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Genetic diversity analysis of *Pinus elliottii* × *P. caribaea* var. *hondurensis* using ISSR markers

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Abstract: Cross-breeding is an important method for generating genetic and phenotypic variation for selecting new varieties. However, because of an uncertainty of phylogenetic relationships, the parents selected for crosses may have a close genetic relationship resulting in hybrid progeny that shows low genetic diversity. Analysis of inter-family genetic diversity was undertaken among eight *Pinus elliottii* × *P. caribaea* var. *hondurensis* full-sib families using inter-sequence simple repeat (ISSR) markers. A total of 480 individuals were analyzed using 10 ISSR primers. Nei's unbiased gene diversity in the families ranged from 0.015 2 to 0.087 2. Shannon genetic diversity index values ranged from 0.021 6 to 0.129 4. Only a small proportion (25.65%) of genetic variation resided within families, whereas the majority of genetic variation (74.35%) accounted for the inter-family genetic differentiation index of G_{st} = 0.743 5. On the basis of estimated genetic distance and UPGMA clustering analysis, the genetic differentiation among the eight families was indicated to be relatively high with low gene flow (N_m = 0.172 5). The low inter-family gene flow may be related to the high genetic heterozygosity of slash pine and Caribbean pine. These findings are expected to provide a foundation for genetic breeding of *Pinus elliottii* × *P. caribaea* var. *hondurensis* hybrids.

Key words: *Pinus elliottii* × *P. caribaea* var. *hondurensis*, families, genetic diversity, ISSR marker

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湿地松×洪都拉斯加勒比松遗传多样性的 ISSR 分析

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摘 要: 杂交育种是产生遗传变异、表型变异及选择新变异的重要方法。然而系统发育不清晰,选择较近的亲缘关系亲本用于杂交子代往往表现出较低的遗传多样性。为探究湿地松、洪都拉斯加勒比松种间杂交后代遗传多样性水平,对 8 个湿地松×洪都拉斯加勒比松家系进行 ISSR 分析。利用 10 条引物共产生 60 个表达清晰可用

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于分析的标记,其中 48 个标记表现为多态性,占总标记数的 80%;湿加松各个家系多态位点百分率在 5%~23.33%之间;各个家系基因多样性指数在 0.015 2~0.087 2之间,Shannon 指数的范围在 0.021 6~0.129 4之间(家系水平为 0.293 4)。8 个家系间的基因分化系数 G_{st}为 0.743 5,即总的遗传变异中有 74.35%的变异存在于家系间,家系内的遗传变异占总遗传变异的 25.65%。采用 UPGMA 法对湿加松的 8 个家系进行了聚类分析,确定了各个家系之间的遗传亲缘关系。8 个家系间的基因流 N_m 为 0.172 5,表明基因流处于较低水平。

关键词：湿加松, 家系, 遗传多样性, ISSR 标记

Pinus elliottii, commonly known as the slash pine, is native to Southeast America, from southern South Carolina west to Southeast Louisiana, and south to Florida Keys. The Caribbean pine, *P. caribaea*, is a hard pine, native to Central America, Cuba, Bahamas, Turks and Caicos Islands. Both species are widely used for building, pulpwood and resin production. *P. elliottii* × *P. caribaea* hybrid was firstly bred in Australia in 1955 and exhibited better growth traits than either parent while combining several complementary characteristics of the parents (Nikles, 1995). In China, slash pine and Caribbean pine were introduced to Guangdong in 1933 and 1964, respectively. Since the early 1990s, more than 600 full-sib families of *P. elliottii* × *P. caribaea* were introduced from the Hongling Seed Orchard to the Guangdong Academy of Forestry. Currently, 26 F_1 families were selected with average annual growth of 1 m height, 1.7 cm diameter at breast height (dbh), and 0.018 5 m^3 volume, and growth rate 100% - 240% higher than that of *P. elliottii* and 10% - 70% higher than that of *P. caribaea* var. *hondurensis*. In addition, a comparative analysis confirmed that the growth performance of superior selected hybrid pedigrees is similar to that of F_1 hybrids bred in Australia (Zhao et al, 2009) and the selections exhibited many superior characteristics, such as fast growth rate, straight trunk, perfect branching pattern, and high yield of oleoresin. Often there is a reduction in genetic variation because of inbreeding and decrease in effective population size during the process of artificial selection. Therefore, there is a need to ascertain the degree of genetic variation and genetic differentiation between hybrids of superior families to aid selection during breeding and promote genetic diversity among hybrid families. Cross-breeding is the main method for enhancing genetic and phenotypic variation from which new varieties may be selected. However, because phylogenetic relationships are often uncertain, the parents in crosses may have a close genetic relationship, and consequently the hybrid progeny may show low genetic diversity. Therefore, it is essential to reveal the phylogenetic relationships between the parents and the hybrids. In the assessment of genetic diversity of pines, attention has been focused on populations (Hamelin et al, 1995; Szmidt et al, 1996; Lerceteau & Szmidt, 1999; Mariette et al, 2001; Shao et al, 2007; Zhou et al, 2008; Dvorak et al, 2009; Ábrahám et al, 2010), seed sources (Feng et al, 2001; Shui et al, 2005; Yang et al, 2005), seed orchards (Ai et al, 2006; Zhang et al, 2008), and hybrids (Tang et al, 2003; Zhang et al, 2011).

Inter-simple sequence repeat (ISSR)-PCR is a technique to generate multilocus markers. ISSR markers arehighly polymorphic, and are useful in studies on genetic diversity, phylogenetic relationships, gene tagging, genome mapping, and evolutionary biology (Godwin et al, 1997; Reddy et al, 2002). In the present study, ISSR markers were used to reveal the genetic variation, relationship, differentiation and gene flow among different *P. elliottii* × *P. caribaea* var. *hondurensis* families.

1 Materials and Methods

1.1 Plant material

Seeds of *P. elliottii* × *P. caribaea* var. *hondurensis* from eight full-sib families-B102 × H7, B118 × Q22,

B118 × R6, B2 × CM64, B2 × H3, B2H7 × S95-20, B97H3-2 × S93-15 and B106H3 × EHA01 were collected in South China. The B102 × H7, B118 × Q22, B118 × R6, B2 × CM64, and B2 × H3 families were the F_1 generation and the other families were the F_2 generation. The seedlings were planted in Guangdong Academy of Forestry in 2010. A total of 60 hybrid offsprings were selected randomly from each family. Fifteen hybrid offsprings were mixed as samples, and a total of 32 samples. One-year-old needles were collected in March 2011 and stored at −20 ℃ for DNA isolation.

1.2 DNA isolation

Genomic DNA was isolated from the needles using the modified CTAB method of Li (2010). Ground fresh tissue (0.2 g) was suspended in 800 μL CTAB and incubated at 65 ℃ for 30−60 min. The suspension was centrifuged at 11 000 r · min^{-1} for 10 min and the supernatant was extracted twice with 600 μL chloroform and precipitated with double volumes of ethanol at −20 ℃. The DNA pellet formed after centrifugation at 10 000 r · min^{-1} for 10 min was washed twice with 75% ethanol. The DNA was then suspended in 100 μL H_2O. Equal amounts of DNA from fifteen individuals of the same family were mixed.

1.3 ISSR-PCR

PCR amplification was performed in a 25 μL reaction volume. The mixture contained 40 ng template DNA (2 μL DNA stock), 2.5 μL of 10 mmol · L^{-1} Tris-HCl buffer, 500 μmol · L^{-1} of each dNTP (1 μL stock), 1.0 U Taq DNA polymerase, and 1 μL of 10 μmol · L^{-1} primers. To make up the volume to 25 μL, 2.5 μL of sterile H_2O was added to each reaction mixture. Ten ISSR primers, named UBC811, UBC817, UBC818, UBC830, UBC846, UBC850, UBC851, UBC873, UBC881 and UBC891, were selected for the analysis. Amplification was carried out in a PTC-200 thermocycler with the following program: 4 min of denaturation at 94 ℃, then 35 cycles of three steps, which were 50 s of denaturation at 94 ℃, 50 s annealing at a temperature specific for each primer (Table 1), and 2 min of elongation at 72 ℃, with a final elongation step of 7 min at 72 ℃ and storage at −20 ℃. The PCR products were separated in a 2.0% agarose gel and fragments sizes were estimated with the DL 2000 ladder marker. A digital image was captured and analyzed using an ultraviolet analysis imaging system.

Table 1 Sequences and amplification band numbers of ten selected primers

Primer	Sequence 5′→3′	No. of amplified bands (No. polymorphic bands)
UBC811	GAG AGA GAG AGA GAG AC	6(4)
UBC817	CAC ACA CAC ACA CAC AA	4(2)
UBC818	CAC ACA CAC ACA CAC AG	7(1)
UBC830	TGT GTG TGT GTG TGT GG	7(7)
UBC846	CAC ACA CAC ACA CAC AGT	7(4)
UBC850	GTG TGT GTG TGT GTG TTC	3(1)
UBC851	GTG TGT GTG TGT GTG TYG	6(4)
UBC873	GAC AGA CAG ACA GAC A	11(7)
UBC881	GGG TGG GGT GGG GTG	5(3)
UBC891	CAT TGT GTG TGT GTG TG	4(1)
Total		60(30)

1.4 Data analysis

Amplified DNA banding patterns generated by ISSR-PCR were scored as (1) for presence or (0) for absence. Using Popgene 32 software, percentage of polymorphic loci, percentage band polymorphism (*PBP*), Shannon's information index (I), observed number of alleles (na), effective number of alleles (ne), gene differentiation coefficient (G_{st}), gene flow (N_m), Nei's genetic distance, and Nei's unbiased gene diversity (h), which is equivalent to expected heterozygosity (H_E) of a population, were calculated. A cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) algorithm was performed based on Nei's genetic distances with NTYSIS 2.01 software.

2 Results and Analysis

2.1 ISSR profiles

For the 32 mixed samples of *P. elliottii* × *P. car-*

ibaea var. *hondurensis* from the eight full-sib families, a total of 60 replicated bands were amplified with the ten primers, of which 30 were polymorphic. The number of bands produced ranged from two to eleven per reaction, with an average six. The size of the amplified fragments ranged from 250−1 800 bp.

2.2 Genetic variation

At the family level, the percentage of polymorphic loci (*PBP*) was 50.00%, whereas that of a single family ranged from 3.33%−23.33%, with an average of 13.75%. At the family level, the average effective number of alleles per locus was 1.083 6. The average expected heterozygosity was estimated to be 0.049 4 within populations (*h*). Shannon's index (*I*) ranged from 0.021 6−0.129 4, with an average of 0.074 1 at the family level. Among the eight families investigated, the B02 × CM64 family revealed higher variability (*PPB*, 23.33%; *na*, 1.1508; *ne*, 0.087 2; *I*, 0.129 4), whereas the B02 × H3 family revealed the lowest variability (*PPB*, 3.33%; *na*, 1.028 5; *ne*, 0.015 2; *I*, 0.021 6; Table 2).

Table 2　Genetic variability among eight families of *Pinus elliottii* × *P. caribaea* var. *hondurensis*

Familie	*na*	*ne*	*h*	*I*	*PBP* (%)
B102 × H07	1.050 0	1.040 2	0.022 1	0.031 7	5.00
B118 × Q22	1.150 0	1.095 6	0.055 9	0.083 1	15.00
B118 × R06	1.200 0	1.108 8	0.065 9	0.100 8	20.00
B02 × CM64	1.233 3	1.150 8	0.087 2	0.129 4	23.33
B02 × H3	1.033 3	1.028 5	0.015 2	0.021 6	3.33
B02H07-1 × S95-20-2	1.233 3	1.132 4	0.079 7	0.120 9	23.33
B97H03-2 × S93-15-1	1.116 7	1.067 1	0.040 6	0.061 5	11.67
B106H03-2 × EHA01-1	1.083 3	1.045 4	0.028 4	0.043 4	8.33
Family level	1.137 5	1.083 6	0.049 4	0.074 1	13.75

Note: ***na***. Observed number of alleles per locus; ***ne***. Effective number of alleles per locus; ***h***. Expected heterozygosity; ***I***. Shannon's infor-mation index; ***PBP***. Percentage of polymorphic loci.

2.3 Genetic relationship and cluster analysis

On the basis of analysis with Popgene 32 software, the genetic identity coefficient among the eight families ranged from 0.655 1 − 0.954 2. The minimum genetic distance was observed between B118 × Q22 and B118 × R6, and the maximum was observed between B102 × H7 and B97H3-2 × S93-15. The result suggested that there was a high genetic similarity among *P. elliottii* × *P. caribaea* var. *hondurensis* families (Table 3).

Nei's genetic identity and distance analysis among the eight families of *P. elliottii* × *P. caribaea* var. *hondurensis* showed that the highest Nei's genetic distance (0.423 0) was between B102 × H7 and B97H3-2 × S93-15, whereas the lowest value (0.046 9) was between B118 × Q22 and B118 × R6 (Table 3). A dendrogram representing relationships among the eight families was constructed using the UPGMA clustering method (Fig. 1). The eight families were divided into three groups with a genetic distance of 0.151. One group included B118 × Q22, B118 × R6, B102 × H7 and B106H3 × EHA01. Another group included B2 × CM64, B2 × H3 and B2H7 × S95-20. B97H3-2 × S93-15 alone made up the third group. These results indicated that families with the same female parent were genetically similar as thus a partial female parent genetic effect was apparent.

2.4 Genetic differentiation and gene flow

Analysis with Popgene 32 software of the genetic differentiation of *P. elliottii* × *P. caribaea* var. *hondurensis* families indicated that the majority of the genetic variation was represented between the families, accounting for 74.35% of the total family-level variation, whereas 25.65% of the total variation occurred within families. The genetic differences among the eight families were high and relatively independent of strain. Gene flow (N_m) is a major factor impacting on the genetic structure and genetic differentiation among families. The gene flow among the *P. elliottii* × *P. caribaea* var. *hondurensis* families was 0.172 5, which indicated there was strong genetic differentiation among families.

Table 3 Nei's genetic identity and distance (above and below the diagonal, respectively) among eight families of *Pinus elliottii* × *P. caribaea* var. *hondurensis*

Family ID	B102 × H7	B118 × Q22	B118 × R6	B2 × CM64	B2 × H3	B2H7-1 × S95-20	B97H3-2 × S93-15	B106H3-2 × EHA01
B102 × H7	—	0.946	0.936 7	0.857 9	0.812 5	0.854 1	0.655 1	0.925 8
B118 × Q22	0.055 5	—	0.954 2	0.852	0.813 4	0.865 8	0.685 2	0.911 3
B118 × R6	0.065 4	0.046 9	—	0.921 6	0.837 6	0.889 3	0.668 2	0.874 4
B2 × CM64	0.153 3	0.160 2	0.081 6	—	0.895 5	0.944 6	0.660 1	0.876 3
B2 × H3	0.207 7	0.206 6	0.177 2	0.110 4	—	0.914 2	0.669 9	0.841 4
B2H7-1 × S95-20	0.157 7	0.144 1	0.117 4	0.057	0.089 7	—	0.745 6	0.887 9
B97H3-2 × S93-15	0.423 0	0.378	0.403 2	0.415 4	0.400 7	0.293 6	—	0.677 4
B106H3-2 × EHA01	0.077 1	0.092 9	0.134 2	0.132	0.172 7	0.118 9	0.389 5	—

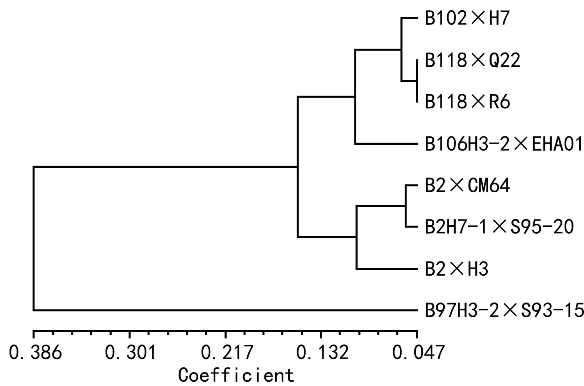

Fig. 1 Dendrogram showing the genetic distance among eight families of *Pinus elliottii* × *P. caribaea* var. *hondurensis*

3 Discussion and Conclusion

Compared with other *Pinus* species, the *PBP* (50.00%) at the family level for *P. elliottii* × *P. caribaea* is much lower than that reported for *P. massoniana* (80.37%; Zhu et al, 2007) and *P. koraiensi* (61.17%) (Feng et al, 2007), and is only higher than that of families of *P. taiwanensis* (*PBP* = 24.10%)(Tang et al, 2 003). Similarly, the *I* value (0.074 1) at the family level for *P. elliottii* × *P. caribaea* var. *hondurensis* is lower than that of *P. massoniana* (0.355 8) and *P. koraiensi* (0.267 4), and is only higher than that of families of *P. taiwanensis* (0.028 6). The study by Tang et al (2003) showed that the levels of genetic diversity among ten families of *P. taiwanensis* were low. Similarly, in the present study a low level of genetic diversity was observed among *P. elliottii* × *P. caribaea* var. *hondurensis* families. However, families generated by artificial pollination are relatively independent of strain and may have certain genetic differentiation.

Analysis of molecular variation indicated high genetic variation among *P. elliottii* × *P. caribaea* var. *hondurensis* families rather than within families (G_{st} = 0.743 5). This might be caused by artificial selection rather than pollen pollution. The N_m of *P. elliottii* × *P. caribaea* var. *hondurensis* was 0.172 5, which indicated gene flow among families was limited. Wright (1931) proposed that gene can flow among the populations. At $N_m>1$ populations would be homogenized, at $N_m<1$ populations may be strongly differentiated, and at $N_m>4$ populations would become a random unit. On the basis of these criteria, strong genetic differentiation among the *P. elliottii* × *P. caribaea* var. *hondurensis* families is indicated. Controlled pollination of the *P. elliottii* × *P. caribaea* var. *hondurensis* hybrids and parental species accessions resulted in limited gene flow among families. The low inter-family gene flow may be related to the high genetic heterozygosity of slash pine and caribbean pine. The results will be helpful for se-

lective breeding of *P. elliottii* × *P. caribaea* var. *hondurensis hybrids*.

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