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Comparative Genome and Transcriptome Analysis Reveals Gene Selection Patterns Along with the Paleo-Climate Change in the *Populus* Phylogeny

You-jie Zhao [1], Chang-zhi Han [2], Yong Cao [1] and Hua Zhou [1,*]

[1] College of Big Data and Intelligent Engineering, Southwest Forestry University, Kunming 650224, China; bioala@swfu.edu.cn (Y.-j.Z.); cncaoyong@swfu.edu.cn (Y.C.)

[2] Department of Science and Technology, Southwest Forestry University, Kunming 650224, China; swfuhcz@163.com

* Correspondence: hzhou@swfu.edu.cn; Tel./Fax: +86-871-6386-3018

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Abstract: Poplars are widely distributed in the northern hemisphere and have good adaptability to different living environments. The accumulation of genome and transcriptome data provides a chance to conduct comparative genomics and transcriptomics analyses to elucidate the evolutionary patterns of *Populus* phylogeny. Transcript sequences of eight *Salicaceae* species were downloaded from public databases. All of the pairwise orthologues were identified by comparative transcriptome analysis in these species, from which we constructed a phylogenetic tree and estimated the rate of divergence. The divergence times of the phylogenetic clades were mainly estimated during the Middle Miocene Climate Transition (MMCT) to Quaternary Ice Age. We also identified all of the fast-evolving sequences of positive selection and found some resistance genes that were related to environmental factors. Our results suggest that drought-, H_2O_2- and cold-stress genes are involved in positive selection along with the paleoclimate change. These data are useful in elucidating the evolutionary patterns and causes of speciation in the *Populus* lineage.

Keywords: paleoclimate change; genus *Populus*; comparative transcriptomics; selective evolution

1. Introduction

Poplars (Genus *Populus*) are widely distributed in the northern hemisphere, ranging from subtropical to northern forests and are the most important source of wood in forests. *Populus* has become an excellent model plant along with the completion of genome projects *Populus trichocarpa* Torr. & A.Gray ex. Hook. [1], *Populus euphratica* Oliv. [2] and *Populus pruinosa* Schrenk [3]. Several studies have focused on this genus, particularly with regard to its phylogenetic relationships [4,5], origin and speciation [6–8]; the timing of diversification events [9–11]; and environmental stress tolerance [12,13]. However, no study explains how poplars have adapted to various climates during its long evolutionary history.

Transcriptome sequencing can rapidly and economically obtain all of the RNA information of organisms at one time, thus playing an important role in identifying molecular markers and functional genes for biology research [14,15]. As the transcriptomes of more species have been generated, comparative transcriptomics has received more attention from researchers [16–20]. Comparative transcriptomics can explain the phylogenetic relationships based on multiple species, as well as determine the functional differences between orthologous genes after species divergence in different living environments.

In this study, the transcript sequences of seven *Populus* and one *Salix* species were downloaded from public databases (Table 1). Comparative genomics and transcriptomics were subsequently

analyzed in eight *Salicaceae* species. A number of positive selection genes were found to be related to environmental factors in the *Populus* lineage.

Table 1. Coding sequences (CDS) in eight *Salicaceae* species.

Salicaceae Species	Data Sources	Number	N50 (bp)	Min (bp)	Max (bp)	Total (Mb)
Populus trichocarpa Torr. & A.Gray ex. Hook.	JGI	35,447	1500	150	16,356	36.87
Populus nigra L.	PlantGDB, NCBI (SRR7538365)	62,740	1344	150	13,161	55.36
Populus deltoides W.Bartram ex Marshall	PlantGDB, NCBI (SRR6960264)	42,207	1284	150	7476	35.86
Populus tremula L.	PlantGDB, NCBI (ERR1352631)	50,902	1167	150	5238	40.06
Populus tremuloides Michx.	PlantGDB, NCBI (SRR6134157)	45,263	1251	150	13,071	37.61
Populus euphratica Oliv.	NCBI (PRJNA178692)	54,527	1785	150	16,377	73.92
Populus pruinosa (Greene) O'Kane & Al-Shehbaz	GigaDB	35,395	1668	150	15,945	40.92
Salix purpurea L.	JGI	37,290	1581	150	16,419	43.57

2. Materials and Methods

2.1. Data Sources

To elucidate the evolutionary pattern of orthologues, the transcript sequences of seven *Populus* and one *Salix* (out-group) species were downloaded from the public databases (Table 1). Sequences of *P. nigra* [21], *P. deltoides*, *P. tremula* and *P. tremuloides* were obtained from PlantGDB [22] and sequence read archive (SRA) of national center for biotechnology information (NCBI) database. The cDNA sequences of *P. trichocarpa* (v3.1) [1], *P. euphratica* [2], *P. pruinosa* [3], and *S. purpurea* (v1.0) were downloaded from genome projects of GigaDB [23], NCBI and the United States department of energy joint genome institute (JGI) [24]. SRA datasets with FASTQ format were filtered to remove raw reads of low quality. Transcriptome assembly was achieved using the short-read assembly program Trinity (version 2.3) [25]. The assembled transcripts ($\geq$300 bp) and expressed sequence tag (EST) sequences of PlantGDB were combined; Coding sequences (CDS) with amino acid sequences ($\geq$50 bp) were extracted by OrfPredictor [26] and clustered with CD-HIT-EST (version 4.0) [27,28]. Sequences with a clustering threshold of0.95 were divided into one class, and the longest sequence of each class was treated as a unigene during later processing.

2.2. Identification of Orthologues among Eight Salicaceae Species

OrthoFinder software (version 2.3.1) [29] was used to identify the putative orthologues among the eight species. Single copy sequences between every pair-wise species were then used to estimate the substitution rates in the subsequent analyses. Single copy sequences shared among all eight species were used to construct the phylogenic tree. The annotations obtained from the NCBI Non-redundant protein database (Nr) were processed through the BLAST2GO program (version 4.0) [30] to obtain relevant Gene Ontology (GO) terms and these were then analyzed by Web Gene Ontology Annotation Plot (WEGO) software (version 2.0) [31] to assign a GO functional classification and illustrate the distribution of the gene functions. A heatmap of orthologues was drawn using the R language (version 3.0).

2.3. Estimation of Synonymous and Non-Synonymous Substitution Rates

To remove the unigenes without open reading frames, pairwise orthologues were searched against plant protein sequences of GenBank using basic local alignment search tool (BLASTX) as previously described [32]. Clustalw software (version 2.1) [33] was used to align the filtered pairwise orthologues, and the output files were formatted to the NUC format for subsequent analysis. The rates of synonymous substitutions (Ka) and non-synonymous substitutions (Ks) were estimated using phylogenetic analysis by maximum likelihood (PAML) software (version 4.7) [34]. Using the fossil calibrations 45 million years ago (Mya) of genera *Salix* and *Populus* [10,11], the rate of substitution

(r) was calculated based on the formula (T = K/2r, T is the time of divergence and K is the rate of non-synonymous substitutions Ks) and the average Ks value 0.121 between *Salix* and *Populus*.

2.4. Phylogenetic Analysis

There were still some inconsistencies in the phylogenetic relationship in previous studies [4,5]. Single copy genes by OrthoFinder were aligned by multiple sequence comparison by log-expectation (Muscle, version 3.8) [35] and formated by Gblock [36]; the maximum-likelihood method was used to build the phylogenetic tree by molecular evolutionary genetics analysis (MEGA, version 6) [37] (bootstrap is 1000 and Kimura 2-parameter model). *S. purpurea* was used as an outgroup to root trees.

3. Results

3.1. Transcript Sequences of Eight Salicaceae Species

There were 35,447; 35,395; 54,527; and 37,290 annotated genes in the genomes of *P. trichocarpa*, *P. pruinosa*, *P. euphratica,* and *S. purpurea*, respectively. These genes respectively made up a total of 37 Mb, 41 Mb, 74 Mb, and 44 Mb cDNA sequences with Contig N50 of 1500 bp, 1668 bp, 1785 bp, and 1581 bp. More than 8153 (23%), 8849 (25%), 16,903 (31%), and 8950 (24%) cDNAs had the length of >1500 bp in *P. trichocarpa, P. pruinosa*, and *S. purpurea* (Figure 1). In contrast, there were 62,740; 42,207; 50902; and 45,263 unigenes in the transcriptomes of *P. nigra, P. deltoides*, *P. tremula,* and *P. tremuloides*, which respectively made up a total of 55 Mb, 36 Mb, 40 Mb, and 38 Mb sequences, with Contig N50 of 1344 bp, 1285 bp, 1167 bp, and 1251 bp. In addition, more than 8156 (13%), 5064 (12%), 5090 (10%), and 5431 (12%) unigenes had the length of >1600 bp in the transcriptomes of *P. nigra*, *P. deltoides*, *P. tremula*, and *P. tremuloides*.

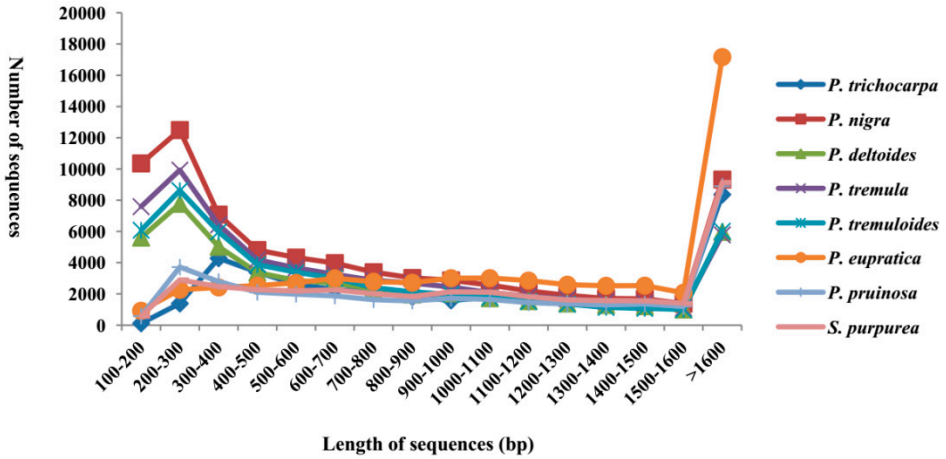

Figure 1. Distribution of coding sequences (CDS) length of eight *Salicaceae* species.

3.2. Orthologue Identification and Functional Characterization among Eight Salicaceae Species

All of the pairwise orthologues were identified by the comparative analysis of eight *Salicaceae* species (Table 2). The results showed that *P. trichocarpa* has the highest average number (8198) of orthologous genes, whereas *P. tremuloides* has the lowest average number (5148). The highest number of orthologous genes (9687) was observed between *P. trichocarpa* and *P. pruinosa*, whereas the lowest (4339) was detected between *P. tremuloide* and *S. purpurea*. One-thousand-eight-hundred-and-thirty-five shared orthologues were found among the eight *Salicaceae* species (Figure 2). The orthologues were functionally annotated using GO terms (Table S1 and Figure S1), and 339 orthologues were involved in biological processes (218), cellular components (113) and molecular functions (273).

Table 2. Number and the rate of non-synonymous substitutions (Ks) peaks of orthologous genes in eight *Salicaceae* species.

	P. trichocarpa	***P. nigra***	***P. deltoides***	***P. tremula***	***P. tremuloides***	***P. euphratica***	***P. pruinosa***
P. trichocarpa							
P. nigra	7532/0.02						
P. deltoides	8141/0.02	5773/0.03					
P. tremula	7540/0.04	5428/0.04	5917/0.04				
P. tremuloides	6679/0.04	4805/0.04	5337/0.04	5131/0.02			
P. euphratica	9625/0.04	5623/0.05	6093/0.05	5676/0.05	5032/0.05		
P. pruinosa	9687/0.04	5242/0.05	5751/0.06	5362/0.06	4713/0.05	7684/0.02	
S. purpurea	8179/0.11	4836/0.12	5232/0.12	4926/0.12	4339/0.12	6373/0.13	6131/0.13

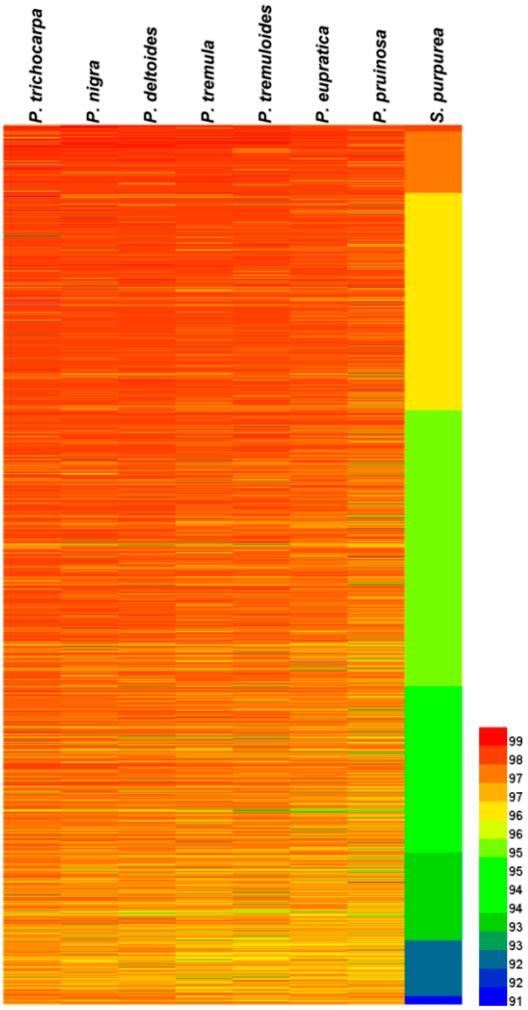

Figure 2. Divergence between orthologues of seven *Populus* and one *Salix* species. The heat map is drawn based on the combined sequences of 1835 putative orthologues in eight species. The orthologues were annotated to a different function with Gene Ontology (GO) (Table S1). Sequence similarity was indicated with different colors from red (highly similar) to blue (weakly similar).

3.3. Phylogenetic Analysis and Divergence Time

Using *S. purpurea* as the outgroup, phylogenetic reconstruction of *Populus* was conducted based on 1835 orthologous transcripts using the maximum likelihood (ML) method (Figure 3). The observed phylogenetic relationship is highly consistent with the phylogenetic tree obtained from single-copy DNA sequences of a previous study [4].

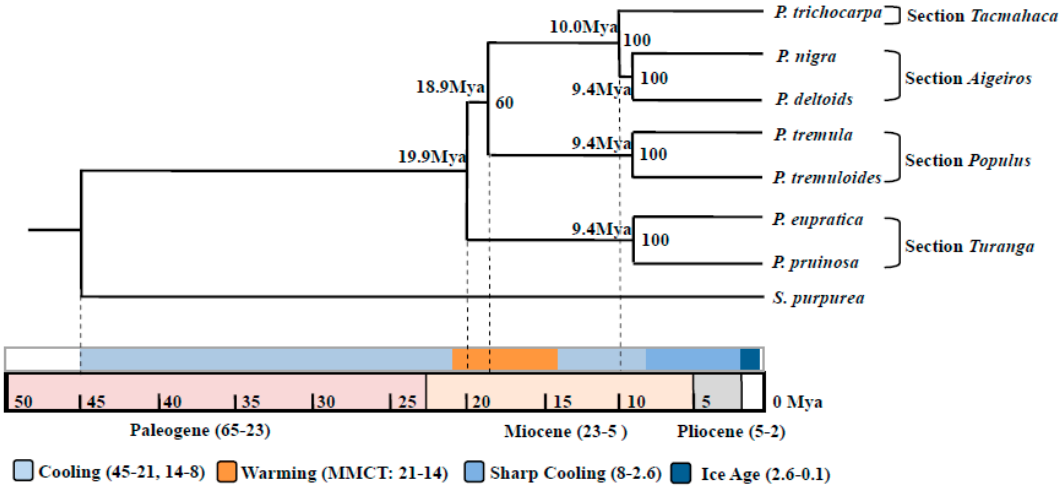

Figure 3. Phylogenetic tree of seven *Populus* and one *Salix* species. Phylogram derived from multiple sequence alignments based on the combined 1835 orthologous transcripts using maximum likelihood (ML) method (Sequences in File S1). Paleoclimate change is presented by different colors.

The genetic distance of species was related to the synonymous mutation rate, which was calculated using the orthologous genes, so the synonymous mutation rates of all of the pairs of orthologues were estimated in the eight *Salicaceae* species (Table 2). The minimum Ks peak is about 0.01 between *P. tremula* and *P. tremuloides* and between *P. euphratica* and *P. pruinasa.* In addition, *P. tricocarpa* has a Ks peak (0.02) with *P. nigra* and *P. deltoides*; 0.04 Ks peak with *P. tremula*, *P. tremuloides*, *P. euphratica* and *P. pruinasa*; and the highest Ks peak 0.11 with the outgroup *S. purpurea* (Figure 4).

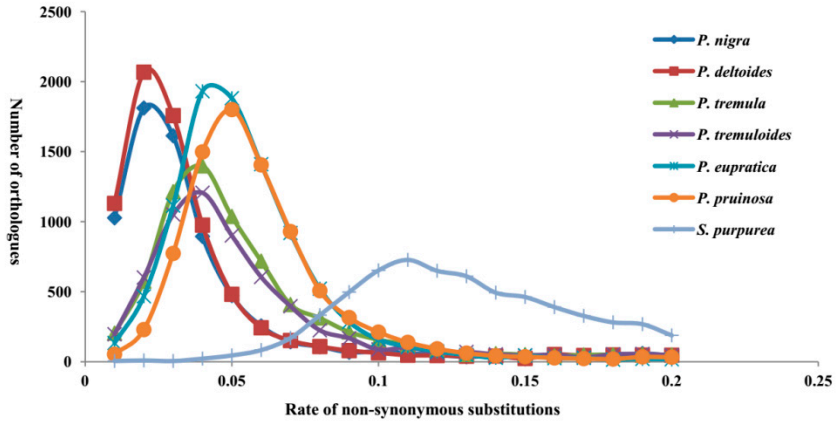

Figure 4. Distribution of the rate of non-synonymous substitutions (Ks) values of orthologous pairs between *P. trichocarpa* and others.

In the phylogenetic tree, the average Ks value is 0.121 between the genera *Populus* and *Salix* (calculated by Table 2), which is highly consistent with the value of 0.12 in previous studies [9]. Based on existing fossil evidence, the divergence time of the genera *Salix* and *Populus* was about 45 million years old (Mya) in the middle Eocene sediments [10,11]. With this time as the separation of the two lineages and K = 0.121, the rate of substitution (r) was calculated to about 1.29×10^{-9} per site and year, which is very close to the previous value of 1.28×10^{-9} [9].

Phylogenetic reconstruction of genus *Populus*, mainly consists of four sections, namely, *Turanga*, *Populus*, *Aigeiros*, and *Tacmahaca*. Using the fossil calibrations (45 Mya) of genera *Salix* and *Populus* [10,11], the divergence times were estimated to be about 19.9 Mya, 18.9 Mya, and 10.0 Mya, respectively, corresponding to the three separated nodes in the *Populus* phylogeny. The minimum

divergence time was about 9.4 Mya between *P. nigra* and *P. deltoides*; *P. tremula* and *P. tremuloides*; and *P. euphratica* and *P. pruinasa*.

3.4. Evolutionary Pattern of Populus spp. Genes

The Ka/Ks rate of orthologous genes could reflect the evolutionary pattern of species. Ka/Ks > 1 indicates that the gene underwent positive selection during evolution. The fast-evolving sequences of positive selection were identified in the genus *Populus* (Table 3), and some resistance genes were found to be related to environmental factors (Table 4).

Table 3. Positive selection orthologues in genus *Populus*.

	P. trichocarpa	*P. nigra*	*P. deltoides*	*P. tremula*	*P. tremuloides*	*P. euphratica*	*P. pruinosa*
P. trichocarpa							
P. nigra	523/HCS						
P. deltoides	555/DCSL	312					
P. tremula	482/D	257	285/D				
P. tremuloides	380/CL	202	254	301/SDL			
P. euphratica	558H	185/H	247/H	159	169/H		
P. pruinosa	290/H	134	151/H	148/DH	124	193	

S: Salt stress; D: Drought stress; H: H_2O_2 stress; C: Cold stress; L: Light stress.

Table 4. Information of resistance genes involved in positive selection in genus *Populus*.

Stress	Nr Annotation	Gene1	Gene2	Ka	Ks	Ka/Ks
Cold	NP_849749.1	Populus_nigra-9391	Populus_deltoides-7168	0.005	0.000	>1
Cold	NP_849749.1	Potri.009G134000.1	Populus_deltoides-7168	0.005	0.000	>1
Cold	NP_849749.1	Potri.T011400.1	Populus_nigra-13623	0.009	0.000	>1
Cold	YP_588274.1	Potri.018G031500.1	tmulo_comp12451_c0_seq1	0.073	1.060	0.077
Drought	BAB68268.1	delto_comp5129_c0_seq1	tmula_comp15883_c0_seq1	0.036	1.977	0.071
Drought	NP_566843.1	Populus_tremula-13647	Populus_deltoides-5749	0.023	0.018	1.289
Drought	AAD11484.1	Potri.005G108900.1	Populus_tremula-3187	0.040	0.032	1.261
Drought	BAC55016.1	Potri.006G265400.1	Populus_deltoides-5551	0.010	0.008	1.214
Drought	AAD11484.1	Ppr_1043.444	Populus_tremula-3187	0.048	0.027	1.772
Drought	BAB68268.1	Potri.005G193600.1	tmula_comp15883_c0_seq1	0.029	2.376	0.068
Drought	BAB68268.1	tmula_comp15883_c0_seq1	tmulo_comp27907_c0_seq1	0.017	2.793	0.048
H2O2	AAG34797.1	delto_comp14938_c1_seq1	euphr_XM_011031397	0.022	1.448	0.032
H2O2	BAE44477.1	nigra_comp12393_c0_seq1	euphr_XM_011032466	0.025	1.008	0.025
H2O2	AAN77157.1	Potri.002G081900.1	Populus_nigra-12261	0.014	0.014	1.000
H2O2	AAG34804.1	Ppr_668.27302	Potri.010G070900.1	0.028	0.019	1.481
H2O2	AAG34804.1	Ppr_668.27302	Populus_deltoides-4453	0.030	0.023	1.339
H2O2	AAG34804.1	Ppr_668.27302	Populus_tremula-16354	0.046	0.041	1.128
H2O2	BAC55016.1	Potri.006G265400.1	euphr_XM_011033131	0.014	1.019	0.015
H2O2	AAG34808.1	Potri.008G175000.1	euphr_XM_011031400	0.024	1.138	0.027
H2O2	AAG34799.1	tmulo_comp24179_c0_seq1	euphr_XM_011032466	0.016	1.960	0.031
Light	NP_188923.1	Potri.008G158200.1	delto_comp18926_c0_seq1	0.013	1.834	0.024
Light	NP_188923.1	Potri.008G158200.1	tmulo_comp16665_c0_seq1	0.040	1.107	0.045
Light	NP_565524.1	tmula_comp31944_c0_seq1	tmulo_comp6817_c0_seq1	0.000	86.546	0.009
Salt	P93209.1	Populus_tremuloides-736	Populus_tremula-11120468	0.004	0.000	>1
Salt	P42652.1	Potri.002G097500.1	Populus_deltoides-3084	0.004	0.000	>1
Salt	P42652.1	Potri.002G103800.1	Populus_nigra-5935	0.003	0.000	>1
Salt	NP_001105414.1	Potri.002G220450.1	Populus_deltoides-3712	0.005	0.000	>1

Potri, Ppr respectively stand for *P. tricocarpa, P. pruinosa*. Sequences show in File S2. Nr: Non-redundant protein database; Ka: the rate of synonymous substitutions; Ks: the rate of non-synonymous substitutions.

In section *Populus*, 301 positively selected genes were identified between *P. tremula* and *P. tremuloides*, which included one salt stress gene (Nr annotation: P93209.1) by producing the 14–3–3 protein [38–40], one drought stress gene (Nr annotation: BAB68268.1) by producing the drought inducible 22 kD protein, and one light stress gene (Nr annotation: NP_565524.1) by producing the SEP protein.

Among various sections, *P. tricocarpa* of section *Tacmahaca* identified 523 and 555 positively selected genes with *P. nigra* and *P. deltoides* of section *Aigeiros*, and the cold stress gene (Nr annotation: NP_849749.1) and salt stress gene (Nr annotation: P42652.1) were found between them by producing

high responses to osmotic stress [41,42] and 14–3–3 proteins. *P. tremula* of section *Populus* harbored 482, 285 and 148 positively selected genes with *P. tricocarpa*, *P. deltoides* and *P. pruinasa*, which included drought stress genes (Nr annotation: AAD11484.1 and NP_566843.1) by producing peroxidase [43–45] and SNF1 kinase proteins [46,47]. H_2O_2 stress genes (Nr annotation: AAG34804.1 and BAC92738.1) were found to be involved in the positive selection between section *Turanga* (*P. euphratica* and *P. pruinasa*) and the other three sections (*P. tricocarpa*, *P. deltoides* and *P. tremula*). These genes produce glutathione S-transferase [48,49] and cytosolic ascorbate peroxidase [50–52].

4. Discussion

4.1. Paleoclimate Changes during the Divergence of Populus Phylogeny

The divergence time of the genera *Populus* and *Salix* was about 45 Mya in the middle of the Paleogene period (66–23 Mya) [53–55]. During the Paleogene period, the global climate went against the hot and humid conditions of the late Mesozoic era and began a cooling and drying trend [56]. As the Earth cooled, tropical plants were restricted to equatorial regions and decreased in number. Deciduous plants became more common as these could survive seasonal climates, during which *Populus* and *Salix* diverged.

Miocene (23–5 Mya) was the main period of section divergence in the *Populus* phylogeny (Figure 3). The divergence times of the four sections *Turanga*, *Populus*, *Aigeiros* and *Tacmahaca* were respectively 19.9 Mya, 18.9 Mya and 10.0 Mya, corresponding to the beginning and end of the Miocene period. During this period, the climate slowly cooled towards a series of the ice age. Although a long-term cooling trend was well underway, there is evidence of a warm period from 21 to 14 Mya, which was named the Middle Miocene Climate Transition (MMCT) [56], during which sections *Turanga* and *Populus* diverged (19.9 and 18.9 Mya). Then, global temperatures decreased and some species became extinct by 14 Mya [57–59]; thus plants and animals had to migrate or adapt to survive. In particular, the climate sharply cooled by 8 Mya and this formed the Quaternary Ice Age (2.6–0.1 Mya) [60]. The climate change during the MMCT to Quaternary Ice Age may play an important role in the divergence of the *Populus* phylogeny.

4.2. H_2O_2-, Drought-Stress Genes and Speciation of Sections Turanga and Populus

The divergence times of sections *Turanga* and *Populus* were about 19.9 Mya and 18.9 Mya during the MMCT, during which the climate was warming. *P. euphratica* and *P. pruinasa* of section *Turanga* are mainly distributed in the deserts of Northern Africa and western China (Table S2). In previous studies, comparisons of the transcriptomes of *P. euphratica* and *P. pruinasa* suggest that these may have caused enough genetic divergence and helped them to adapt to these different desert habitats [12,13]. Our results show that the H_2O_2 stress gene was generally identified to be involved in positive selection between section *Turanga* and the other three sections. The H_2O_2 stress gene can help plants develop abiotic resistance to adapt to the complex environment. It is suggested that selective evolution of H_2O_2 stress genes should play an import role in the speciation of section *Turanga.* Meanwhile, *P. tremula* and *P. tremuloides* of section *Populus* are mainly distributed in the cooler and drought region of Northern America, Europe and Asia (Table S2). Our results show that drought stress genes are involved in positive selection between *P. tricocarpa* (section *Tacmahaca*) and *P. tremula* (section *Populus*), and *P. deltoides* (section *Aigeiros*) and *P. tremula* (section *Populus*). Speciation of the section *Populus* might be related to selective evolution of the drought stress genes during the MMCT.

4.3. Cold-, Salt-Stress Genes and Speciation of Sections Tacmahaca, Aigeiros

Previous research had suggested that Pleistocene (1.8–0.1 Mya) glacial cycles acted as drivers of speciation of *Populus balsamifera* and *P. tricocarpa* [8]. The same results were found in *Amborella trichopoda*, that two main genetic groups of Amborella were shaped by the divergence of two ancestral populations during the last glacial maximum [61]. In our work, cold stress genes were found to be

involved in positive selection between *P. nigra* (section *Aigeiros*) and *P. tricocarpa* (section *Tacmahaca*), and *P. deltoides* (section *Aigeiros*) and *P. tricocarpa* (section *Tacmahaca*). In addition, their divergence time was about 10.0 Mya–9.4 Mya at the beginning of cooling period. Our results show that the start time affected by the cooling climate may be earlier than the Quaternary Ice Age (2.6–0.1 Mya) during the divergence of the *Populus* phylogeny. *P. tricocarpa* of section *Tacmahaca* was mainly distributed in coastal western North America. Our results show that salt stress genes were involved in positive selection between *P. tricocarpa* and *P. nigra*, between *P. tricocarpa* and *P. deltoides.* This may be related to different geographical distribution of these *Populus* species.

5. Conclusions

In this study, we completed the comparative analysis based on transcript sequences of eight *Salicaceae* species from public databases. All the pairwise orthologues were identified in these species, from which we constructed a phylogenetic tree and estimated the rate of substitutions. The divergence times were estimated by the comparative transcriptomic analysis, and this suggested the speciation of *Populus* was involved in the period from the MMCT to Quaternary Ice Age. Furthermore, a number of positive selection genes were found to be related to environmental factors. In particular, cold-, salt-, drought- and H_2O_2-stress genes may be the driving force of species formation in the *Populus* phylogeny. The study shows that the paleoclimate change and selective evolution played an important role in the divergence of *Populus* phylogeny.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/2/163/s1. Table S1. GO annotation of shared orthologues in eight Salicaceae species, Table S2. Main geograhoical distribution of seven *Populus*, Figure S1. GO classes of shared orthologues in eight Salicaceae species, File S1. Sequences of 1835 shared orthologues in eight Salicaceae species, File S2. Sequences of resistance genes in genus *Populus*.

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

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