 bp (mean length: 646.45 bp–967.28 bp), and the median length of unigenes was 353 bp–427 bp (mean length: 574.69 bp–763.58 bp), with contig N50s ranging from 899 bp to 1562 bp for transcripts and from 787 bp to 1334 bp for unigenes (Table 1). BUSCO results showed that the completeness of de novo assembly reached 88.1%,
80.8%, 84.8%, 87.1%, and 83.7% for *S. amara*, *S. amurensis*, *S. pachyneura*, *S. salwinensis*, and *S. velutina*, respectively (Figure 1), suggesting high-level quality.

Table 1. Summary table of assembly information for five transcriptomes of *Saussurea* species.

| Species       | Type    | Total Sequence Number | Total Sequence Base (bp) | Percent GC (%) | Largest (bp) | Average (bp) | N50 (bp) |
|---------------|---------|-----------------------|--------------------------|----------------|--------------|--------------|----------|
| *S. amara*    | Unigenes| 92,247                | 70,437,963               | 39.69          | 15,736       | 763.58       | 1334     |
|               | Transcripts | 185,952         | 179,868,433              | 39.95          | 15,736       | 967.28       | 1562     |
| *S. amurensis*| Unigenes| 147,303               | 84,653,535               | 44.78          | 14,904       | 574.69       | 787      |
|               | Transcripts | 249,898         | 161,547,729              | 43.63          | 14,904       | 646.45       | 899      |
| *S. pachyneura*| Unigenes| 91,980                | 65,877,634               | 40.21          | 13,829       | 716.22       | 1196     |
|               | Transcripts | 176,265         | 151,495,643              | 40.44          | 13,829       | 859.48       | 1354     |
| *S. salwinensis*| Unigenes| 120,849               | 87,535,736               | 39.30          | 16,755       | 724.34       | 1154     |
|               | Transcripts | 270,778         | 237,345,971              | 39.83          | 16,755       | 876.53       | 1349     |
| *S. velutina* | Unigenes| 106,591               | 78,896,059               | 39.81          | 15,517       | 740.18       | 1186     |
|               | Transcripts | 228,331         | 209,268,034              | 39.97          | 15,517       | 916.51       | 1422     |

**BUSCO Assessment Results**

![BUSCO assessment results](image)

Figure 1. BUSCO assessment results for the five assembled transcriptomes of *Saussurea*. Bar charts produced with the BUSCO plotting tool show proportions classified as complete (C, blues), complete single-copy (S, light blue), complete duplicated (D, dark blue), fragmented (F, yellow), and missing (M, red).
TransDecoder identified 16,851, 28,182, 15,906, 19,979, and 19,978 protein-encoding regions for *S. amara*, *S. amurensis*, *S. pachyneura*, *S. salwinensis*, and *S. velutina*, respectively, consisting of 16.53–19.13% of unigenes for each species (Table 2). All the unigenes were annotated based on similarity to the public NR, Swiss-Prot, Pfam COG, KEGG, and GO databases. NR had the highest proportion of successful annotations, while KEGG had the lowest proportion (Table 2). The two top hits for the three species in the NR database are *Cynara cardunculus* var. *scolymus* and *Helianthus annuus* (Figure 2). Three functional categories: cellular component, molecular function, and biological process were classified based on the GO terms of all unigenes (Supplementary Figures S1–S5).

Figure 2. The proportion of the unigenes of *S. amara*, *S. amurensis*, *S. pachyneura*, *S. salwinensis*, and *S. velutina* annotated to NCBI non-redundant protein database. For species distributions, the top 10 matched species are provided.
2.2. Estimation of Divergence Time

A total of 1136 single-copy orthologous genes were used for phylogenetic reconstruction. The resulting phylogeny (Figure 3) is consistent with known phylogenetic relationships within Asteraceae. The topology shows that the two lowland species form a clade and together are sisters to the three alpine species. Among the alpine species, *S. velutina* is sister to *S. pachyneura* and *S. salwinensis* (Figure 3). According to the MCMC-Tree analysis, the crown group of *Saussurea* diverged at ca. 9.50 million years ago (Mya) (95% highest posterior density (HPD): 6.08–13.76 Mya), and all *Saussurea* species are estimated to have originated in the late Miocene to the early Pliocene (4.41–9.50 Mya). The divergence time between *Saussurea* and *Cynara* is estimated to be ca. 22.30 Mya (95% HPD: 13.95–32.37 Mya). The divergence time of Asteraceae species is estimated to diverge at ca. 56.26 Mya (95% HPD: 40.70–72.81 Mya) (Figure 3).

Figure 3. Estimated time tree of *Saussurea* based on 1136 single-copy orthologous gene sequences. Mean node ages are provided, and node bars provided are 95% HPD intervals for mean node ages. The three alpine species and two lowland relatives are in the blue and pink background, respectively.
Table 2. Summary table of annotation information for five transcriptomes of *Saussurea* species. Unigenes were annotated based on similarity to the public databases, including NCBI non-redundant protein (NR), Swiss-Prot protein (SWISS-PROT), PFAM, Cluster of Orthologous Groups (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO).

| Species      | ORF (%) | NR (%) | SWISS-PROT (%) | PFAM (%) | COG (%) | KEGG (%) | GO (%)  |
|--------------|---------|--------|----------------|----------|---------|----------|---------|
| *S. amara*   | 16,851  | 21,595 | 12,683         | 9826     | 10,440  | 8110     | 12,543  |
| *S. amurensis* | 28,182  | 31,705 | 22,320         | 15,886   | 21,901  | 14,179   | 22,081  |
| *S. pachyneura* | 15,906  | 21,642 | 12,819         | 9474     | 10,726  | 8204     | 12,674  |
| *S. salwinensis* | 19,979  | 26,176 | 14,722         | 11,017   | 11,890  | 9479     | 14,577  |
| *S. velutina* | 19,978  | 24,748 | 15,182         | 11,268   | 12,359  | 10,008   | 15,021  |

2.3. Evolutionary Analysis

OrthoVenn2 identified 17,380 orthologous clusters (containing at least two species) among five *Saussurea* species, which includes 1514 core orthologs (shared by all five species) (Figure 4). The number of lineage-specific gene families for three alpine species, *S. pachyneura*, *S. salwinensis*, and *S. velutina*, are 52, 126, and 127, respectively. GO enrichment was then conducted on the lineage-specific gene families by annotating them with UniProt. In total, 14 significant GO terms (*p*-value < 0.05) are enriched (three for *S. pachyneura*, five for *S. salwinensis*, and six for *S. velutina*) (Table 3). These GO terms included genes involved in oxidoreductase activity (GO: 0016705; GO:0016491), pectin catabolic process (GO:0045490), lipid transport (GO:0006869), and polysaccharide metabolic process (GO:0005976), which may play important roles in defense of hypoxia and freezing temperatures of the QTP.

2.4. Genes under Positive Selection

To detect genes that might contribute to the high-altitude adaptation of the alpine species (*S. pachyneura*, *S. salwinensis*, and *S. velutina*), we used the branch model to screen positively selected genes (PSGs) by estimating the dN/dS ratio on each branch of the alpine species using orthologous genes of five *Saussurea* species as input. In total, 250 genes across all three high-altitude species displayed signs of positive selection, of which 215 genes were successfully annotated with at least one homologous gene matched to *A. thaliana* (Supplementary Table S3). Functional enrichment analysis revealed 136 GO processes and 24 KEGG pathways significantly enriched (Supplementary Table S4). The top 20 clusters with their representative-enriched terms (one per cluster) are shown in Figure 5. Numerous PSGs show significant enrichment in biological functions associated with harsh environment defense, such as stabilization of membrane potential (e.g., GO:0030322, GO:0042391), regulation of transmembrane transport (e.g., GO:0098662, GO:2000576, GO:0032890), reproductive process (e.g., GO:0048544, GO:0051754), response to temperature (GO:0009266) and light stimulus (GO:0009416), and are also involved in important metabolic processes associated with nutrient or energy intake, such as glutamine metabolic process (GO:0006541, GO:0009064), sulfur metabolism (KEGG: ko00920, GO:0000096) and tryptophan biosynthesis (KEGG: M00169, GO:0000162). In addition to the significantly enriched pathways, many PSGs are annotated to homolog proteins of *A. thaliana* that are important for species adaptation. For example, RAD54, RUP2, TT5, and ATSG35735 are involved in DNA repair, response to UV-B, flavonoid biosynthesis, and cellular response to hypoxia.
Figure 4. Gene family comparison among five *Saussurea* species. Numbers on the graph represent the count of gene families. Gene Ontology (GO) enrichment for lineage-specific gene families of three alpine species (*S. pachyneura*, *S. salwinensis*, and *S. velutina*) are provided in Table 3.

Table 3. GO enrichments based on the annotations in UniProt of lineage-specific gene families of three alpine *Saussurea* species.

| Species       | GO Term                | GO Term Definition                                               | p-Value  |
|---------------|------------------------|------------------------------------------------------------------|----------|
| *S. pachyneura* | GO:0016705             | Oxidoreductase activity, acting on paired donors, with inclusion or reduction of molecular oxygen | 0.0065   |
|               | GO:0050896             | Response to stimulus                                             | 0.0250   |
|               | GO:0016491             | Oxidoreductase activity                                           | 0.0140   |
| *S. salwinensis* | GO:0045490             | Pectin catabolic process                                          | 0.0000   |
|               | GO:0070072             | Vacuolar proton-transporting V-type ATPase complex assembly      | 0.0001   |
|               | GO:005615              | Extracellular space                                               | 0.0001   |
|               | GO:0009908             | Flower development                                                | 0.0038   |
|               | GO:0005976             | Polysaccharide metabolic process                                  | 0.0360   |
| *S. velutina*  | GO:0016707             | Hydrolase activity                                                | 0.0045   |
|               | GO:0016491             | Oxidoreductase activity                                           | 0.0250   |
|               | GO:0016209             | Antioxidant activity                                              | 0.0210   |
|               | GO:0006725             | Cellular aromatic compound metabolic process                      | 0.0054   |
|               | GO:0005896             | Response to stimulus                                              | 0.0120   |
|               | GO:0006869             | Lipid transport                                                   | 0.0020   |
Figure 5. Functional enrichment analysis of genes under positive selection. The top 20 clusters with their representative enriched terms (one per cluster) of GO biological processes (B) and KEGG pathways (K) are shown.

3. Discussion

Despite de novo assembly due to the lack of whole-genome sequence, transcriptomes of Saussurea generated in this study are of high quality. BUSCO results showed a minimum of 81% completeness (up to 88%) for the five transcriptomes (Figure 1), providing a strong foundation for our following comparative transcriptomic analyses. Using single-copy orthologs identified in genomic sequences, we added transcriptomic evidence of the divergence history of Saussurea. Our dated time tree showed the divergence of Saussurea occurred in the Miocene (Figure 3), which was consistent with our parallel study using whole plastome sequences (ca. 8.3–15.35 Mya) [33] and the result based on the nuclear internal transcribed spacer (ITS) dataset (ca. 11.7–14.4 Mya) [30]. A much older divergence time was provided by the study of Xu et al. [32] (ca. 17.1 Mya) using plastid protein-coding genes. Additionally, the estimation of divergence time between Saussurea and Cynara (ca. 22.3 Mya) was younger than the study of Barres et al. [34] (ca. 28.3 Mya), which may be attributed to the sparse sampling of related genera of Saussurea in the present study. Nonetheless, all these studies suggest that the rapid diversification of Saussurea was related to the uplift of the QTP and adjacent mountains (e.g., Hengduan Mountains). Overall, our results support a Miocene diversification of Saussurea triggered by extensive orogenic movements of the QTP and subsequently mediated by genomic evolution to adapt to the fluctuant environments.
Orthologous cluster analysis of *Saussurea* transcriptomes detected some important gene families specific to the alpine species (Figure 4). Subsequent functional characterization provided genetic evidence of high-altitude adaptation. For example, some gene families specific to alpine species are related to oxidation-redox reactions (oxidoreductase activity; GO:0016705, GO:0016491), which is one of the most important types of reactions in the biological process. Alpine species may recruit more members involving in the oxidoreductase activity to strengthen the adaptive capability under a hypoxic environment. In high-altitude environments, cold acclimation is vital for the survival of species. A key function of cold acclimation is to stabilize membranes against freezing injury [35]. Multiple mechanisms have been documented to be involved in this stabilization, such as changes in lipid composition and accumulation of sugar content [35–37]. Some biological processes we detected specific to alpine species, such as the pectin catabolic process (GO:0045490), lipid transport (GO:0006869), and polysaccharide metabolic process (GO:0005976), may protect the plant from cold damage by regulating the physical state of the plasma membrane. Moreover, numerous PSGs functionally enriched in the stabilization of membrane potential (GO:0030322), response to freezing (GO:0050826), and response to temperature stimulus (GO:0009266) pathways may help plants avoid freezing injury from cold climate by regulating cell membrane permeability.

Positive selection analysis in phylogenetic context with the branch model showed most of the genes displaying a $dN/dS$ ratio <1, indicating that most genes are undergoing purifying selection. This is true for species inhabiting all altitude distributions. Functional annotation revealed that those PSGs are closely related to the abiotic stresses of high-altitude environments produced by strong UV radiation, hypoxia, or fluctuate temperatures. The highly energetic UV radiation in the QTP is harmful to all living species since it causes direct damage to the DNA, RNA, and proteins of organisms. Some PSGs were annotated to homolog proteins of *A. thaliana* that are involved in DNA repair and response to UV-B radiation, such as RAD54, a DNA repair/recombination protein essential in homologous recombination for repairing DNA double-strand breaks [38], RUP2 involved in balancing UV-B-specific responses and ensuring normal plant growth [39], and TT5, a chalcone-flavone isomerase family protein involved in flavonoid biosynthesis. Flavonoids function as antioxidants that reduce DNA damage caused by reactive oxygen species (ROS) accumulation, which is induced by abiotic stresses such as extreme temperatures, UV radiation, and drought, and thus is crucial for species adapting to extreme environments [40]. These results were consistent with previous studies of high-altitude adaptation [6,22,41], suggesting that DNA-repair and UV-B-response related PSGs play vital roles for alpine species to survive in harsh environments.

Furthermore, extreme environmental conditions may impact several key biological processes including nutrient or energy intake, photosynthesis, and reproductive process [41]. Our functional annotation showed many PSGs involved in the nitrogen utilization, reproductive process, growth, and metabolic process (Supplementary Table S3). PSGs participating in metabolic processes of important compounds, such as glutamate and sulfur, were annotated in GO biological processes and KEGG pathways. Glutamate plays a central role in plant nitrogen metabolism and is crucial for many metabolic functions, including protein and glutathione synthesis, energy production, maintenance of optimal antioxidant status, and immune function [42]. Nitrogen utilization is vital for alpine species of *Saussurea* because nutrient deficiency, especially nitrogen deficiency is a typical feature of the alpine screes habitats [43,44]. The uptake and assimilation of sulfur and nitrogen are strongly related, which is an important issue in plant nutrition [45]. Moreover, sulfur plays an important role in fundamental processes such as electron transport, structure, and regulation, and is also associated with photosynthetic oxygen production, abiotic and biotic stress resistance, and secondary metabolism [45,46]. Reproductive success is critical to the continuation of species. Genes involved in the meiotic process (e.g., GO:0051754, GO:0070601, GO:0033046), recognition of pollen (GO:0048544), floral organ formation (GO:0048449), and seed dormancy process (GO:0010162, GO:0022611) were detected under
positive selection. These genes provide evidence that alpine species of *Saussurea* have evolved specific reproductive strategies via positively selected genes associated with the meiotic, flowering, and seed dormancy process for adapting to the short growing season in the harsh environments of the QTP.

4. Materials and Methods

4.1. Sample Collection and Transcriptome Sequencing

Five *Saussurea* species were sampled for RNA-seq, of which three (*S. pachyneura*, *S. salwinensis*, and *S. velutina*) were collected from the QTP alpine zone of Baima Snow Mountain, De Qin County, Yunnan Province, China (between 4550 and 4620 m), and the other two (*S. amurensis* and *S. amara*) were collected from the Changbai Mountain, Jilin Province, China (between 150 and 350 m) (Supplementary Table S1). To obtain a sufficient level of transcripts, multiple tissues, including stems, fresh flowers, and leaves, were collected from living plants, mixed and stored in liquid nitrogen. Total RNA was extracted using the TRIzol® Reagent (Invitrogen, Shanghai, China). The concentration of the total RNA was detected by Nanodrop 2000, and the quality was checked using 2% agarose gel electrophoresis. For each species, a paired-end cDNA library was constructed using TruSeq Stranded mRNA Library Prep Kit (Illumina), as described in the Illumina manual, and was sequenced using the HiSeq 2000 sequencing platform.

4.2. Transcriptome Assembly and Annotation

Raw RNA-seq reads were cleaned for quality using Cutadapt v.1.16 [47] to remove potential adapter sequences and low-quality regions with options “-q 20 -m 20”. The clean reads were checked with FastQC v.0.11.4 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed on 5 June 2021) and were de novo assembled using Trinity v.2.6.6 [48] with default parameters. Redundant contigs were eliminated for the raw assemblies (transcripts) using CD-Hit v.4.6 [49] to obtain non-redundant transcripts (hereafter unigenes). TransRate v.1.0.3 [50] was used to calculate the statistics for evaluating the de novo transcriptome assemblies. BUSCO v.4.1.4 [51] was used to assess the completeness of the assemblies by employing the embryophyta_odb10 database. TransDecoder v.5.5 [13] was utilized to predict coding regions of all unigenes and translate them into protein sequences with a minimum open reading frame (ORF) length of 100 amino acids. For functional annotation, all unigenes were annotated based on similarity to the public NCBI non-redundant protein database (NR), Swiss-Prot protein database (Swiss-Prot, http://www.expasy.ch/sprot, accessed on 5 June 2021), Pfam (https://pfam.xfam.org/, accessed on 5 June 2021), Cluster of Orthologous Groups database (COG, http://www.ncbi.nlm.nih.gov/COG/, accessed on 5 June 2021) and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/, accessed on 5 June 2021) using BLASTP v.2.2.25 [52] with an e-value cut-off of $1 \times 10^{-5}$. The NR annotation results were used to obtain the Gene Ontology (GO) term assignments of the unigenes with the BLAST2GO v.3.1 [53] (with e-value < $1 \times 10^{-5}$) program.

4.3. Estimation of Divergence Time

To estimate the divergence time of *Saussurea* species, the amino acid sequences of seven Asteraceae species (*Artemisia annua*, *Carthamus tinctorius*, *Chrysanthemum nankinense*, *Cynara cardunculus*, *Helianthus annuus*, *Lactuca sativa*, *Mikania micrantha*) and other two outgroups (*Coffeea canephora*, *Solanum lycopersicum*) were downloaded from the National Center for Biotechnology Information (NCBI) and Phytozome (version 12.1; https://phytozome.jgi.doe.gov/, accessed on 5 June 2021). Orthologous groups of these species were determined by OrthoFinder v.2.4.1 [54]. Amino acid sequences of single-copy orthologous genes were aligned using MAFFT v.7.470 [55]. Ambiguous positions of the alignments were deleted in TrimAL v.1.2 [56] with the “-automated1” command. The sequence alignments were then concatenated and were used to construct a maximum likelihood (ML) phylogenetic tree using IQ-TREE v.1.6.12 [57] with ModelFinder [58] deter-
mining the best-fit substitution model. Divergence time was estimated in the MCMCtree program of PAML v.4.9 [59] using the approximate likelihood method with an independent substitution rate (clock = 2) and the birth-death prior. The ML tree and the concatenated translated nucleotide alignments of 1136 single-copy orthologs (see Results) were used as input of MCMCtree. In our analysis, the MCMCtree discarded the first 1000,000 iterations as burn-in, and then it sampled every 50 iterations until it gathered 200,000 samples. In total, the MCMCtree ran 1000,000 + 50 × 200,000 = 11,000,000 iterations. Two runs were conducted for checking convergence, and Tracer v.1.7.1 [60] was to assess the effective sample size (ESS > 200) of each parameter. The divergence time between Asteraceae and outgroups was calibrated to 93–107 Mya based on the TimeTree database (http://www.timetree.org/, accessed on 5 June 2021) [61].

4.4. Evolutionary Analysis

OrthoVenn2 web platform [62] was used to perform comparison and annotation of orthologous gene clusters among five *Saussurea* species, with the following parameters: e-value < 1 × 10^{-5} and an inflation value 1.5. A Venn diagram was generated to show the distribution of shared gene families among selected species. The lineage-specific gene families of three alpine species (*S. pachyneura*, *S. salwinensis*, and *S. velutina*) were annotated using the non-redundant protein database in UniProt and the corresponding GO enrichment was performed in OrthoVenn2.

4.5. Phylogenetic Tests of Positive Selection

To detect signs of positive selection on protein-coding genes along specific lineage, we used the branch model [63] in the codeml program of PAML [58] to estimate the ratio \( \omega \) of the nonsynonymous substitution rate \( \text{d}N \) to the synonymous substitutions rate \( \text{d}S \). We used orthologous groups of five *Saussurea* species as input. Three analyses were conducted, in which a foreground branch was specified as the clade of *S. pachyneura*, *S. salwinensis*, and *S. velutina*, separately. We deleted all gaps (clean data = 1) from the alignments to lower the effect of ambiguous bases on the inference of positive selection. Likelihood ratio tests were used to determine whether positive selection occurred on the foreground branch. A gene with a p-value <0.05 was considered as a positively selected gene (PSG). Protein sequences of PSGs were then annotated to the proteome of *A. thaliana* downloaded from the Arabidopsis Information Resource (TAIR11) database using BLASTP with an e-value cut-off of 1 × 10^{-5} and -max_target_seqs of 5. Functional enrichment analysis was conducted in Metascape web-based portal [63]. Pathway terms with a p-value <0.01 and an enrichment factor >1.5 (the enrichment factor is the ratio between the observed counts and the counts expected by chance) were collected and grouped into clusters based on their membership similarities [64].

5. Conclusions

Despite a fundamental understanding of the taxonomy, phylogeny, and ecology of *Saussurea* [25,30–32], our knowledge about the genetic mechanism behind adaptation to high-altitude habitats is still lacking. Transcriptomic data generated by the present study provided a good chance to investigate the adaptive evolution of *Saussurea*. Through comparative and evolutionary genomic analyses, we identified the gene families specific to alpine species and genes showing signs of adaptive evolution. Functional annotations of these genes elucidate that they potentially possess specific traits of adaptive significance, such as membrane stabilization, response to radiation, DNA repair, and organic metabolism, demonstrating genetic signatures under the adaptation of *Saussurea* to the low temperature, high UV radiation, and hypoxia environments on the QTP. Overall, our study provided reference-level genomic resources for alpine plants while gaining important insights into how plants adapt to harsh and extreme environments.
Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10081715/s1, Figures S1–S5. GO terms of all unigenes of five Saussurea transcriptomes. Table S1. Sample information of five species of Saussurea. Table S2. Summary of reads information of sequenced transcriptomes. Table S3. Annotation information of positively selected genes to TAIR11 database. Table S4. Summary of functional enrichment analysis of positively selected genes.

Author Contributions: X.Z., H.W. and H.S. designed the study. X.Z., Y.S., J.B.L., H.W. and H.S. drafted the manuscript. X.Z., J.S. (Jun Shen) and H.Z. performed the experiments and analyzed the data. T.K., W.S., J.S. (Jiao Sun), B.B.T. and T.D. collected materials. All authors discussed the results and helped shape the final manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Second Tibetan Plateau Scientific Expedition and Research (STEP) program (2019QZKK0502), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA20050203), the Key Projects of the Joint Fund of the National Natural Science Foundation of China (U1802232), and the Major Program of the National Natural Science Foundation of China (31590823).

Data Availability Statement: The raw RNA sequencing data have been deposited in the SRA under Bioproject number PRJNA566024.

Acknowledgments: We thank Haicheng Zhou for helping with transcriptome material collection.

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

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