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Crossbreeding of *Phyllostachys* species (Poaceae) and identification of their hybrids using ISSR markers

X.C. Lin[1], Y.F. Lou[1], J. Liu[2], J.S. Peng[3], G.L. Liao[3] and W. Fang[1]

[1]The Nurturing Station for the State Key Laboratory of Subtropical Silviculture, Zhejiang Agriculture and Forestry University, Zhejiang, China
[2]Forest and Water Conservancy Bureau of Yuhang District of Hangzhou, Zhejiang, China
[3]Jiangxi Forestry Academy of Sciences, Jiangxi, China

Corresponding author: W. Fang
E-mail: zjfufw@163.com

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ABSTRACT. Crossbreeding is an efficient means to increase production and quality in plants; however, hybridization is seldom reported in bamboo. We crossbred two bamboo species *Phyllostachys kwangsiensis* (female parent) and *Phyllostachys bambusoides* (male parent) for the first time, and obtained suspected bamboo hybrids. We identified two bamboo hybrids from the above parents using PCR/ISSR. We concluded that ISSR markers are useful to identify bamboo hybrids, and that breeding between bamboo species is possible and useful.

Key words: Crossbreeding; Suspected hybrid; Bamboo; ISSR; Hybrid identification

INTRODUCTION

The bamboos are a family of woody grasses, including 88 genera and more than 1400 species; there are 34 genera and 534 species in China (Wu and Raven, 2006). With characteristics of short rotation, and marketable culms, bamboos are the fastest growing plants (Kassahun, 2000; Franklin, 2006). Besides young edible shoots and culms used for timber, furniture, handicrafts, and raw material for pulping, bamboo is an efficient agent for preventing soil erosion and conserving soil moisture (Christanty et al., 1996, 1997; Mailly et al., 1997; Kleinhenz and Midmore, 2001). In recent years, worldwide interest in bamboo as a source of biofuel or bioenergy has rapidly increased (Scurlock, 2000).

Crossbreeding and gene transformation are two important and efficient approaches for increasing plant productivity and quality. However, there are no scientific reports on gene transformation in bamboos because bamboo gene transformation depends on a stable and efficient regeneration system, which is not yet completely defined or established (Zhuo and Liu, 2004; Lu et al., 2009). In addition, bamboo flowering is unpredictable and occurs at long intervals; bamboos usually die *en masse* after flowering. As a result, bamboo crossbreeding is difficult to accomplish. However, several bamboo hybrids have been generated by crossbreeding *Bambusa pervariabilis* with *Dendrocalamus latiflorus*, *D. hamiltonii* with *D. latiflorus*, *B. textilis* with *D. latiflorus*, *B. pervariabilis* with *D. latiflorus* or *B. textilis* (Zhang and Cheng, 1980, 1986; Zhang, 2000; Wang et al., 2005), *Pleioblastus simonii* with *Phyllostachys violascens*, *Sasa tokugawana* with *S. borealis*, and *Sinobambusa tootsik* with *Pl. distichus* (Lu et al., 2009).

A reliable method to identify bamboo hybrids is indispensable for controlled breeding. The traditional method for hybrid bamboo identification, which is based on morphological characteristics, is usually affected by environmental factors, and it is difficult to identify hybrids at an early stage. Molecular markers, such as RFLP, SSR, ISSR, and AFLP, used to detect DNA polymorphism, are currently widely used for the identification of artificial and natural hybrids in many plants (Wolfe et al., 1998; Rajora and Rahman, 2003; Ruas et al., 2003; Shasany et al., 2005). We crossbred two bamboo species, *Phyllostachys kwangsiensis* (female parent) and *Ph. bambusoides* (male parent), for the first time in Jiangxi province, and obtained three suspected bamboo hybrids.

MATERIAL AND METHODS

Crossbreeding

The female parent, *Ph. kwangsiensis*, and the male parent, *Ph. bambusoides*, were cultivated in Xingguo, Jiangxi province. Pollen was collected from anthers picked from the male parent. Before fertilization, stamens were removed from florets on the female parent. The pollen was then sprayed on the stigmas of florets of the female parent. The pollinated florets were labeled, and seeds were collected after three months.

Hybrid identification

Seeds obtained from the above crossbreeding were planted in soil, and three sus-

pected bamboo hybrids were obtained. Fresh and clean young leaf samples were collected from the suspected bamboo hybrids as well as from their parents. The leaves were then washed with water and their surface sterilized, as previously described (Lin et al., 2009).

Total DNA was isolated from each leaf sample (0.5-1.0 g) using the CTAB (cetyltrimethylammonium bromide) method, as previously described (Lin et al., 2009). DNA concentration in each sample was determined using a NanoDrop1000 spectrophotometer (Thermo Scientific, USA) and DNA purity was verified by electrophoresis on a 0.8% agarose gel in 1X TAE buffer. Each DNA sample was diluted to a working concentration of 50 ng/μL.

Inter-simple sequence repeat (ISSR) primers (Table 1) were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Each amplification reaction in a 20-μL volume consisted of 60 ng genomic DNA, 10 mM primers, 0.2 mM dNTPs, 1.0 U Taq polymerase, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, and 2% deionized formamide. All amplifications were performed with a 9700 polymerase chain reaction (PCR) thermocycler (Applied Biosystems, USA) with initial denaturing at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 50-55°C for 45 s and 72°C for 90 s, and a final elongation at 72°C for 7 min. PCR products were separated by electrophoresis on a 1.6% agarose gel in 1X TAE buffer, and then stained with ethidium bromide for visualization and photographed under Image Master^R VDS (Amersham Phamacia, USA).

Only clear and intense DNA fragments on the gel were scored as presence (1) or absence (0). Genetic similarity was determined using the software package NTSYS-PC V2.10E (Rohlf, 2000) based on Dice's coefficient (Dice, 1945) with ISSR data, and the genetic similarity (GS) index was converted to the genetic distance (GD) index using its complement (GD = 1 - GS). The dendrogram was constructed by UPGMA (unweighted pair-group method with arithmetic mean) based on the similarity index.

RESULTS

We collected hybrid seeds from the female parent plant three months after crossbreeding, and planted these seeds in soil. We obtained three suspected bamboo hybrids crossbred between the bamboo species *Ph. kwangsiensis* (female parent) and *Ph. bambusoides* (male parent).

To determine genetic relationship between the above three bamboo hybrids and their parents, we used the PCR/ISSR technique with eight different primers (Table 1). Based on the PCR/ISSR results, these three hybrids were genetically related to their parents. A representative ISSR fingerprint is shown in Figure 1, but the other ISSR fingerprint data are not shown. In the band pattern shown in Figure 1, the hybrids each share some bands with their parent, but also display their own bands that are not shared with the parents. Hybrid H1 shared 18 bands with both parents, 20 bands with the female parent, and 11 bands with the male parent, and displayed 5 unshared bands (Table 2). Similarly, hybrid H2 shared 18 bands with both parents, 8 bands with the female parent, and 17 bands with the male parent, and had 6 unshared bands. Hybrid H3 shared 20 bands with both parents, 28 bands with the female parent, and 1 band with the male parent; it had 1 unshared band.

Table 1. List of inter-simple sequence repeat (ISSR) primers used for determination of bamboo hybrids.

Primer	Sequence (5'→3')
ISSR-3	$(AC)_8TT$
ISSR-23	$(AC)_8TA$
ISSR-33	$(AG)_8AT$
ISSR-34	$(AG)_8AA$
ISSR-35	$(AG)_8TA$
ISSR-56	$(AG)_8TT$
ISSR-74	$(ACTG)_4$
ISSR-76	$(AGTC)_4$

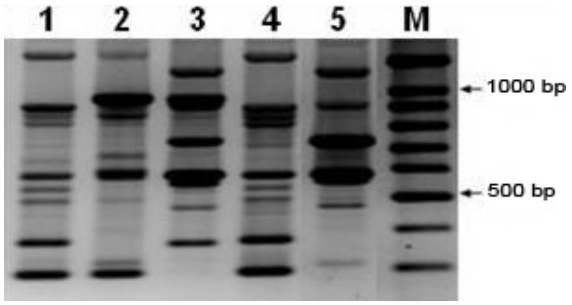

Figure 1. Inter-simple sequence repeat (ISSR) profile of three suspected hybrids and their parents generated by the primer ISSR-3. *Lane 1 = Phyllostachys kwangsiensis* (female parent); *lanes 2-4* = hybrids H1, H2, and H3, respectively; *lane 5 = Ph. bambusoides* (male parent); *lane M* = DNA ladder (marker).

Table 2. Statistical analysis of ISSR bands of suspected bamboo hybrids.

Suspected hybrid	Bands shared with female parent	Bands shared with male parent	Bands shared with both parents	Bands for hybrid only
H1	20 (37.0%)	11 (20.4%)	18 (33.3%)	5 (9.3%)
H2	8 (16.3%)	17 (34.7%)	18 (36.7%)	6 (12.2%)
H3	28 (56.0%)	1 (2.0%)	20 (40.0%)	1 (2.0%)

Data are reported as number with percent in parentheses.

To further determine the genetic relationships between these three hybrids and their parents, we measured genetic distances among these five bamboos. Genetic distances among the species ranged from 0.03 to 0.60, and the genetic distance between the two parents was 0.60 (Table 3). The genetic distances between hybrid H1 and the female parent and between hybrid H2 and the female parent were 0.26 and 0.47, respectively, and both genetic distances were less than the maximum genetic distance between the two parents. Similarly, the genetic distances between hybrid H1 and the male parent and between hybrid H2 and the male parent were 0.47 and 0.33, respectively, and both genetic distances were also less than the maximum genetic distance between the two parents. These data demonstrate that hybrids H1 and H2 are truly crossbreeds between the bamboo species *Ph. kwangsiensis* and *Ph. bambusoides*. However, the genetic distance between hybrid H3 and the female parent was only 0.03, while the genetic distance between hybrid H3 and the male parent was 0.60,

which is the maximum genetic distance between two independent species. These data suggest that hybrid H3 is likely an intraspecies offspring of the species *Ph. kwangsiensis* instead of a hybrid between *Ph. kwangsiensis* and *Ph. bambusoides*. Abundant genetic variation was observed between hybrids and their parents (Figure 2 and Table 3). This demonstrates that breeding between bamboo species is viable.

Table 3. Genetic distances of suspected bamboo hybrids and their parents.

	Phyllostachys kwangsiensis (female parent)	H1	H2	H3	*Phyllostachys bambusoides* (male parent)
Phyllostachys kwangsiensis	0.00				
H1	0.26	0.00			
H2	0.47	0.48	0.00		
H3	0.03	0.27	0.49	0.00	
Phyllostachys bambusoides	0.60	0.47	0.33	0.60	0.00

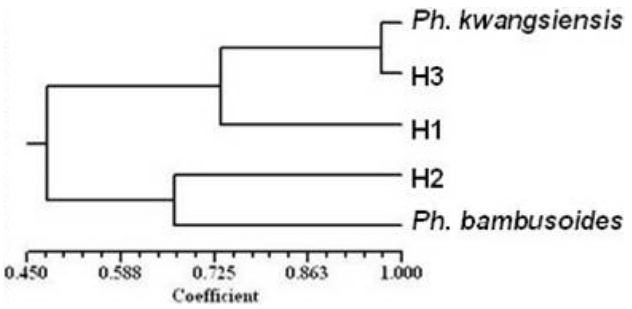

Figure 2. UPGMA dendrogram of three suspected hybrids (H1, H2, H3) and their parents (*Phyllostachys kwangsiensis* and *Phyllostachys bambusoides*) generated by the ISSR data.

DISCUSSION

Traditionally, morphological and cytological methods are widely used to identify hybrids, but identification of hybrids by these methods is ambiguous, tedious, and time-consuming, particularly in bamboo, and it is incapable of quantification of genetic relationships, such as determination of genetic distances between the species. Bamboos rarely blossom, so that the morphology of their culm sheath is widely used for their classification (Wu and Raven, 2006). However, it is very difficult to identify bamboo hybrids using morphology of the culm sheath, which also appears only during a short period of time. It is difficult to identify bamboo hybrids by examining numbers of chromosomes in cells because bamboos are divided into two groups, monopodial and sympodial. Monopodial bamboos usually have 48 chromosomes, while most of the sympodial bamboos have 72 chromosomes (Li et al., 1999, 2001).

In recent years, molecular markers, such as RFLP, SSR, ISSR, and AFLP, which are usually used to detect DNA polymorphism, were also used for identification of hybrids (Rajora and Rahman, 2003; Ruas et al., 2003; Shasany et al., 2005). Simple sequence repeat (SSR) markers are very advantageous for hybrid identification, but the development of SSR markers is time-consuming and costly, and it requires known corresponding genomic information.

Compared to SSR markers, ISSR primers, which are composed of a microsatellite sequence anchored by 2-4 arbitrary nucleotides at the 3' or 5' end, are easy to synthesize. They target SSRs (microsatellites) that are abundant throughout eukaryotic genomes, but do not require known DNA sequences. For these reasons, ISSR markers have been widely used for hybrid identification (Zietkiewicz et al., 1994; Reddy et al., 2002).

We used the ISSR technique with eight different primers to identify three suspected bamboo hybrids. We found that only two hybrids were authentic hybrids crossbred from the bamboo species *Ph. kwangsiensis* and *Ph. bambusoides*. The other was likely an intraspecies offspring of *Ph. kwangsiensis*. However, it cannot be definitely excluded that this progeny is also a hybrid, because hybrids often have preference for one of their parents (Duan et al., 2002; Chen and Zhang, 2004). However, this possibility needs other approaches for verification. Hybrid H1 exhibited genetic preference for the female parent, while hybrid H2 exhibited genetic preference for the male parent (Figure 2). Taken together, these data suggest that the ISSR technique is beneficial for the identification of bamboo hybrids and that breeding between bamboo species is resourceful.

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