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Genetic diversity and relationships within populations of *Dendrocalamus giganteus* Wall. ex Munro and *Ochlandra stridula* Moon ex Thwaites in Sri Lanka using RAPD

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Abstract: The selection of useful species or varieties of bamboos for planting requires an understanding of the heterogeneity within populations. Random Amplified Polymorphic DNA (RAPD) was used to study the genetic diversity within a population of *Dendrocalamus giganteus* and *Ochlandra stridula* in Sri Lanka. *D. giganteus*, introduced as a single plant to Sri Lanka in 1856, showed a relatively low genetic diversity of 0.092 ± 0.027. Although seeding in this species is rare, seed-raised plants showed the highest genetic distances and contributed significantly to the genetic diversity. The species is mainly propagated vegetatively and the presence of groups of individuals with no polymorphism indicated clones. *O. stridula*, an endemic annually flowering species, showed a relatively higher mean genetic distance of 0.446 ± 0.210.

Key words: *Dendrocalamus giganteus*, *Ochlandra stridula*, RAPD, genetic distance.

INTRODUCTION

The subfamily Bambusoideae of the family Poaceae includes both herbaceous and woody bamboos. Of these, the woody bamboos of the tribe Bambusae are among the most useful of the forest products. They are naturally adapted to forest habitats and mostly grow in wild stands. The main centres of diversity appear to be the monsoon belt of South-East Asia with Southern China, and coastal regions of the Atlantic side of South America (Ohrnberger, 1999). They have been introduced to different new habitats where they have become naturalized.

The traditional and industrial utilization of bamboo and its potential as a wood substitute have brought about an increasing demand for the woody bamboos. This makes their taxonomy and identification important. Although molecular data have been used to show relationships between the temperate, tropical New World and Old World woody

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bamboos, further resolution within the sub-family Bambusoideae is needed (Clark, 1997; Kobayashi, 1997). Compared to other members of the grass family, taxonomic studies in bamboo are few. In majority of bamboo descriptions, species are not clearly described. There are many reasons for this lapse. Their identification is mainly based on vegetative features of the culm, culm sheath, leaf, bud, branch and rhizome. The differences among these characters can be minute, especially when it comes to differentiating species within a genus. These vegetative traits can be influenced by environmental factors unlike floral characters, which are more uniform. But flowering in most species is rare or may not take place at all. Bedell (1997) points out that another reason for shortcomings in identification is the use of materials from a limited range of distribution. Heterogeneity within species is not well understood and it is thus necessary to use materials from a wide range of distribution. Identification has also been based on collections of descriptions reported by different investigators who studied different parts of the plant, leading to incorrect identification. Thus, lack of adequate reference material and usable identification keys make it difficult to identify species correctly (Kumar, 2002).

The genetic code is least affected by the environment and can help in tracing the evolutionary history. Molecular systematics based on methods of identifying DNA polymorphism between individuals or species can overcome problems of identification and classification in bamboo. Very few reports are available on the use of DNA polymorphism in bamboo. Friar and Kochert (1991,1994) used nuclear Restriction Fragment Length Polymorphism (RFLP) to study the variation and evolution of *Phyllostachys*, a group of temperate bamboos. Geilis *et al.*(1997) applied Random Amplified Polymorphic DNA (RAPD) to identify unknown genotypes and assessed genetic variation in *Phyllostachys*. A wider selection of bamboo and related species has been used to study the phylogeny and evolution of the bamboos (Clark, 1997; Kobayashi, 1997).

The present investigation is an attempt to use RAPD to detect genetic variation within a population of an introduced species, *Dendrocalamus giganteus* Wall ex Munro and an endemic species, *Ochlandra stridula* Moon ex Thwaites in Sri Lanka. It is reported that the latter has two varieties, one with green culm colour and the other, *O. stridula* var. *maculata*, with green culms banded or blotched with dark purple. The purple colour is reported to be lost under cultivation (Sumithraarachchi *et al.*, 1993). The variety we studied did not show purple colouration. The species description is in accordance with Senaratne (1956) in the population studied. They formed dense thickets and were in flower.

MATERIALS AND METHODS

Plant material

Fresh young leaves collected from 92 individual clumps of *D. giganteus* within a radius of 40 km around Kandy, including those in the Royal Botanic Gardens (RBG), Peradeniya and of *O. stridula* (sub tribe Melocanninae), from six individual clumps in Awissawella, about 100 km west of Kandy, were used to extract DNA.

The individual clumps were labeled and their positions located in a map for future reference. Further descriptions of morphological traits and flowering were noted in separate data sheets for all individuals.

DNA extraction

The leaves were cleaned, cut into small pieces and ground to a fine powder using liquid nitrogen. DNA extraction was carried out following the method of Weising and Kahl (1997), a simplified Doyle and Doyle (1990) protocol. About 1 to 1.5 g of leaf powder was homogenized in 8 ml extraction buffer (2% CTAB, 1.4 M $NaCl_2$, 20 mM Na_2EDTA and β-mercaptoethanol added last to a final concentration of 0.2%) at 65°C for over 30 min. The incubated sample was mixed gently with 15 ml of chloroform: isoamyl alcohol (24 : 1). This was repeated using the top aqueous layer that separated after centrifugation. The crude DNA in the top aqueous layer was precipitated in 0.6 volumes of isopropanol and washed in a solution of 76 per cent ethanol and 10 mM ammonium acetate. RNA was removed by incubating the precipitate with RNAse at 37°C and polysaccharides removed in 7.5 M ammonium acetate. The DNA was precipitated in absolute ethanol, washed in 70 per cent ethanol and finally suspended in TE (10 mM Tris-HCl: 1mM EDTA). DNA was diluted to a working concentration of 15 ng μl^{-1}.

RAPD

Amplification was carried out in a 25 µl reaction volume with 60 ng DNA, 5 pM random 10-mer primer (Operon Technologies, California), 125 µM each of dATP, dTTP, dGTP and dCTP (Promega Corporation, USA), 0.25 μL (5units μL^{-1}) Taq DNA polymerase in 1× polymerase buffer, 2.5 mM $MgCl_2$ (Promega Corporation, USA) and overlaid with 25 µl mineral oil. Amplification was performed in a Sanyo DNA thermal cycler (MIR-D30) programmed for 45 cycles with the first cycle at 94°C for 4 min, 36°C for 1min and 72°C for 2 min, followed by 44 cycles at 94°C for 1 min, 36°C for 1min and 72°C for 2 min.

Initially, a total of 41 random 10-mer primers were tested with DNA extracted from 10 individuals of *D. giganteus*. Out of these, six primers that generated eight or more bands were selected (Table 1) and tested repeatedly to make sure of the consistency of RAPD band patterns. DNA extracted from the 92 individuals of *D. giganteus* was

used to generate RAPD bands with these six selected primers. These primers were tested with *O. stridula* and four were selected to generate bands.

The amplified products were separated by electrophoresis in 1.5 per cent agarose gels in 0.5 × TBE (45 mM Tris-Borate, 1 mM EDTA) buffer. The gels were stained with ethidium bromide and photographed on Polaroid 665 films under UV light.

Data analysis

The Random Amplified Polymorphic DNA fragments (bands) generated were visually scored as 1 for presence or 0 for absence. The computer freeware package RAPDistance version 1.04 was used to analyse these data (Armstrong *et al.*, 1994). The monomorphic bands (all 1's or all 0's) were deleted before the genetic distances were computed, as these do not contribute to the genetic relatedness of samples. The pair-wise genetic distances between individuals were calculated according to Nei and Li (1979). This distance matrix was used to construct a neighbor-joining tree based on the method of Saitu and Nei (1987). A PTP (Permutation Tail Probability) test was performed to ensure that the tree generated was not an artifact. Data analysis was carried out separately for *D. giganteus* and *O. stridula* populations.

RESULTS

The randomly amplified DNA fragments generated varied according to the combination of primer and DNA sample. Of a total of 41 primers tested with *D. giganteus*, four did not give amplification. The other primers generated a range of 2 to 12 bands. A total of 58 bands from six selected primers that generated eight or more bands were used in the *D. giganteus* population (Table 1). Four primers that generated 29 bands were used for *O. stridula* population.

Table 1. Random 10-mer primers used to detect polymorphism in *D. giganteus* and *O. stridula*

Primer	Sequence	No. of DNA fragments (bands)	
		D. giganteus	*O. stridula*
OPF 09	CCAAGCTTCC	12	-
OPF 14	TGCTGCAGGT	08	-
OPG 19	GTCAGGGCAA	08	08
OPH 04	GGAAGTCGCC	10	07
OPI 05	TGTTCCACGG	10	08
OPJ 20	AAGCGGCCTC	10	06
Number of polymorphic bands		51	11

Genetic diversity in *D. giganteus* population

The distance matrix of the *D. giganteus* population showed that the genetic distance among individuals was low ranging from 0 to 0.299 with a mean of 0.092 ± 0.027. The dendrogram (Fig. 1) shows two main branches that had a distance of only 0.011 between them indicating that they were a single clade. The individuals collected from different locations are shown in Table 2. Individuals from all locations were dispersed in the dendrogram and did not form groups according to their locations or spatial distribution. Many individuals had identical band patterns. These occupy the

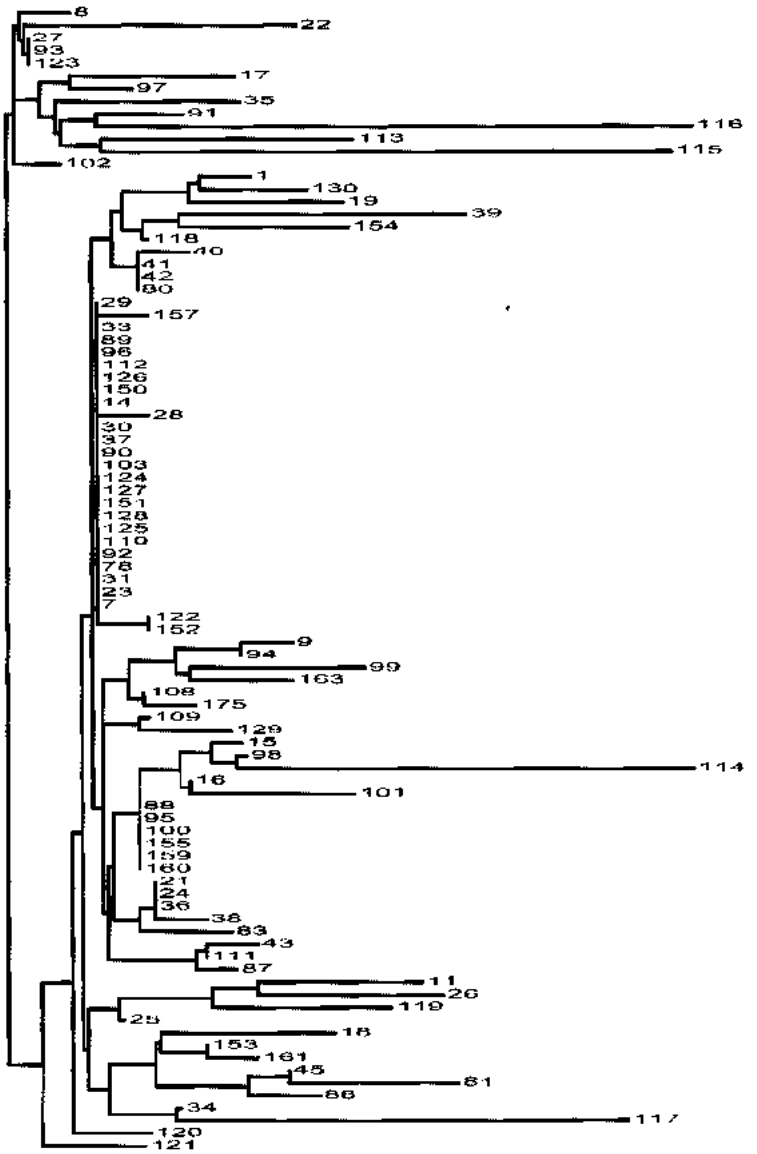

Figure 1. Dendrogram of *D. giganteus* population (individuals are designated by numbers).

Table 2. Individuals of *D. giganteus* (numbered) collected in five locations within the Kandy district

Locations				
Royal Botanic Gardens	Kandy	Kandy (S)	Matale	Kandy (N)
7, 8, 9, 11,	1, 18, 19*, 26	78,80, 81, 83	108,109, 110, 111	96, 97,98, 99
14, 15, 16, 17	27, 28, 29, 30	86,87, 88,89	112', 113'',114'',115''	100,101
21, 22, 23, 24	31, 33, 34, 35	90, 91, 92, 93	116'', 117'', 118, 119	
25, 150, 151, 152	36, 37, 38, 39	94, 95, 175	120, 121, 123, 124	
153, 154, 155, 157	40, 41, 42, 43			
159, 160, 161, 163	45, 102, 103, 126			
	127, 128, 129, 130*			

112' flowered and set seed; '' seed raised offspring of 112 ; 19* 130* Tissue culture raised plants from 1(parent).

same position in the dendrogram (Fig. 1). The highest genetic distance (0.299) was between 114 and 116, which were two offsprings developed from seeds of 113. The next highest distance of 0.282 was also between 114 and another sibling, 115. Four individuals 114, 115, 116 and 117, were raised from seeds when 113 flowered and set seed. All these were from a farmer's field in Matale. Although they did not show any morphological variations, they were genetically not similar as manifested by RAPDs. In the dendrogram (Fig. 1) the two siblings 116 and 115 were close to each other and to the parent clump 113.

D. giganteus is the largest of the bamboos and it grows to over 15 m in height. The population studied showed that many of the clumps were massive with more than 200 culms. The vegetative characters are in accordance with description of the species in Sumithraarachchi *et al.* (1993) and Senaratne (1956), except for one clump that differed in having variegated leaves and was raised *in vitro*. The mean culm girth in the population was 60 cm. Of a total of 130 clumps observed, 20 were flowering at the time of study. The leaf sheath, which is a specific character used in species identification, did not show any variation. Two clumps of *D. asper* in the Royal Botanic Gardens (RBG), which appeared similar to *D. giganteus* were accidentally included in the *D. giganteus* population, formed a separate branch when the dendrogram was computed and had a higher genetic distance of 0.356 from the other individuals. Further investigations especially on leaf sheath characters led to their identity and these were omitted from the genetic distance studies of the *D. giganteus* population. This indicated that RAPD technique could distinguish between these two species.

Genetic diversity in *O. stridula* population

Figure 2 is the dendrogram of *O. stridula*. The genetic distance among individuals ranged from 0.111 to 0.800 with a mean of 0.446 ± 0.210.

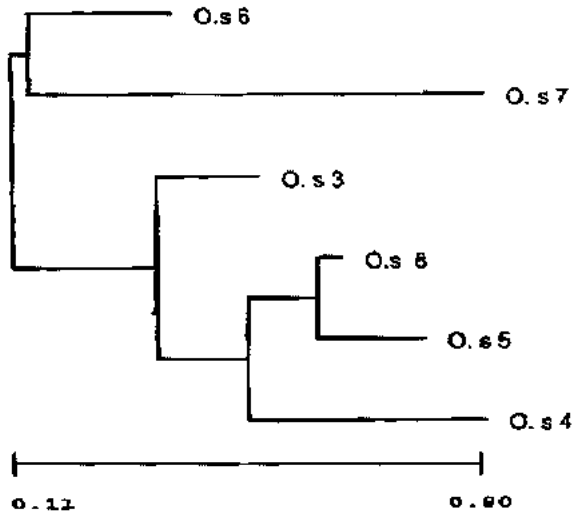

Figure 2. Dendrogram of *O. stridula* population (individuals are designated as Os and number).

DISCUSSION

Genetic diversity in *D. giganteus* population

Seed-derived plants of *D. giganteus*, 114, 115, 116 and 117, showed the highest genetic distance from others in the population. Flowering in *D. giganteus* is rare (Ramanayake and Yakandawala, 1998). Of the 130 individuals observed, only 20 clumps were flowering at the time of study. When the parent clump, 113 in Matale, flowered and set seed, there were no other flowering clumps in this location. Therefore, the seeds which may have developed after self-pollination have given rise to offspring that were genetically different. It is possible that the parent (113) was heterozygous. This study indicates that offspring from seed contributed to genetic diversity in the population.

D. giganteus was introduced to Sri Lanka in 1856 (McMillan, 1908; Petch, 1925). Petch (1925), a former curator of the RBG, Peradeniya, reported that numerous plants were propagated vegetatively from a single plant of *D. giganteus* received by RBG from Kolkota, India.. These were set out in the RBG, Peradeniya or distributed to other parts of the Island. Thus, the present population originated from a single plant. The low genetic diversity shown in this investigation reflects this narrow genetic base. Many plants that did not show polymorphism could be clones derived by vegetative propagation (Fig. 1). The RAPD band patterns were identical in many of the individuals with no distance between them. A group of genetically identical individuals that comprised a relatively large number is, 29, 33, 96, 112, 126, 150, 14, 30, 37, 90, 103, 124, 127, 151, 128, 125, 110, 92, 78, 31, 23, 7 (Fig. 1). These consist of individuals from all locations including some from the RBG (Table 2). Our studies

as well as reports of past curators of the RBG, showed that some of the plants in the RBG flowered, set seed and did not die at the end of flowering (McMillan, 1908; Petch, 1925; Ramanayake and Yakandawala, 1998). Therefore, some of the plants present in the RBG may be the survivors of the original clone, now more than 150 years old. Later, plants raised from seeds too could have been planted in the RBG as well as in other locations. These could have contributed to the present diversity seen in this population.

The two plants 130 and 19, which were raised *in vitro* by axillary shoot proliferation from axillary buds of individual number 1 located in Kandy, are very close in the dendrogram but do not occupy identical positions, and showed polymorphism unexpected in clonally propagated individuals. During continuous axillary shoot proliferation, some of the shoots developed variegated leaves (Ramanayake and Yakandawala, 1997). The individual 130, which was developed from these shoots is about 10-years-old and the character of variegated leaves has persisted. Thus, genetic variations could even develop during rapid axillary shoot proliferation, a method of vegetative propagation. Albinism in *Phyllostachys bambusoides* is reported to be due to a recessive character (Smith, 1977). Thus, it is possible that the mother plant was heterozygous for this character.

We observed that death of old culms in the centre caused division of clumps into sectors in a few large clumps of *D. giganteus* in the RBG. This is the natural method of vegetative propagation. Vegetative propagation over a long period of time could lead to separation of the clump into many parts. If mutant characters develop, they could separate in this manner and contribute to genetic diversity in the population. This is possible in plants that grow over long periods without sexual reproduction.

Genetic diversity in *O. stridula* population

O. stridula showed a higher mean genetic distance of 0.446 ± 0.210 compared to the introduced species *D. giganteus* with a mean distance of 0.092 ± 0.027 (Fig. 2). Unlike *D. giganteus*, sexual reproduction is more prevalent as the species flower and set seed annually. Although the reproductive biology of the species in Sri Lanka is not documented, the genus *Ochlandra* consists of cross-pollinating species (Koshy and Harikumar, 2001). Being an endemic species, it has been able to evolve in its native habitat over a longer period of time. The higher genetic diversity shown by this species in comparison to *D. giganteus* may be related to these factors.

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