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RELATIONSHIP BETWEEN HETEROSIS AND PARENTAL GENETIC DISTANCE BASED ON RAPD AND EST-SSR MARKERS IN *PINUS TAEDA* L. × *P. CARIBAEA* MORELET

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Abstract

The assessment of parental genetic distances (GDs) and their correlations with progeny performance have largely been circumvented by DNA marker development. Both strong and weak correlations between progeny performance and parental GDs have been detected in numerous plant species. ESTs (expressed sequence tags) in the pine genome can be functionally annotated, providing ideal makers to explain heterotic effects. We found stronger correlations between F_1 hybrid performance and their parental GDs based on 4 EST-simple sequence repeat (SSR) primer pairs with 19 alleles compared to random amplified polymorphic DNA (RAPD) primers with 46 bands. The correlation coefficients of parental GDs based on EST-SSR and/or RAPDs with the better parental heterosis (BPH) of seedling diameter at ground line (DGL) were 0.288 ($p = 0.049$) and 0.290 ($p = 0.050$), respectively. In contrast, there was no significant correlation between parental GDs based on RAPD markers and F_1 hybrid growth traits. Our results demonstrate that appropriate molecular markers more effectively illustrated heterotic effects than randomized markers. We expect that functional gene markers from sequenced genomes will provide a starting point for heterosis research in pines.

Introduction

Hybrids in breeding are primarily used an alternative to increase genetic diversity within taxa that have low or almost null genetic variability for certain traits or as a tool to combine desirable characteristics (Del *et al*. 2012). In pines, hybrids have provided an attractive approach for improvement. Despite some biological problems with crossability, there have been a number of successes (Dungey 2001). Slash pine (*Pinus elliottii* Engelman var. *elliottii*) and loblolly pine (*P. taeda* L.) were first introduced to China in the 1930s. Afterwards, *P. caribeae* Morelet was introduced in Chinese tropical and subtropical monsoon forest plantations as a pure species or a parental species for hybridization (Wang *et al*. 1999). The plantations established with the exotic pines now exceed 3 million hactare. The production of potential hybrids to address the severe timber shortage was a primary focus in recent years. This involved hybridizing Caribbean and loblolly pines in addition to slash pine. The results showed that the loblolly pine × Caribbean pine hybrid had a relatively higher growth volume and similar survival rate as the female loblolly pine parent. This revealed that specific hybrids can be planted in north of 27°N latitude (Luan *et al*. 2013).

Quantitative genetic theory suggests that there is a linear relationship between heterosis of a hybrid and the genetic distance (GD) between its parents considering all loci underlying the quantitative trait of interest. Consequently, predicting hybrid performance based on GDs between parents has been suggested (Reif *et al.* 2012). However, predicting heterosis with estimates of GDs

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between the parental lines based on different DNA markers has not always been successful (Cheres *et al.* 2000; Lanza *et al.* 1997, Reif *et al.* 2003, Uaatav *et al.* 2014). Many molecular markers have been developed to estimate GD and construct genetic linkage maps, such as random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), inter-simple sequence repeat (ISSR) and restriction fragment length polymorphisms (RFLP) (Lu *et al.* 2012). However, like RFLPs and RAPDs, SSRs and AFLPs can be found in either the coding or noncoding regions of the genome. Recently, emphasis has been given towards the use of expressed sequence tags (ESTs) that only score the expressed region of the genome. EST-SSRs offer the following advantages over other genomic DNA-based markers: (1) These should detect variation in the expressed portion of the genome, thus gene tagging provide "perfect" marker-trait associations, (2) these can be used to develope at no cost from the EST databases (genomic SSRs are expensive to develop) and (3) once developed, these can be used across a number of related species (unlike genomic SSRs) (Gupta *et al.* 2003).

The weak relationship between heterosis and parental GD-based DNA markers can be explained by a poor association between heterozygosity estimated from marker data and heterozygosity at quantitative trait loci affecting the trait of interest (Hua *et al.* 2002). EST-SSR markers should yield "perfect" marker-trait associations; thus, the GD between the parents estimated by the EST-SSRs must be more related to the heterosis of the EST-related traits within the hybrid offspring. A few studies have assessed this given the lack of pine functional gene markers (e.g., ESTs). Fortunately the first pine genome assembled provides a foundation to study conifer biology. A large set of EST sequence data from loblolly pine was recently generated (Zimin *et al.* 2014). In this article, EST-SSR markers related to tree stem growth were developed from ESTs of *P. taeda*. The relationships between heterosis and parental GD based on the EST-SSR and RAPD markers in *P. taeda* × *P. caribaea* were studied to guide the optimized selection of parents to produce hybrids and verify the predictive effectiveness of heterosis using randomized and functional gene markers.

Materials and Methods

Controlled pollination was carried out in a clonal seed orchard of loblolly pine at Changle Forestry Farm (30.33°N,119.86°E), Hangzhou, China, where the *P. caribaea* species cannot survive due to the cold winter temperature. We randomly selected 47 clones (labeled from FEM1 - FEM47) in the loblolly orchard as the maternal parents of the crosses. *P. caribaea* var. *hondurensis* pollen mixture (labeled with Mal) from several ramets of one best clone as male parent was collected from a Zhanjiang tree breeding farm located in southern China (21.25°N,110.25°E). The pollen collection and pollination procedures were described previously (Tighe 2005). Pollination was conducted in April 2010, with subsequent seed collection including corresponding open-pollinated seeds from loblolly pine mother trees. The collected seeds of 47 hybrid families (labeled HY1 to HY47) and 1 mixture (labeled with parent) of 3 superior open pollinated families were used to raise seedlings under similar growing conditions in the Changle Forestry Farm nursery. We employed a randomized complete block design with 5 replicates and 20 seed plots. Seed spacing was usually 10 × 10 cm. Fifty seedlings for each family located at the inner area of each plot were randomly selected for the data collection. The diameter at ground line (DGL) and height for selected seedlings were measured at the age of one year.

The *P. taeda* EST sequences used in this study were retrieved from Pine Gene Index (PGI) (downloaded from ftp://occams.dfci.harvard.edu/pub/bio/tgi/data/**)**, also see the introduction from http://compbio.dfci.harvard.edu/tgi/**.** More than 20,000 non- redundant EST sequences were selected to find the sequences related to stem growth traits according to the annotation of

corresponding ESTs. All the selected ESTs related to stem growth traits (such as extension growth and secondary growth) were searched to identify SSRs using the Simple Sequence Repeat Identification Tool (SSRIT) available at http://archive.gramene.org/ db/markers/ssrtool. A subset of sequences from the SSRs containing unigenes were selected for primer design using Primer3web software (version 4.0.0), which is available at http://primer3.ut.ee/.

From 100 randomly chosen RAPD primers purchased from Sangon Biotech (Shanghai, China), we selected S193, S199, S336, S377, S390, S477, S486, and S496, these generated high polymorphisms.

Polymerase chain reaction (PCR) amplification and band detection pine needles of each loblolly pine (maternal parents) and the mixture of three Caribbean pines (paternal parents) were collected for DNA extraction, EST-SSR and RAPD amplification according to the method described by Nkongolo (1999).

The better parental heterosis (BPH) of each trait was calculated as: BPH = ([F_1-BP]/BP), where, F_1 is the mean for each hybrid family of 50 seedlings and BP is the mean for the mixture of 3 superior open-pollinated families.

Data analysis of RAPD and EST-SSR markers was performed as described by Zhang *et al.* (2013). Briefly, each size of PCR-products was treated as a unit character and scored in a binary code of either 1 or 0 for presence or absence, respectively. To keep things simple, We assume that the dissimilarity coefficient using the Nei and Li (1979) was equally to the GDs in this paper. NTSYS-pc (Numerical Taxonomy and Multivariate Analysis for Personal Computers) version 2.1 software was used to calculate the GDs between maternal parental clones (loblolly pine) and male parental clone (Caribbean pine) based on the RAPD (GD1) and EST-SSR markers(GD2) respectively, as well as their combined results (GD3).

Pearson's correlation coefficients between GDs (RAPD and EST-SSR markers and the combined results) and F_1 progeny traits, as well as correlation coefficients between GDs and BPH were calculated using PROC CORR in SAS software (SAS Institute Inc., Cary, NC, USA). Analyses of variance were computed for the two seedling traits using a general linear model (GLM) with Type III SS (SAS Institute Inc. 1997). $p < 0.05$ was considered significant.

Results and Discussion

There were significant differences ($p < 0.01$) among F_1 families for the traits of DGL and F_1 hybrid height at the age of one year. The mean values of all F_1 hybrids for seedling height and DGL were 26.6 cm and 6.0 mm, respectively which were slightly lower than the better parental mixture. On the other hand, the BPHs for DGL and height were mostly negative. Howerever, 15 and 18 of the 47 F_1 hybrids exhibited positive heterosis for seedling height and DGL, respectively (Table 1).

Eight RAPD markers that generated high polymorphisms were selected from 100 randomly chosen RAPD primers purchased from Sangon Biotech. A total of 46 bands was detected with an average of 5.8 bands per primer. We identified 39 (84.78%) polymorphic bands (Table 2).

We tried 13 primer pairs for EST-SSR molecular marker analysis related to growth for *Pinus* L. (Table 3). It was found that only 4 primer pairs reliably amplified polymorphic PCR products from the genomic DNA of both loblolly and Caribbean pines. The 4 polymorphism primer pairs were TC97515, TC101746, TC105392 and BX252926 (bolded values in Table 3) amplified 4, 6, 3, and 6 alleles, respectively. The number of polymorphic allele was 18 (94.73%).

Table 1. Mean and BPH for DGL and height.

F_1 hybrids and parent	Seedling height (cm)			DGL (mm)		
	Mean	Std Dev	BPH	Mean	Std Dev	BPH
HY1	25.8	8.1	–2.1	5.8	1.6	–0.3
HY2	24.2	5.0	–3.7	5.8	1.1	–0.3
HY3	24.3	9.3	–3.6	6.2	1.5	0.1
HY4	27.3	6.8	–0.6	6.0	1.5	0.0
HY5	25.8	6.9	–2.1	5.5	1.5	–0.5
HY6	27.0	9.1	–0.9	6.0	2.1	–0.1
HY7	28.6	8.0	0.7	6.0	2.2	–0.1
HY8	26.2	6.9	–1.7	5.6	1.5	–0.4
HY9	26.3	6.0	–1.6	5.9	1.7	–0.2
HY10	29.4	7.1	1.5	5.7	1.7	–0.3
HY11	27.0	7.7	–0.9	5.6	1.8	–0.5
HY12	29.2	7.9	1.3	6.2	1.6	0.2
HY13	29.0	10.6	1.1	6.6	1.8	0.5
HY14	24.9	7.9	–3.0	6.4	1.6	0.3
HY15	23.1	9.1	–4.8	5.8	1.5	–0.3
HY16	27.7	7.6	–0.2	6.2	1.7	0.1
HY17	24.4	6.9	–3.5	6.0	1.9	–0.1
HY18	22.6	6.0	–5.3	5.8	1.7	–0.2
HY19	25.7	6.9	–2.2	5.5	1.8	–0.5
HY20	30.4	8.6	2.5	6.1	1.1	0.1
HY21	27.2	7.4	–0.7	5.7	1.7	–0.4
HY22	30.6	7.9	2.7	6.9	1.7	0.8
HY23	29.4	8.3	1.5	5.9	1.7	–0.1
HY24	26.4	6.3	–1.5	6.1	1.8	0.1
HY25	29.5	11.4	1.6	6.7	2.3	0.7
HY26	23.9	7.5	–4.0	6.0	2.0	–0.1
HY27	29.9	8.0	2.0	6.8	1.6	0.7
HY28	24.6	7.8	–3.3	5.7	1.9	–0.3
HY29	24.4	7.7	–3.5	5.2	1.5	–0.9
HY30	22.4	7.0	–5.5	5.7	1.4	–0.3
HY31	26.2	6.9	–1.7	6.0	1.8	–0.1
HY32	25.8	6.1	–2.1	5.7	1.5	–0.3
HY33	32.2	8.0	4.3	6.9	2.0	0.9
HY34	28.0	8.8	0.1	6.5	1.7	0.4
HY35	29.1	5.6	1.2	7.0	1.6	1.0
HY36	24.3	5.7	–3.6	5.4	1.5	–0.7
HY37	27.3	5.7	–0.6	5.9	1.7	–0.1
HY38	22.4	5.3	–5.5	5.5	1.4	–0.5
HY39	27.9	12.3	0.0	6.2	1.9	0.1
HY40	23.9	7.2	–4.0	5.4	1.6	–0.6
HY41	24.1	8.7	–3.8	6.0	2.2	–0.1
HY42	22.6	6.1	–5.3	5.1	1.3	–0.9
HY43	29.6	9.5	1.7	5.9	1.8	–0.1
HY44	21.9	6.8	–6.0	5.7	1.2	–0.3
HY45	29.8	6.0	1.9	7.1	1.7	1.0
HY46	32.1	9.5	4.2	6.0	1.4	–0.1
HY47	25.5	7.1	–2.4	5.8	1.5	–0.2
Parent	27.9	8.4	0.0	6.1	1.7	0.0
Average	26.6	7.6	–1.3	6.0	1.7	–0.1

BPH = Better parental heterosis. DGL = Diameter at ground line.

Table 2. Screened RAPD primers with polymorphic products.

Primers	Sequence (5'-3')	Total bands	Polymorphic bands
S193	GTCGTTCCTG	5	5
S199	GAGTCAGCAG	6	5
S336	TCCCCATCAC	7	7
S377	CCCAGCTGTG	4	4
S390	TGGGAGATGG	7	7
S477	TGACCCGCCT	2	1
S486	GAGCGCCTTG	5	3
S496	AGTGCAGCCA	10	7
Total	-	46	39
Average	-	5.8	4.8

Table 3. EST-SSR primer-pairs related to growth traits developed from loblolly genome data.

Primer	Motif	Forward primer(5'-3')	Reverse primer(5'-3')	Product size
TC90336	$(TG)_3$	TCTGGGGAAGAAACAGATCG	CAGCCGAAAAGTTGGTCATT	203
TC97514	$(CT)_7$	GAGGGGAGGAATTACGTGGT	ACGAGGTGCAGCCTTAATGT	227
TC97515	**$(TG)_6$**	**GATAGCAAACAATGGCAGCA**	**CAACAACAGGACTCCTTGACAG**	**280**
TC100162	$(TG)_3$	TCTTCTGTGGGCACTGATAATG	GGCATCGAGGAACTTGAGAG	387
TC101746	**$(TG)_6$**	**TGTTCTTAGCGCAAATCAGG**	**AAACTGGGGCTTCAGGCTAC**	**299**
TC104928	$(TA)_3$	GGGCAAGCAGTGGTTGTATT	GTTGTTGTAACAGGGGCACA	286
TC105392	**$(AG)_4$**	**GCGGTGAAGTTATTCGCTCT**	**ATCGGTGTGTTCTCCGAATC**	**296**
TC107430	$(TG)_3$	CAAGTGCTGTGCAAAGTGTC	CAGAATATAGCAATTACAATGCAAC	246
TC110661	$(GT)_3$	AAATCAGGGGATCACTGGAA	CATTAAACTGGGGCTTCAGG	291
ST55D12	$(AT)_3$	TTGTAGAAGAGGAACGGCTTTT	CCCCTCTTTTGGTTTTCCAG	266
BX252926	**$(AC)_3$**	**CATATCCCGATAGCAAGGACA**	**AAACTGTGCAAAGGACACACA**	**284**
CT580901	$(AT)_3$	GAGCAAATATATTGCCTTCTTGC	GGAACACAACGACATTTGGA	249
CT580717	$(AT)_6$	AAGGATGGCTTCCTCTGGA	GGCACAGGGTGAAATTCAAA	290

EST-SSR = Expressed sequence tags-simple sequence repeat.

The NTSYS-pc program was used to calculate GD between maternal loblolly pine and male Caribbean pine based on RAPDs and/or EST-SSRs. The average GD based on RAPDs, EST-SSRs and both were 0.3174 (GD1), 0.4790 (GD2) and 0.3308 (GD3), respectively with corresponding ranges were 0.1351 - 0.6552, 0.200 - 0.750 and 0.1698 - 0.5897 (Table 4).

GDs between parents based on EST-SSR and/or RAPD markers was significantly correlated ($p < 0.05$) with the BPH for seedling DGL of the F_1 hybrids with low correlation coefficients (0.288 and 0.290, respectively, (Table 5, Fig. 1). In contrast, the correlation coefficients of GD of parents based on RAPD to the BPH for seedling DGL was 0.236 ($p = 0.111$).

Table 4. Genetic distance between male and female parents based on RAPDs and/or EST-SSRs.

Parents	GD based on		
	RAPD	EST-SSR	RAPD and EST-SSR
MALE			
FEM1	0.2973	0.5000	0.3191
FEM2	0.3488	0.5000	0.3585
FEM3	0.4054	0.7143	0.4348
FEM4	0.3750	0.7143	0.4146
FEM5	0.3529	0.3333	0.3333
FEM6	0.3500	0.3333	0.3333
FEM7	0.2941	0.7143	0.3488
FEM8	0.2105	0.3333	0.2245
FEM9	0.2353	0.5000	0.2727
FEM10	0.1500	0.7143	0.2245
FEM11	0.2000	0.5000	0.2400
FEM12	0.2000	0.5000	0.2400
FEM13	0.2381	0.5000	0.2692
FEM14	0.2093	0.5000	0.2453
FEM15	0.3182	0.5000	0.3333
FEM16	0.2857	0.7143	0.3333
FEM17	0.2195	0.5000	0.2549
FEM18	0.2558	0.7143	0.3077
FEM19	0.1351	0.7143	0.2174
FEM20	0.1500	0.5000	0.2000
FEM21	0.4667	0.3333	0.4146
FEM22	0.4444	0.7143	0.4667
FEM23	0.3171	0.4000	0.3208
FEM24	0.2558	0.5556	0.2963
FEM25	0.3023	0.5000	0.3208
FEM26	0.3023	0.5000	0.3208
FEM27	0.3182	0.5556	0.3455
FEM28	0.3023	0.7143	0.3462
FEM29	0.3846	0.7143	0.4167
FEM30	0.5135	0.5556	0.5000
FEM31	0.4500	0.2727	0.3962
FEM32	0.4054	0.7143	0.5652
FEM33	0.4706	0.7500	0.5000

(Contd.)

(Contd.)

FEM34	0.4375	0.5000	0.4286
FEM35	0.6552	0.5000	0.5897
FEM36	0.5882	0.2000	0.4783
FEM37	0.5000	0.2000	0.4167
FEM38	0.2273	0.2000	0.2143
FEM39	0.2273	0.2000	0.2143
FEM40	0.1707	0.2000	0.1698
FEM41	0.1707	0.2000	0.1698
FEM42	0.2093	0.2000	0.2000
FEM43	0.3500	0.3333	0.3333
FEM44	0.2857	0.3333	0.2830
FEM45	0.3023	0.5000	0.3208
FEM46	0.3158	0.3333	0.3061
FEM47	0.3158	0.3333	0.3061
Average	0.3174	0.4790	0.3308
Range	0.1351-0.6552	0.2000-0.7500	0.1698-0.5897

EST-SSR = expressed sequence tags-simple sequence repeat. GD = Genetic distance.

Table 5. Correlations between parental GD and F_1 hybrid performance.

	GD1-RAPD Corr.C (p)	GD2-EST-SSR Corr.C (p)	GD3-RAPD and EST-SSR Corr.C (p)
Height (cm)	0.102 (0.495)	0.202 (0.172)	0.149 (0.318)
DGL (mm)	0.236 (0.110)	0.281 (0.056)	0.287 (0.050)
BPH height	0.102 (0.494)	0.205 (0.167)	0.150 (0.314)
BPH DGL	**0.236 (0.111)**	**0.288 (0.049)**	**0.290 (0.048)**

BPH = Better parent heterosis. Corr. C = Correlation coefficient. DGL = Diameter at ground line.
GD = Genetic distance.

A number of successful inter-specific *Pinus* hybrids have been developed and most of the heterotic effects were explained by the performance of parents and their crosses (Dungey 2001). In the past few decades, the assessment of parental GDs and the correlations with progeny performance have largely been circumvented by the development of DNA markers. Both strong and weak correlations between progeny performance and parental GDs have been detected in numerous plant species. It is widely considered that selection and application of an ample number of adequate molecular genetic markers with appropriate characteristics will enhance the efficiency of molecular-based progeny prediction and a prior parent choice (Quackenbush *et al.* 2001). Unfortunately, commonly used markers like RFLPs, RAPDs, SSRs, and AFLPs can be found in either coding or noncoding regions of the genome. Even in the coding regions, these markers do

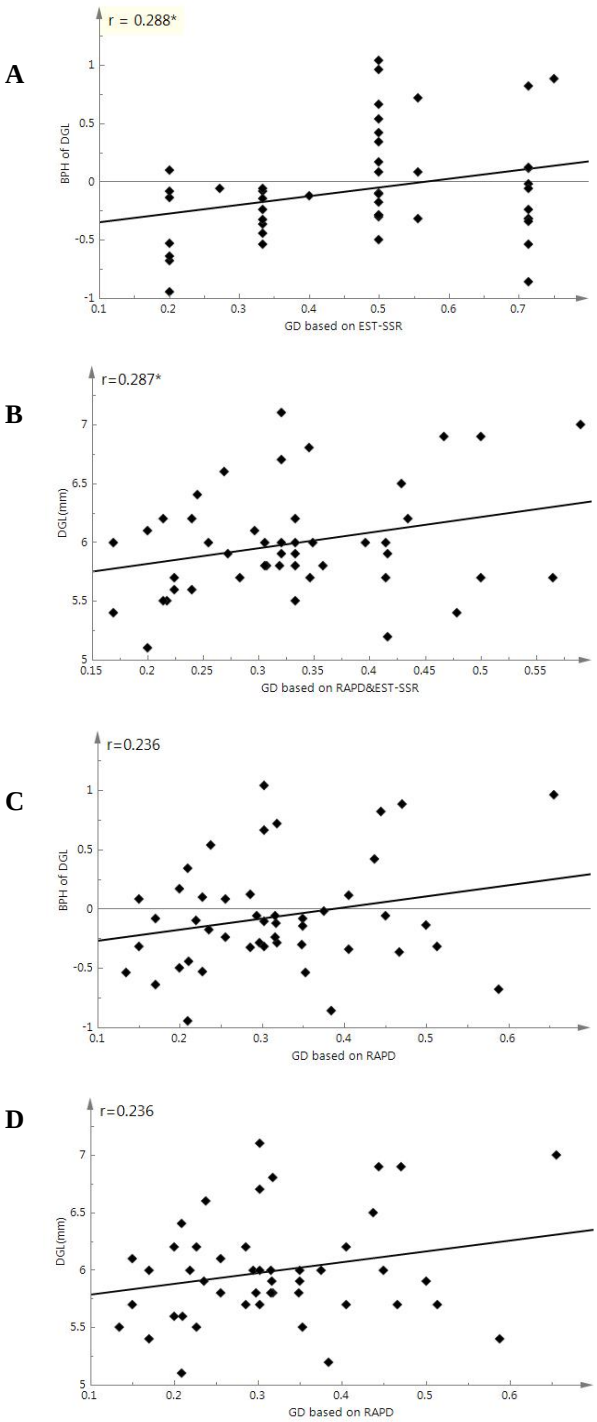

Fig. 1. The plot line for Pearson's correlation between GD based on RAPD (C, D) and EST-SSR markers (A), (B) with F_1 hybrid performance. *Indicates significance at $p < 0.05$ in each case.

not always score specific traits. Thus, effective molecular genetic markers related to specific characteristics in pines were not available until the first pine genome was assembled. Pine ESTs only score the expressed region of the genome and can be functionally annotated, making them ideal makers for explaining heterotic effects. We observed stronger correlation between F_1 hybrid performance and parental GDs based on only 4 EST-SSR primer pairs with 19 alleles than that based on 8 RAPD primers. The results showed that the molecular markers bearing appropriate characteristics would more effectively illustrated the heterotic effects than random markers such as RAPD

Without considering the genetic backgrounds of the materials, many researchers have realized that good numbers of DNA markers that are highly related to corresponding characteristics are needed to predict heterosis. Liu and Wu (1998) divided the SSR alleles into favorable and unfavorable groups and were able to significantly affect yield heterosis in hybrid rice breeding. Hua *et al*. (2003) and Xiao *et al*. (1995,1996) reported that complementary dominance associated with specific traits was responsible for a majority of heterosis. Another group selected "key" markers from common SSR and RAPD markers and suggested that using key marker-loci was an efficient method for predicting and improving heterosis in the breeding of hybrid crop varieties (Cho *et al.* 2004). With the development of recent genome sequencing and genome-wide association studies, a growing set of favorable or "key" markers such as EST-SSR, ESTP, and SNPs is available for enhancing the efficiency of the molecular-based progeny prediction and a priori parent choice. Pine breeding has faced many difficulties related to breeding group construction and DNA marker development. Few results from heterosis research on pine hybrids based on the GDs of DNA markers have been reported. Among those that have been described, the correlation coefficients of heterosis to the GD were not high. We expect that functional gene markers such as ESTPs, EST-SSRs, and SNPs screened from recently sequenced genomes will provide a starting point for developing research questions related to heterosis in pines.

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