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A novel set of single-copy nuclear DNA markers for the genetic study of Salicaceae

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ABSTRACT. Species of *Populus* are widely distributed worldwide, playing a significant role in both ecology and economy. However, the lack of single-copy nuclear markers limits knowledge about the phylogeny and population genetics of this genus. In the present study, primer pairs of 15 single-copy nuclear markers were developed through bioinformatic methods based on complete genomic sequences of *Populus trichocarpa* and *Salix arbutifolia*. Twenty individuals of *Populus davidiana* Dode and *Salix matsudana* Koidz were used to evaluate the basic application of these markers with respect to marker length and diversity indices, respectively. The utility of single-copy nuclear markers is anticipated to facilitate further studies about the phylogeny, population genetics, and phylogeography of this genus, in addition to providing information about the evolutionary dynamics of Salicaceae.

Key words: *Populus*; *Salix*; Single-copy nuclear markers; Diversity indices

INTRODUCTION

The genus *Populus* is widely distributed from subtropical to boreal forests in the northern hemisphere. This genus contains some of the most commercially exploited trees, and has become a model organism for the study of tree biology (Cronk, 2005; Jansson and Douglas, 2007). Studies about the phylogeny and population genetics of *Populus* have received particular focus (Smith, 1988; Smith and Sytsma, 1990; Hamzeh and Dayanandan, 2004; Hamzeh et al., 2006; Lee et al., 2011; Levsen et al., 2012). Chloroplast DNA (cpDNA) and nrDNA internal transcribed spacer (ITS) sequences have been used in the phylogeny of *Populus* (Rajora and Dancik, 1995; Leskinen and Alström-Rapaport, 1999; Shi et al., 2001; Hamzeh and Dayanandan, 2004; Wei et al., 2010). Nevertheless, the phylogenetic relationships among species in this genus remain poorly resolved for several reasons, including the slow rate of sequence divergence, maternal inheritance of cpDNA, and inadequate sequence variation of ITS (Sang, 2002; Small et al., 2004), along with the high level of morphological variation and the extensive interspecific hybridization. Recently, low-copy nuclear genes were used to reconstruct phylogenetic relationships in plants (Peng and Wang, 2008; Yang et al., 2012). However, this procedure often requires much time and effort to obtain these sequences. To resolve the genetic relationship among closely related species of *Populus*, efficient and highly variable genetic markers with high interspecific diversity are needed. The completion of the *P. trichocarpa* genome sequence (Tuskan et al., 2006) made it possible to search for orthologous single-copy nuclear sequences, which are expected to serve as useful markers for genetic study of poplar. Here, we reported a novel set of 15 single-copy nuclear markers that were developed based on the genome sequence of *P. trichocarpa*. Furthermore, to expand the scope of application of these markers, we modified the primers to make them useful for a broader range of Salicaceae species.

MATERIAL AND METHODS

Biological material

The biological material was sampled from 1 *Populus davidiana* population located in Xinjiang, China, and 1 *Salix matsudana* population (including 1 *varietas* and 3 *forma*) located in Beijing, China. In 2012, 20 individuals from each population were sampled. Fresh leaves were collected and stored in plastic bags with silica gel. Four other species (namely *P. lasiocarpa*, *P. laurifolia*, *P. nigra*, and *P. euphratica*), which represented the other 4 sections (*Leucoides*, *Tacamahaca*, *Aigeiros*, and *Turanga*) of *Populus* were used to test the utility of these markers in the *Populus* genus. In addition, *Salix arbutifolia* and 3 randomly selected willow species (*Salix babylonica*, *S. triandra*, and *S. raddeana*; Table 1) were used to test the broader utility of the modified markers in the Salicaceae family.

Marker procedure

Populus-specific orthologous single-copy nuclear loci were found using the following bioinformatic methods: first, we searched GENE (http://www.ncbi.nlm.nih.gov/gene) using the single-copy nuclear locus tag provided by Duarte et al. (2010), then linked to the

KEGG webpage to obtain the amino acid sequence coded by the locus. We then accessed Phytozome (http://phytozome.net/search.php), selected the angiosperm node on the tree in the home page as the search target, and used BLAST as the search tool. After BLAST with the amino acid sequence, we checked the "family view" option with the lowest E-value. On the next gene family page, we clicked the gene page of *P. trichocarpa*, and copied the sequence as the reference sequence. Consequently, we ran the nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the reference sequence with "other (nr) databases" and "somewhat similar sequence" options, to check whether the locus we chose was a single-copy in the *P. trichocarpa* genome.

Table 1. Geographic position of sample site.

Species	Site name	N° of samples	Latitude	Longitude	Altitude (m asl)
Populus davidiana	Xinjiang	20	47°23'N	87°50'E	514
P. lasiocarpa	Hubei	2	30°16'N	109°29'E	478
P. laurifolia	Xinjiang	2	47°20'N	87°38'E	529
P. nigra	Germany	2	53°33'N	9°50'E	4
P. euphratica	Xinjiang	2	47°21'N	87°43'E	545
Salix matsudana f. pendula f. tortuosa f. umbraculifera var. pseudo-matsudana	Beijing	20	39°51'N	116°24'E	49
Salix arbutifolia	Hebei	2	40°40'N	117°14'E	433
S. babylonica	Zhejiang	2	30°16'N	120°09'E	18
S. triandra	Zhejiang	2	30°15'N	120°09'E	20
S. raddeana	Hebei	2	40°38'N	117°14'E	410

DNA isolation, amplification, and sequencing

Total genomic DNA was extracted from 25 mg leaf tissue from each individual using the methods of Doyle and Doyle (1987). Polymerase chain reactions (PCR) was performed in a total volume of 30 μL containing 5 to 50 ng genomic DNA, 2.4 μM of each primer, 0.8 μM of each dNTP, 2.0 mM $MgCl_2$, and 0.15 U ex Taq DNA polymerase (TaKaRa, Shiga, Japan). Amplification was carried out in a temperature gradient 96 U thermocycler (Applied Biosystems, Forster City, CA, USA) as follows: 4 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 50° to 60°C (depending on the annealing temperature of the specific primers and the length of the amplified regions), 90 s at 72°C, and a final extension at 72°C for 8 min. Products were examined by electrophoresis on agarose gel and purified using a DNA Purification kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The purified DNA was directly sequenced using an ABI 3730 DNA analyzer (Applied Biosystems). The same primers were used for both PCR amplification and sequencing.

Primers were designed using the Primer Premier 5.0 software (Premier Biosoft International, Silicon Valley, CA, USA), based on the reference sequences, which are listed in Table 2. We BLASTed (Altschul et al., 1997) the reference sequences in the draft genome of the *S. arbutifolia*, and assembled the sequence using Illumina next-generation sequencing platform (Du SH, Wang ZS and Zhang JG, unpublished results) to modify the primer pairs for *S. matsudana*, which are listed in Table 3.

Table 2. Characteristics of 15 single-copy nuclear markers of *Populus davidiana*.

Loci	Primer sequences (5'-3')	Location of chromosome	PCR product (bp)	Ta (°C)	GenBank accession No.
DSH 1	F: ATTGAGGCTTTTGTTCAGCGGTTAT R: CCTGTACTTGTTTGTCTGGCTTTGT	II	550-589	58	KC706924-KC706943
DSH 2	F: CATCTTTTGCCTTATTGTCTGCT R: TGCGTTAAATGATCTTTCTGGTA	IV	410-451	56	KC706944-KC706963
DSH 3	F: TCTGCTTTCCACTTCTTGC R: CATACTCTCCCATTGTCCC	VI	654-680	55	KC706964-KC706983
DSH 4	F: CCACCGCTACTCCTCCG R: TCCACCCCTCCATCCAC	X	401-465	58	KC706984-KC707003
DSH 5	F: TGGCAGAATCACCAGACCCTC R: CCAATTTAGCATCTTCAGCCTCAT	XII	583-622	59	KC707004-KC707023
DSH 6	F: GCCTCCTGATTATTATGC R: TATTACAAGCCCTTCCAG	XV	456-526	54	KC707024-KC707043
DSH 7	F: TGTCCACAAACGCATCC R: CAAACTTTACCACCCCA	XVI	512-574	58	KC707044-KC707063
DSH 8	F: GTTTGTTGTTCTGTTGATTGT R: GGCTTCTCTTCTCTGATATTT	XVIII	531-586	56	KC707064-KC707083
DSH 10	F: TACAAAAGCATTAAAGATCACCACT R: GGAGCACACTTATCAATAAAACTAC	I	780-842	54	KC707244-KC707263
DSH 11	F: GTGGCAAGACCAGCTGCTAGT R: ATGGAGGAAGGGTGGACAATG	I	912-1005	55	KC707264-KC707283
DSH 12	F: CACCACATCCCGCTTTCTCTCTTCACTT R: TAAACCCCAGGAGGCAAAACAGCACCAG	II	496-558	57	KC707284-KC707303
DSH 14	F: TGTTTGATGGACCTGGCTGCT R: CGGTTTATTGCCTTGTGGAGA	III	845-921	55	KC707304-KC707323
DSH 15	F: CTGAAAGGGAAAATAGTGGACAGTCAA R: GGATAACAGTAGCATGGAGATATGGAT	III	798-861	56	KC707304-KC707323
DSH 19	F: AAGTCTGGTCAAGGCAGTGGTC R: TCTGTGCTGTGATGTTTGGGGG	XIV	796-845	54	KC707344-KC707363
DSH 21	F: CATGCTTATGAAGGTGTGGGCTT R: TGCAAACATCTCACTGGTGACTG	XVII	701-775	53	KC707364-KC707383

Table 3. Characteristics of 15 single-copy nuclear markers of *Salix matsudana*.

Loci	Primer sequences (5'-3')	PCR product (bp)	Ta (°C)	GenBank accession No.
DSL 1	F: ATTGAGGCTTTTGTTCAGCGGTTAT R: CCTGTACTTGTTTGTCTGGCTTTGT	470-484	58	KC707084-KC707103
DSL 2	F: CCCTTGGAACCTAGCATGTATTCC R: TAGTTCTCATGGCTAAGATATT	404-445	56	KC707104-KC707123
DSL 3	F: TCTGCTTTCAACTTCTTGC R: CATACTCTCCCATTGTCCC	564-635	55	KC707124-KC707143
DSL 4	F: CCACCGCTACTCCTCCG R: TCCACATCTCCATCCAC	451-489	58	KC707144-KC707163
DSL 5	F: GCCTGGAAAAAACCTAATTTTCAG R: CCAGGAGCAGAAGAAAGAACCCG	636-658	59	KC707164-KC707183
DSL 6	F: GCCTCCTGATTATTACGC R: TATTACAAGCCCTTCCAG	573-600	54	KC707184-KC707203
DSL 7	F: GGACAAATACCGTCCACAA R: CCTCCAAACTTTACCACCC	415-460	58	KC707204-KC707223
DSL 8	F: GTTTGTTGTTCTGTTGATTGT R: GGCTTCTCTTCTCTGATATTT	556-616	56	KC707224-KC707243
DSL 24	F: TACAAAAGCATTAAAGATCACCACT R: GGAGCACAGTTATCGATAAAACTAC	1050-1126	54	KC707384-KC707403
DSL 25	F: GTGGCAAGACCAGCTGCTAGT R: AAGGAGGGGCAAAGCAAAGCTGTGG	671-730	55	KC707404-KC707423
DSL 26	F: TCTCTCTTCACTTGCATATTTATACGCA R: TAAACCCCTGGAGGCAAAACAGCACCAG	495-535	57	KC707424-KC707443
DSL 28	F: TGTTTGATGGACCTGGCTGCT R: CGGTTTATTGCCTTGTGGAGA	1024-1082	55	KC707444-KC707463
DSL 29	F: CTGAAAGGGAAAATAGTGGACTGTCAA R: GGATAACAGTAGCATGGAGATATGGAT	779-826	56	KC707464-KC707483
DSL 33	F: AAGTCTGGTCAAGGCAGTGGTC R: TCTGCTCTGTGATGTTTGGGTGA	521-579	54	KC707484-KC707503
DSL 35	F: CATGCATATGAGGGTGTGGGCTT R: TGCAAACATCTCATTGCTAACTG	745-786	53	KC707504-KC707523

Data analysis

The assembled contigs of each individual were aligned using CLUSTAL X (Thompson et al., 1997) and refined manually in BioEdit (Hall, 1999). The number of haplotypes per locus (A), the number of polymorphic sites (S), the average number of nucleotide differences (κ), the nucleotide diversity (π), and nucleotide polymorphism (θ_w) were analyzed using Dnasp5.10.0 (Librado and Rozas, 2009). The expected heterozygosity (H_E), the gene diversity (H_O), and the Tajima D-test were calculated using Arlequin 3.5.1.3 (Excoffier et al., 2005).

RESULTS AND DISCUSSION

After performing BLAST with the reference sequences of *P. trichocarpa*, all of the searched 30 single-copy nuclear markers were also found to be single copies in the *S. arbutifolia* genome, of which 8 did not work in amplification and 7 did not work in the sequencing. This result may be attributed to frequent indels in the sequences; hence, these 15 markers were abandoned in the following steps. The other 15 markers performed well in both amplification and sequencing, in which 2 loci (DSH 1 / DSL 1, DSH 8 / DSL 8) were the same, and 13 loci were modified, because of mutations in the primer region. The success rates for the amplification and sequencing of these markers in *P. lasiocarpa*, *P. laurifolia*, *P. nigra*, and *P. euphratica*, *S. arbutifolia*, *S. babylonica*, *S. triandra*, and *S. raddeana* were 100% (data not shown).

After manual refining, the sequences used in analysis were 414-948 bp. The number of haplotypes per locus ranged from 4 to 17, with a mean of 11. An average of 11 polymorphic sites was obtained. The average number of nucleotide differences ranged from 1.23 to 5.78, with an average of 3.72. The genetic polymorphism parameters of π, θ_w, H_E, and H_O were, on average, 0.00603, 0.00410, 0.338, and 0.792, respectively. This result indicated that *P. davidiana* had high genetic diversity. The results of the Tajima D-test showed that all loci were consistent with the neutral theory at $P > 0.05$ (Table 4).

Table 4. Results of initial primer screening in *Populus davidiana*.

Locus	N	Length (bp)	A	S	π	θ_w	κ	H_E	H_O	Tajima D
DSH 1	20	537	10	17	0.0108	0.00747	5.78	0.309	0.774	1.45
DSH 2	20	414	17	15	0.0134	0.00854	5.55	0.350	0.913	1.83
DSH 3	20	643	9	11	0.00417	0.00404	2.67	0.219	0.767	0.0951
DSH 4	20	428	9	6	0.00556	0.00330	2.37	0.371	0.745	1.60
DSH 5	20	559	10	10	0.00636	0.00422	3.54	0.326	0.796	1.51
DSH 6	20	474	8	6	0.00451	0.00297	2.14	0.358	0.760	1.39
DSH 7	20	529	4	3	0.00232	0.00133	1.23	0.409	0.539	1.61
DSH 8	20	566	9	8	0.00536	0.00332	3.03	0.379	0.677	1.75
DSH 10	20	804	17	16	0.00525	0.00468	4.22	0.264	0.931	0.394
DSH 11	20	948	8	9	0.00258	0.00223	2.45	0.272	0.728	0.460
DSH 12	20	528	10	10	0.00709	0.00445	3.75	0.375	0.863	1.77
DSH 14	20	881	17	14	0.00601	0.00374	5.30	0.374	0.874	1.92
DSH 15	20	834	14	16	0.00641	0.00449	5.37	0.331	0.873	1.38
DSH 19	20	826	11	15	0.00670	0.00421	5.54	0.369	0.751	1.82
DSH 21	20	739	11	8	0.00385	0.00255	2.84	0.365	0.883	1.45
Mean	20	647	11	11	0.00603	0.00410	3.72	0.338	0.792	1.36

The length of 15 sequenced loci in *S. matsudana* was 417-1096 bp, with an average of 634 bp. The average number of haplotype and polymorphic sites were 10 and 11, respectively,

which were similar to those of poplar. The average number of nucleotide differences ranged from 0.322 to 12.0, with an average of 4.34, which was higher compared to poplar. The values of π, θ_w, H_E, and H_O also showed the presence of high genetic diversity in *S. matsudana*. Moreover, the D values of 9 loci (DSL 1, DSL 3, DSL 5, DSL 7, DSL 24, DSL 28, DSL 29, DSL 33, and DSL 35) significantly deviated from that expected by the neutral model ($P < 0.05$) (Table 5). Given that 1 *varietas* and 3 *forma* were included in the analysis, it was expected that alleles existed in high frequencies in different loci that had been derived from divergent taxonomic units. Alternatively, balancing selection might have been present in these loci. Therefore, further research should be conducted about these loci.

Table 5. Results of initial primer screening in *Salix matsudana*.

Locus	N	Length (bp)	A	S	π	θ_w	κ	H_E	H_O	Tajima D
DSL 1	20	471	11	19	0.0193	0.00948	9.09	0.479	0.878	3.40
DSL 2	20	417	10	9	0.00417	0.00510	1.73	0.192	0.833	-0.534
DSL 3	20	584	10	24	0.0206	0.00966	12.0	0.500	0.897	3.81
DSL 4	20	445	9	8	0.00383	0.00423	1.70	0.213	0.603	-0.266
DSL 5	20	605	7	9	0.00685	0.00350	4.15	0.461	0.833	2.80
DSL 6	20	564	4	3	0.00213	0.00125	1.20	0.401	0.650	1.54
DSL 7	20	427	14	12	0.0125	0.00661	5.35	0.446	0.910	2.76
DSL 8	20	532	3	2	0.000600	0.000880	0.322	0.161	0.309	-0.596
DSL 24	20	1096	18	8	0.00310	0.00172	3.38	0.425	0.901	2.29
DSL 25	20	709	10	8	0.00403	0.00265	2.86	0.357	0.863	1.48
DSL 26	20	508	6	9	0.00105	0.00417	0.938	0.104	0.395	-1.63
DSL 28	20	1065	11	7	0.00279	0.00155	2.97	0.424	0.894	2.22
DSL 29	20	806	11	20	0.0122	0.00583	9.79	0.490	0.897	3.58
DSL 33	20	547	12	19	0.0163	0.00817	8.92	0.469	0.908	3.28
DSL 35	20	743	7	11	0.00099	0.00348	0.732	0.0666	0.396	-2.18
Mean	20	634	10	11	0.00736	0.00455	4.34	0.346	0.744	1.46

CONCLUSIONS

We presented a novel set of 15 single-copy nuclear markers that were specifically developed for Salicaceae, and utility in *P. davidiana* and *S. matsudana.* The combined phylogenetic inference of *Populus* when using these markers as outgroups, along with the markers of other willow species, is currently in progress, and the preliminary results are promising. Although previous studies have focused on the phylogeny of *Populus*, there has been extensive debate on the relationships among species and the placement of some species (which might have originated through hybridization). This set of efficient and highly resolved molecular markers is anticipated to be of particular use in phylogenic and population genetics, as well as for elucidating the evolutionary dynamics of *Populus* species and the Salicaceae family.

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