# AFLP as an efficient molecular marker for detecting varietal differences in antioxidant activity in edible shoots of Dendrocalamus hamiltonii

Sayanika Devi Waikhom[1*], Bengyella Louis[1,2], Subhabrata Ghosh[3], Swati Sen-Mandi[3], Narayan Chandra Talukdar[1]

[1]Institute of Bioresources and Sustainable Development (IBSD), Takyelpat, Imphal-795001, Manipur, India.

[2]Department of Biochemistry, University of Yaoundé I, BP 812-Yaoundé, Cameroon.

[3]Division of Plant Biology, Bose Institute, 93/1, APC Road, Kolkata-700009, West Bengal, India

*Email: sayanikawaikhom@gmail.com

## Abstract

Juvenile bamboo shoots are traditional food delicacies in North-East India for its rich nutritive content. Shoots from *Dendrocalamus hamiltonii* are widely eaten in processed and fermented forms. Nonetheless, some landraces of *D. hamiltonii* are rich in cynogenic glycoside and poses toxicity threat upon consumption and must be identified. Genetic variability in landraces of *D. hamiltonii* was studied in two major fermented bamboo shoots production districts of Manipur, India using fluorescence-based AFLP markers. The study shows that the populations were divided into major groups. Principal coordinate analysis based on the AFLP data clearly separated the populations according to their genetic diversity and antioxidant activity. Our findings indicate that the genotype (U-IEP8) contained high antioxidant activity with low total cyanide content and it could be used as superior genotype with desirable trait for mass cultivation by farmers.

Key words: AFLP marker, antioxidant activity, *Dendrocalamus hamiltonii*, edible bamboo shoots, total cyanide content

## Introduction

Young succulent bamboo shoots are consumed as seasonal vegetable or in fermented forms mostly by the ethnic communities of North- East India. Fermented bamboo shoots are available throughout the seasons in the local market and also exported to the neighboring countries like Myanmar and Thailand. Hence, there is good scope for commercial utilization of bamboo shoots in the food-processing sector.

**Theme: Biology, Morphology and Taxonomy**

Regardless of its palatable and delicious taste, young bamboo shoots possess enormous nutritive values such as macro- and micronutrients, antioxidant properties and high fibre content (Nirmala et al. 2007; Park et al. 2010; Nirmala et al. 2011; Sayanika devi et al. 2013). Nonetheless, edible bamboo shoots also possess toxic cyanogenic glycosides content (Haque and Bradbury 2002; Sayanika devi et al. 2013). Interestingly, the nutritive properties and cyanogenic glycoside content varied in bamboo shoots based on their geographical positions i.e., altitude as reported by Sayanika devi et al. (2013). Landraces are heterogeneous plant varieties that are reproduced by farmers as populations that are subject to both artificial and natural selection. Landraces are distinguished by farmers who usually gave them a name that is associated with their specific traits, and different farmers might use the same landrace with the cultivation of different populations (Bellucci et al. 2013). Thus, in a landrace, the diversity is structured between and within populations (at the field/farmer level). It is hypothesized that the landraces of a single conventional species of bamboo may vary for desirable nutritional trait as well as low content of undesirable toxic cyanogenic glycosides. *Dendrocalamus hamiltonii* is an economically important bamboo species mainly used for fermented shoots preparation because of its good taste and low water content. The two popular traditional fermented bamboo shoots (*shoibum*) production centers of Manipur, India are located in the Chandel and Imphal-East districts (of Manipur).

Here, we evaluated the genetic relations between the landraces of *D. hamiltonii* using automated DNA sequencer AFLP technique. Furthermore, we determined the total cyanide content and antioxidant activity in bamboo shoots of *D.hamiltonii* and finally analysed the specific association between AFLP markers and biochemical traits such as antioxidant activity and total cyanide content for use in marker-assisted breeding.

## Materials and methods

Sampling strategy: We analysed ten accessions of *D. hamiltonii* Nees & Arn. ex Munro that were collected from two populations, i.e., Imphal-East and Chandel districts of Manipur, India, during July-August of 2009-2010. Collection sites were chosen based on the distribution of *D. hamiltonii* and each accession of a population (Imphal-East and Chandel) was 10-50 km apart. This region often received an average rainfall of 1320±3mm and temperature of 23±3$^{\circ}$C during the months of July-August. Their name, geographical features and collection numbers are shown in Table 1. The herbarium specimens of the collected samples were deposited to the Botanical Survey of India, Howrah, West-Bengal, India, for future references. For biochemical analysis, 15-days old bamboo-shoots (from the day of emergence) of the 10 accessions were collected from three randomly chosen culms at each collection site. Subsequent to harvest, the bamboo-shoots were directly frozen in liquid nitrogen in a thermocol box and transported to the laboratory where they were stored at −80$^{\circ}$C for downstream analysis.

DNA extraction: Young leaves were harvested for DNA extraction following the modified protocol of Aras et al. (2003). DNA quantity and quality were determined by Nano Drop$^{Tm}$ spectrophotometry (Nano Technology) and evaluated by agarose gel electrophoresis, respectively.

AFLP marker: The AFLP technique was performed on 500 ng genomic DNA with the AFLP Plant mapping kit for average sized genomes. The AFLP procedure followed the manufacturer's protocol. The method is based on Vos et al. (1995), but the primers are labelled with nonradioactive fluorescent dye. In all, 20 selective primer combinations were tested, of which four were chosen that gave good amplifications and that showed polymorphisms. The combinations were *Eco*RI-AAG/*Mse*I-CTC, *Eco*RI-ACG/*Mse*I-CAG, *Eco*RI-AAC/*Mse*I-CTG and *Eco*RI-ACC/*Mse*I-CTG. Selective amplification was conducted on a Thermocycler (Applied Biosystems Viriti, USA) following the programmed as: $94^0$C for 2 min; 10 cycles of $94^0$C for 20s, $66^0$C ($-1^0$C/cycle) for 30s and $72^0$C for 2 min; 20 cycles of $94^0$C for 20s, $56^0$C for 30s and $72^0$C for 2 min; $60^0$C for 30 min and $4^0$C for infinity. The samples were loaded onto a 6% (29:1) polyacylamide gel on an ABI Prism 377 Sequencer (Applied Biosystems, USA). An internal size standard (GS-500 ROX, Applied Biosystems, USA) was included in each sample to facilitate fragment sizing and GeneScan Analysis Software 2.1 was used to estimate fragment size. Hereafter the GeneScan scoring table was constructed and exported to Microsoft excel. Markers that failed in displaying good amplification in any of the individuals were excluded for further analysis.

Antioxidant activity: The scavenging effect of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical was monitored according to Mensor et al. (2001) method as follows: 1 mL of 0.3 mM DPPH ethanolic solution was added to each 3.0 mL of the samples with different concentrations of bamboo shoots extract. The mixtures were vortexed for 1 min and then left to stand at room temperature in the dark. After 30 min absorbance was read at 517 nm in a UV-visible spectrophotometer (UV-1700, Shimadzu). The scavenging activity of the DPPH radical was calculated using the following equation: Scavenging activity (%) = 100 x ($A_{Control} - A_{Sample)} / A_{Control}$, where $A_{Control}$ is the absorbance of the control reaction (containing all reagents except the test compound) and $A_{Sample}$ is the absorbance of the test compound. The inhibition concentration ($IC_{50}$) was defined as the amount of extract required for 50% reduction of free scavenging activity. The $IC_{50}$ values were obtained from the inhibition curves.

Principal Coordinate analysis: Principal Coordinate analysis (PCoA) was carried out based on the pairwise genetic similarity matrix using the DCENTER and EIGEN procedures of NTSYS pc software version 2.2.

## Results and discussion

### Molecular characterization and assessment of genetic diversity with AFLP marker:

The selected combinations of primers generated a total of 1684 peaks of which 95.7% were polymorphic (Table 2). The UPGMA cluster analysis revealed that the ten accessions were clustered into 2 groups (Fig. 1). The group 1 consisted mainly of populations from Imphal-East district with a mean similarity index (Si) 0.592, while the Group 2 contained populations from chandel district with a mean similarity index (Si) 0.563.

Scavenging activity on DPPH radicals: The Figure 2 shows the dose-response curve for the radical scavenging activity of water extract of bamboo shoots (WEB) of the ten *D. hamiltonii* accessions. A significant variation in terms of free radical scavenging activity of WEB was observed among accessions of *D. hamiltonii* (26.54-43.45%). Strong DPPH activity of WEB at 400 µg/ml was observed in U-IEP8 (43.45%), followed by U-IEP9 (38.9%), while the lowest was obtained in U-CHP6 (26.54%). The $IC_{50}$ value of WEB in U-IEP9 was 0.114 mg/ml compared with the $IC_{50}$ of Ascorbic acid of 0.001 mg/ml. Two cultivated edible bamboo species of Korea viz. *Phyllostachys pubescens* and *Phyllostachys nigra* possessed strong antioxidant activity in their bamboo shoot extracts have been reported in a study carried out by Park and Jhon (2010). The present study was the efforts for the elaborate work on the screening of scavenging activity of WEB on the DPPH free radicals of *D. hamiltonii* accessions grown in two districts- the major fermented bamboo shoots production region of Manipur.

Total cyanide content using the picrate method: Total cyanide content of three portions i.e. tip, middle and base in bamboo shoots of the ten accessions of *Dendrocalamus hamiltonii* are shown in Table 3. The total cyanide levels are highest in the tip and lowest at the base of the bamboo shoot. And the highest cyanide content in the middle portion was found in the U-CHP3 (1123 ppm) and the lowest was obtained in U-IEP8 (940 ppm). The total cyanide levels decreased from the tip to the base of bamboo shoots which were in agreement with the previous studies (Haque and Bradbury 2002). There is a significant variation of total cyanide content in bamboo shoots amongst accessions. Hence, the present study reports on the elaborative study of total cyanide content determination within genotypes of bamboo species.

Principal Coordinate analysis: In a Principal Coordinate analysis with all samples, the first two axes explained 25.22% and 16.17%, respectively, of the variation in the matrix and differentiated the accessions according to their genetic diversity and antioxidant activity (Fig. 3). Geographically, the genetic groups 2 distributed in the same areas i.e. different geographical sites of Chandel district. Conversely, the genetic group 1, containing 4 accessions, is geographically isolated from the first ones and covers the cultivating areas of Imphal-East district. Basically, all *D. hamiltonii* accessions of the same geographical location clustered in identical group. The two major groups (Group 1 and 2) revealed by UPGMA cluster analysis were confirmed by PCoA. This analysis allowed discrimination among the accessions and established the relevance of the bands to each Principal Coordinate, also substantiating their status in terms of antioxidant activity.

Multiple regression analysis: Multiple regression analysis (MRA) was analysed to determine the association of AFLP markers with biochemical attributes i.e. antioxidant activity of WEB and total

cyanide content in different accessions of *Dendrocalamus hamiltonii*. A total of 4 alleles generated by primers *Eco*RI-AAG (Joe)/*Mse*I-CTC, *Eco*RI-ACC (Ned)/*Mse*I-CTG, *Eco*RI-AAC (Ned)/*Mse*I-CTG and *Eco*RI-ACG (Joe)/*Mse*I-CAG showed positive correlation with antioxidant activity (Table 4). If the relationship between independent variables and the dependent variable is not linear, the results of the regression analysis will under-estimate the true relationship. To ascertain the relation between selected AFLP markers and antioxidant activity, markers selected at the first three consecutive steps of MRA were tested for the linear relationships with high antioxidant activity. It showed that U-IEP8 accession with highest antioxidant activity in bamboo shoots extract occupied the range between 0.8 and 1.0 in the x-axis and 10 and 15 in the y-axis for the AFLP markers (Fig. 4). On the other hand, bamboo shoots extract of U-IEP9, U-CHP2, U-CHP1 and U-CHP4 accessions with the moderate antioxidant activity occupied the central position at 0-0.2 in the x-axis and -5-0 in the y-axis. The rest accessions with low antioxidant activity occupied the position in the negative range (both in the x- axis and y-axis) of the regression plot. In case of total cyanide content, step-wise regression identified four alleles corresponding 346 bp, 377bp, 434bp and 282 bp generated with the four primers viz. *Eco*RI-AAG/*Mse*I-CTC, *Eco*RI-ACG/*Mse*I-CAG, *Eco*RI-AAC/*Mse*I-CTG and *Eco*RI-ACC/*Mse*I-CTG respectively (Fig: 5). The GRC marker-trait association identification has several advantages over the linkage-based identification of markers associated with genes/QTLs because of the following reasons (i) this allows the detection of QTL that varies across a wide spectrum of biodiversity rather than just between two planned parental genotypes (ii) this requires less inputs of time, labor and financial resources, compared to the linkage-based QTL identification and (iii) this technique plays an important role in plant MAS/QTL breeding programs, especially in orphan crops and long-juvenile woody plants with heterozygosity when no other genetic information such as linkage maps and Quantitative Trait Loci are available. Such studies of germplasm-regression-combined (GRC) analysis were also carried out to identify the associations of molecular markers with desirable traits in various crops and woody plants with great success, such as Asia rice (Mishra and Sen-Mandi 2004), mulberry (Karl et al. 2008) and birch (Wang et al. 2008; Xia et al. 2008).

Conclusion

The AFLP markers associated with the biochemical traits, identified through MRA, could be of great use in marker-assisted breeding programs for identification of superior genotypes of *Dendrocalamus hamiltonii* which are having high antioxidant activity with low total cyanide content.

# References

Affifi, A.A.; Clark, V. 1984. Computer-aided multivariate analysis. Van Nostrand Reinhold, New York.

Aras, S.; Duran, A,; Yenilmez, G. 2003. Isolation of DNA for RAPD analysis from dry leaf material of some Hesperis L. specimens. Plant Molecular Biology Reporter 21,461a-461f.

**Theme: Biology, Morphology and Taxonomy**

Bellucci, E.; Bitocchi, E.; Rau, D.; Nanni, L.; Ferradini, N.; Giardini, A.; Rodriguez, M.;, Attene, G.; Papa, R. 2013. Population structure of barley landrace populations and gene-flow with modern varieties. Plos One 8(12), e83891. doi: 10.1371/journal.pone.0083891.

Haque, M.R.; Bradbury, J.H. 2002. Total cyanide determination of plants and foods using the picrate and acid hydrolysis methods. Food Chemistry 77, 107-114

Kar, P.K.; Srivastava, P.P.; Awasthi, A.K..; Urs, S.R. 2008. Genetic variability and association of ISSR markers with some biochemical traits in mulberry (*Morus* spp.) genetic resources available in India. Tree Genetics & Genomes 4, 75-83.

Mensor, L.L.; Menezes, F.S.; Leitao, G.G.; Reis, A.S.; Santos, T.S.; Coube, C.S. et al. 2001. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytotheraphy Research 15, 127-130.

Mishra, R.K.; Sen-Mandi, S. 2004. Molecular profiling and development of DNA marker associated with drought tolerance in tea clones growing in Darjeeling. Current Science 87, 60-66.

Nirmala, C.; Bisht, M.S.; Haorongbam, S. 2011. Nutritional properties of bamboo shoots: potential and prospects for utilization as a health food. Comprehensive Reviews in Food Science and Food Safety 10(3), 153–168.

Nirmala, C.; David, E.; Sharma, M.L. 2007. Changes in nutrient components during ageing of emerging juvenile bamboo shoots. International Journal of Food Sciences & Nutrition 58, 345–352.

Park, E-J.; Jhon, D-Y. 2010. The antioxidant, angiotensin converting enzyme inhibition activity, and phenolic compounds of bamboo shoot extracts. LWT-Food Science and Technology 43,655-659. doi:10.1016/j.lwt.2009.11.005

Ruan, C.J.; Li, H.; Mopper, S. 2009. Characterization and identification of ISSR markers associated with resistance to dried-shrink disease in sea buckthorn. Molecular Breeding 24, 255-268.

Sayanika, D.W.; Louis, B.; Chandradev, S.K.; Kumari, P.; Bharat, G.S.; Wakambam, S.M.; Talukdar , N.C. 2013. Grappling the high altitude for safe edible bamboo shoots with rich nutritional attributes and escaping cyanogenic toxicity. BioMed Research International. http://dx.doi.org/10.1155/2013/289285.

Virk, P.S.; Ford-Lloyd, B.V.; Jackson, M.T.; Pooni, H.S.; Clemeno, T.P.; Newbury, H.J. 1996. Predicting quantitative variation within rice germplasm using molecular markers. Heredity 76, 296-304.

Vos, P.; Hogers, R.; Bleeker, M.; Reijans, M.; van de Lee, T.; Hornes, M. et al. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23, 4407–4414.

Wang, D.; Wei, Z.G.; Yang, C.P.; Liu, G.J. 2008. Analysis and identification of SCAR molecular markers associated with birch fiber length trait. Journal of Forestry Research 19, 288-292.

**Theme: Biology, Morphology and Taxonomy**

Xia, D.A.; Wei, Z.G.;Yang, C.P.; Liu, G.J. 2008. Analysis of ISSR and SCAR Markers associated with birch fiber length trait. Journal of Northeast Forest University 39: 1-4 (in Chinese with an English abstract).