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Original Article

Species identification of economic bamboos in the genus *Dendrocalamus* using SCAR and multiplex PCR

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Abstract

Taxonomic and systematic studies of bamboos are traditionally based on floral morphology, but this can lead to difficulties in identification because of the irregular reproductive cycle of the bamboos. To overcome such problems several molecular-marker approaches have been used. In this study, eight species of the woody bamboos belonging to the genus *Dendrocalamus* were employed. For each species, DNA samples of 20 individual plants from different localities were isolated and then pooled to make eight bulks of DNA. Fifty RAPD primers were used to screen all bulked DNA samples. Only five primers yielded consistent and reproducible RAPD band patterns across all 160 individuals. The amplicons were present among five species of *Dendrocalamus*, but were absent in the other three species. They were cloned, sequenced and subsequently, five pairs of SCAR primers were designed. All SCAR primers were combined in multiplex PCR reactions to unequivocally discriminate five species of *Dendrocalamus*.

Keywords: bamboo, *Dendrocalamus*, multiplex PCR, RAPD, SCAR

1. Introduction

Bamboos are classified in the subfamily Bambusoideae which consists of 1,439 described species in 116 genera (Bamboo Phylogeny Group, 2012). The bamboos are recognized as one of the most important natural resources in Southeast Asia. They have been widely used in paper industries, medicines, traditional handicrafts as well as construction industries (Gami *et al.,* 2015).

The genus *Dendrocalamus* Nees (woody bamboos) belonging to the tribe Bambuseae comprises about 50 species that are distributed from southern China to India, Myanmar, Thailand, Cambodia, Laos PDR, Vietnam, Malaysia and Papua New Guinea (Ohrnberger, 1999). Thirteen species have been reported to occur in Thailand (Sungkaew, 2008). These woody bamboos flower at irregular intervals (ranging from 3 to 120 years) and rarely set seed (Janzen, 1976). This causes problems in traditional taxonomy and identification of bamboo species based on floral morphology. Consequently, bamboo species identification has been shifted from reproductive to vegetative characters (Bhattacharya *et al.,* 2006; Sharma *et al.,* 2008). These vegetative traits however, are likely to be influenced by ecological factors.

In recent years, the application of molecular technology for systematic and taxonomic studies of bamboos has become predominant. These techniques include random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP), sequence characterized amplified region (SCAR) and DNA sequencing of different loci. These DNA-based markers provide precise information on genetic diversity (Desai *et al.,* 2015; Goyal *et al.,* 2015; Hodkinson *et al.,* 2000; Shalini *et al.,* 2013; Tian *et al.,* 2012; Waikhom *et al.,* 2012), species-specific marker (Das *et al.,* 2005) as well as phylogenetic relationships of the bamboos (Goh *et al.,* 2010, 2013; Sungkaew *et al.,* 2009). The results of these studies are necessary both for breeders to ensure protection of intellectual property rights and also for propagators and consumers.

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Moreover, they are essential in decision-making for conservation and sustainable management of the bamboo species.

This study aimed to identify *Dendrocalamus* species present in Thailand using several PCR-based techniques, including RAPD, SCAR and multiplex PCR. It is essential to develop robust SCAR primers based on sequences of cloned RAPD fragments that were used effectively in multiplex PCR reactions to discriminate the species of *Dendrocalamus* under study.

2. Materials and Methods

2.1 Plant material

Fresh, healthy leaf samples that were free of any visible surface contaminants were obtained from eight taxa of *Dendrocalamus*, namely *D. asper*, *D. brandisii*, *D. copelandii*, *D. giganteus*, *D. hamiltonii*, *D. latiflorus*, *D. membranaceus*, and *Dendrocalamus* sp., which are economically important. The *Dendrocalamus* sp. used under study was an unidentified species having the common Thai name as "Pai Sang Mon". The leaf samples were collected from four sampling sites, including Queen Sirikit Park (Bangkok Metropolitan Administration) and other well established commercial bamboo plantations in Kanchanaburi, Prachinburi, and Chonburi provinces (Figure 1). *Dendrocalamus* species cultivated as the living plant collection at Queen Sirikit Park have been officially identified by bamboo specialists. Our previous molecular study of these bamboos provided nucleotide sequences of two regions of nuclear DNA: (i) the internal transcribed spacer (ITS) region and (ii) the granule-bound starch synthase (*GBSSI*) gene (unpublished data). The nucleotide sequences of representative members of these bamboos have been deposited in the GenBank database with the accession numbers KY-296045–KY296060. Other ITS- and *GBSSI*-derived sequences from published data of *Dendrocalamus* species as well as our sequences were used as references to determine species boundaries of the *Dendrocalamus* samples obtained from Kanchanaburi, Prachinburi, and Chonburi provinces. These molecular data were employed, in addition to morphological taxonomy, as a means to authenticate the species under study.

In order to cover a range of genetic diversity present in each species, five fresh leaf samples from different clumps were collected in each site. Therefore, for eight species studied 160 samples were obtained.

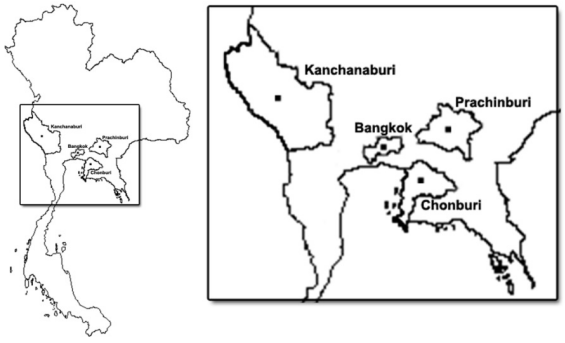

Figure 1. Sampling sites for *Dendrocalamus* species.

2.2 DNA extraction

The leaf samples were surface sterilized with 1% sodium hypochlorite, cut into small pieces, and ground to a fine powder using liquid nitrogen. Total genomic DNA was extracted from leaf tissues of each species using DNeasy Plant Mini Kit (QIAGEN, Germany).

For all 160 DNA samples obtained, the concentration and purity of the individual samples were determined using a spectrophotometer (UV-1700 PharmaSpec, Shimadzu). Each sample was diluted to a concentration of 100 ng/µL. Twenty DNA samples belonging to each species were pooled to make a bulked DNA sample and a total of eight bulked DNA samples were stored at -20 °C until use.

2.3 HAT-RAPD amplification

In order to increase the reproducibility of the RAPD technique, a high annealing temperature (HAT)-RAPD was used for amplification of the genomic DNA. Fifty decamer primers from RAPD primer kits (NAPS Unit, University of British Columbia Biotechnology Laboratory) were employed. All bulked DNA samples from the eight species were used for PCR reactions. Each amplification was carried out in a final volume of 15 µL containing 25 ng DNA template, 150 µM of each dNTP, 2 mM $MgCl_2$, 0.6 µM primer, and 0.4 U TopTaq DNA Polymerase (QIAGEN, Germany). In a thermal cycler (Eppendorf Mastercycler Gradient 5331), after 2 min of initial heating at 94 °C, 35 amplification cycles were conducted under the following conditions: 94 °C for 1 min, 48 °C for 1 min, and 72 °C for 2 min with a final extension reaction of 72 °C for 7 min. For each round of the PCR amplification, a negative control was prepared with all required PCR components except the DNA template. PCR amplification products were analyzed by gel electrophoresis on 1.5% (w/v) agarose gel and stained with ethidium bromide. The gels were visualized and photographed under UV light. To ensure the reproducibility of polymorphic bands obtained from each RAPD primer, the PCR amplifications were carried out twice. In addition, the presence of each polymorphic band was verified against individual plants of different species. Only those polymorphic bands which appeared consistently across all individuals were considered.

2.4 Cloning and sequencing of informative RAPD fragments

All reliable polymorphic RAPD bands observed among different species were excised from the gels. They were purified with QIAquick Gel Extraction Kit (QIAGEN, Germany). The purified products were subsequently ligated into the pGEM-T Easy Vector System II (Promega, USA) following the manufacturer's instructions, and then introduced into the *Escherichia coli* strain JM 109 competent cells via chemical transformation. A number of white colonies were selected from Luria-Bertani (LB) agar plates containing 100 µg/mL ampicillin, 40 µg/mL X-gal, and 1 mM IPTG. The plasmids were isolated using the alkaline lysis method (Sambrook & Russell. 2001). The sizes of DNA inserts were confirmed by *Eco*RI restriction digestion followed by separation on 1% (w/v) agarose gel. The bacterial colonies

with desired DNA inserts were cultured in LB broth containing 100 µg/mL of ampicillin. The recombinant plasmids were isolated using QIAGEN Plasmid Mini Kit (QIAGEN, Germany). The DNA inserts were sequenced at Bio Basic Canada Inc. using the T7 and SP6 promoter primers for forward and reverse directions.

2.5 Conversion of RAPD markers to SCAR markers

To construct a SCAR marker, complete sequences of the RAPD marker obtained from different clones with the same insert size were aligned using the Clustal X version 2.0. program (Larkin *et al.,* 2007). The positions of the T7 and SP6 promoter primers were identified and then removed from the sequences to determine the actual length of the DNA insert. A pair of oligonucleotide primers was designed using Primer3 (Untergasser *et al.,* 2012).

Single PCR reactions were performed in a final volume of 25 µL containing 100 ng DNA template, 0.2 mM each of dNTP and 1.5 mM $MgCl_2$, and 0.66 µM each of forward and reverse primers and 1.25 U HotStarTaq DNA Polymerase (QIAGEN, Germany). The amplification included the following parameters: initial heating at 95 °C for 15 min, 30 cycles at 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min 30 sec, and a final extension at 72 °C for 2 min. The PCR products obtained were examined using 1% (w/v) agarose gel electrophoresis.

2.6 Multiplex PCR and validation of SCAR primers

In order to amplify species-specific fragments simultaneously, a combination of SCAR primer pairs was employed in a multiplex PCR. The PCR components were performed in a final volume of 25 µL containing 100 ng DNA template, 0.2 mM each of dNTP, 1.5 mM $MgCl_2$, and 0.8 µg/µL bovine serum albumin (BSA) and 0.66 µM each of forward and reverse primers for long products but 0.22 µM for short products and 1.25 U HotStarTaq DNA Polymerase (QIAGEN, Germany).

The PCR amplification steps involved an initial 15-min heat denaturation at 95 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min 30 sec, extension at 72 °C for 1 min 30 sec, and final extension at 68 °C for 15 min. The PCR amplicons were analyzed on 1% (w/v) agarose gel.

3. Results

3.1 Screening of differential RAPD bands

The amplification profiles of the total genomic DNA from eight bamboo species with 50 RAPD primers generated 255 fragments ranging in size from 0.2–2.0 kb. Out of these 50 random primers, only 15 produced distinct polymorphic bands. These potential primers were then used to evaluate the individual DNA samples from different pools of DNA. Five primers showed consistent and reproducible amplicons in all individuals of each species. The primers, NAPS197, NAPS305, NAPS311, NAPS328, and NAPS378, generated different sizes of RAPD amplicons, i.e. 1100, 600, 950, 800, and 500 bp, respectively, and were selected for further study. These amplicons were present among five species of *Dendrocalamus* (*Dendrocalamus* sp., *D. asper*, *D. copelandii*, *D. hamiltonii*, and *D. latiflorus*) but were absent in the other three species.

3.2 Cloning, sequencing and designing of SCAR primers

All five RAPD fragments, i.e. 1100, 600, 950, 800, and 500 bp, were purified and cloned. Several positive colonies containing the inserts of the same sizes were selected for sequencing. All the sequences obtained were edited and their actual lengths were revealed as 1041, 560, 905, 789, and 479 bp (Table 1). They were deposited in the GenBank database with the accession numbers KY296040–KY296044. Sequence comparison showed that these sequences did not have any similarity with other sequences deposited in any of the databases. Based on these sequenced RAPD fragments, five pairs of SCAR primers were designed. Their primer sites were located as shown in Figure 2. Single PCR reactions using the individual SCAR primer sets of Bam197F-Bam197R, Bam 305F-Bam305R, Bam311F-Bam311R, Bam328F-Bam328R, and Bam378F-Bam378R yielded the expected SCAR markers of 804, 502, 825, 681, and 402 bp, respectively (Table 2 and Figure 3).

Table 1. Five successful RAPD primers, actual lengths of amplicons and the presence of RAPD markers in *Dendrocalamus* species.

RAPD primer	Sequence (5' ---> 3')	Actual length of amplicon (bp)	Presence of RAPD marker
NAPS197	TCCCCGTTCC	1041	*Dendrocalamus* sp.
NAPS305	GCTGGTACCC	560	*D. asper* and *D. copelandii*
NAPS311	GGTAACCGTA	905	*D. asper*
NAPS328	ATGGCCTTAC	789	*Dendrocalamus* sp. and *D. latiflorus*
NAPS378	GACAACAGGA	479	*D. hamiltonii*

Figure 2. Nucleotide sequences of cloned RAPD fragments. Boldfaced nucleotides represent RAPD primers. Underlined nucleotides indicate SCAR primer pairs: (A) Bam197F-Bam197R, (B) Bam305F-Bam305R, (C) Bam311F-Bam311R, (D) Bam328F-Bam328R, (E) Bam378F-Bam378R.

Table 2. SCAR primers, their respective SCAR markers and the existence of SCAR markers in *Dendrocalamus* species.

RAPD primer	SCAR primer	Sequence (5' ---> 3')	SCAR marker (bp)	Existence of SCAR marker
NAPS197	Bam197F	GGCGTGGTCATAATGTGCTA	Bam197 (804)	*Dendrocalamus* sp.
	Bam197R	CCCGTTCCATCACTCAAGTT		
NAPS305	Bam305F	GCTGTCTTGGTTGTCCACAC	Bam305 (502)	*D. asper* and
	Bam305R	TGTGCTTGACCCTATCATCG		*D. copelandii*
NAPS311	Bam311F	CGTGTGTGGTGAACAGGGTA	Bam311 (825)	*D. asper*
	Bam311R	TGACTCGACTGTAAGCCACCT		
NAPS328	Bam328F	GCCACCCCTACAACAGTTACA	Bam328 (681)	*Dendrocalamus* sp.
	Bam328R	CCTTACCACACAACACATAC		and *D. latiflorus*
NAPS378	Bam378F	CGCCAAGGAGAAGGGAGT	Bam378 (402)	*D. hamiltonii*
	Bam378R	CGCATGCGGACTACCACT		

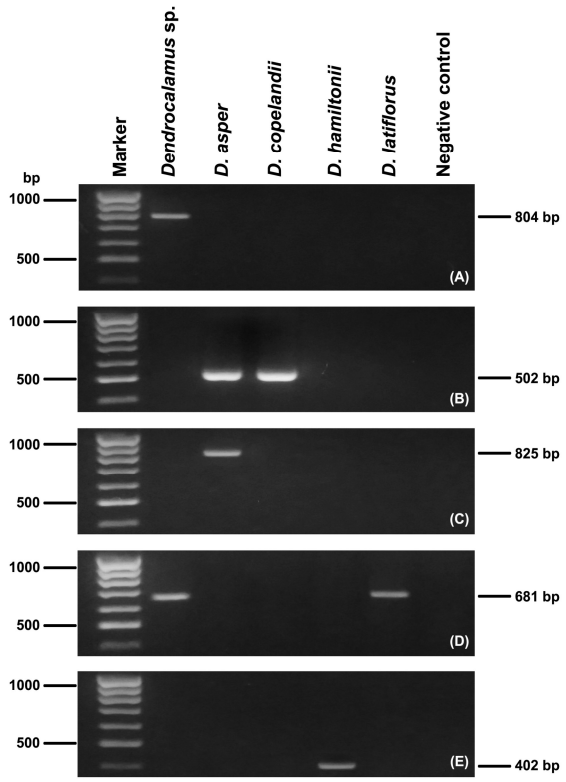

Figure 3. Electrophoretic profile of single PCR reactions using SCAR primer pairs: (A) Bam197F-Bam197R (B) Bam 305F-Bam305R (C) Bam311F-Bam311R (D) Bam328F-Bam328R and (E) Bam378F-Bam378R.

3.3 Multiplex PCR using SCAR primers

A combination of all five pairs of the SCAR primers was used in the multiplex PCR assay of five species of *Dendrocalamus*. The multiplex PCR electrophoretic profile showed that the SCAR markers could efficiently identify each species with their specific banding patterns (Figure 4).

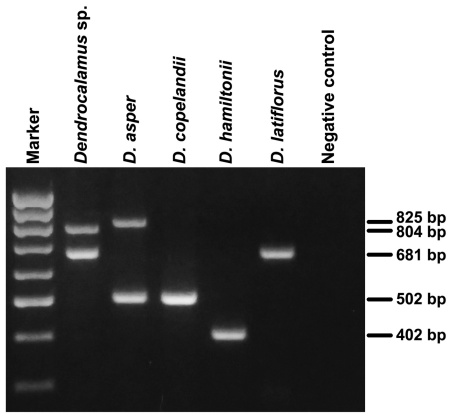

Figure 4. Multiplex PCR electrophoretic profile of five polymorphic SCAR markers.

4. Discussion

Characterization of bamboos is known to be difficult due to the lack of morphological differences and infrequent flowering. Thus, the traditional methods of identifying species by morphological characters are gradually being replaced by reliable DNA profiling.

Bamboo reproduction is mostly asexual by its rhizomes. The rhizomes produce offspring that are identical to the maternal plant and to each other. This reproduction system can affect the genetic structure of a population (Murawski & Hamrick, 1990). Based on their vegetative propagation low genetic diversity is expected within some bamboo populations where they form large clonal stands. The RAPD and ISSR analyses of the woody bamboos revealed that low genetic variation was observed in the populations of *Phyllostachys pubescens* (Lai & Hsiao, 1997), *Guadua angustifolia* (Marulanda *et al.,* 2002), and *Dendrocalamus giganteus* (Ramanayake *et al.,* 2007). In addition, Bhattacharya *et al.* (2006) and Yang *et al.* (2012) reported lower genetic diversity among the populations of *Bambusa tulda* and *D. membranaceus*. Such genetic uniformity within the species might be caused by the naturally long vegetative phase and low seed set over the life cycle which are typical features of the woody bamboos

(Zhang & Ma, 1990). Moreover, certain species have been subjected to severe reduction in their natural distribution (Yang *et al.,* 2012). As a result, only a few clones of individual species might act as the genetic donor leading to the low level of genetic variability both within and among populations.

The tribe Bambuseae is divided into temperate, neotropical, and palaeotropical groups. Phylogenetic analyses of the multi-gene regions indicated that Bambuseae was not monophyletic because the tribe Olyreae (herbaceous bamboos) was placed as a sister group only to the tropical Bambuseae and not to the whole tribe (Sungkaew *et al.,* 2009). In addition, within the tropical Bambuseae, *Dendrocalamus* formed a complex with *Bambusa* and *Gigantochloa* (Goh *et al.,* 2010, 2013). These three genera are very similar in morphology and it is difficult to separate them without flowers.

In this study, the identity of each species of *Dendrocalamus* was confirmed by the nucleotide sequences of the ITS region and *GBSSI* gene. Representative clones of five species, namely *D. asper*, *D. copelandii*, *D. hamiltonii*, *D. latiflorus*, and *Dendrocalamus* sp. obtained from different localities in the central part of Thailand were distinguished using molecular approaches. From a total of 50 arbitrary primers screened, only 15 (30%) were polymorphic. Of these 15 RAPD primers, only five (33.33%) generated accurate and consistent RAPD patterns for every sample used, while 10 primers showed non-reproducible results. This lack of reproducibility, which is one of the major disadvantages of the RAPD technique, limits the use of RAPD markers. To overcome this problem, the sequences of the amplified fragments of the five polymorphic RAPD primers were converted to SCAR markers. In their single PCR reactions, the SCAR primers produced the expected sizes of the amplicons, including (i) 804-bp fragment for *Dendrocalamus* sp., (ii) 502-bp fragments for *D. asper* and *D. copelandii*, (iii) 825-bp fragment for *D. asper*, (iv) 681-bp fragments for *Dendrocalamus* sp. and *D. latiflorus*, and (v) 402-bp fragment for *D. hamiltonii*.

The multiplex PCR reactions which were conducted in the present study consisted of five SCAR primer sets. As mentioned earlier, these primer sets produced distinct bands but only three pairs were unique for the three species of *Dendrocalamus*. The other two pairs yielded PCR products for more than one species. Thus, the PCR reactions with a combination of all five pairs of the primers were carried out. This approach would certainly be more cost-effective and less time-consuming compared to many rounds of the single PCR reactions. Several studies using the multiplex PCR to identify plant species and cultivars or strains of mushrooms included Polashock and Vorsa (2002), Moon *et al.* (2010) and Wu *et al.* (2010). Although these primer sets could distinguish all five species simultaneously, optimization of the parameters in the multiplex PCR was required. Initially, long amplification products (804 bp and 825 bp) were rather weak; therefore, we modified the multiplex PCR protocol based on the single PCR by increasing the final extension time from 2 min to 15 min, decreasing the annealing temperature from 56 °C to 55 °C, and decreasing the temperature for the final extension step from 72 °C to 68 °C. In addition, 0.8 µg/µL of BSA was added to increase the efficiency of the PCR, and the proportions of various primers were adjusted in the reaction with an increase in the amount of primers for weak loci and a decrease in the amount for strong loci. These parameters were adjusted according to Henegariu *et al.* (1997).

Our study provided the ready-to-use SCAR primers which generated discrete bands in the multiplex PCR assays compared to other PCR-based techniques such as ISSR and AFLP that normally produce a large number of amplified bands that can be difficult to analyze and are not specific to a particular species. DNA barcoding, which involves the use of short DNA sequences that can be amplified by PCR and sequenced, has proved to be useful in species diagnosis of some organisms. Nevertheless, it has created controversial issues among taxonomists. Decision-making for DNA regions to be used is essential, i.e. short DNA fragments (400–800 bp) should be reliable with true orthologous sequences, and should contain appropriate mutation rates for different taxonomic levels (reviewed in Ali *et al.,* 2014).

In conclusion, this study showed that the RAPD technique was rapid and efficient for the screening of polymorphism among the species of *Dendrocalamus*. However, this technique is known to be error-prone due to its sensitivity to changing conditions. Therefore, five sets of the SCAR primers were developed based on the sequences of the RAPD fragments. These primer sets, when incorporated in the multiplex PCR assay, were able to discriminate all five species effectively. To the best of our knowledge, this is the first report that emphasizes the relevance of molecular applications for the determination of *Dendrocalamus* species. Further studies are required to develop a full set of SCAR markers for the identification of all Thai *Dendrocalamus*.

Acknowledgements

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