VOLUME 5

Biology and Taxonomy

Table of Contents

Preface

Everyone knows how ecologically, economically and/or traditionally important bamboos are. They can be used as an alternative to wood in a variety of ways. To be able to study any more-advanced research topics or utilization, however, it is necessary to understand the fundamentals on bamboo biology and taxonomy, including systematics.

This session is contributing the most basic but the most important part among bamboo researches. It is chaired by De Zhu Li and co-chaired by Sarawood Sungkaew. D.Z. Li is a well-known bamboo taxonomist at Kunming Institute of Botany, Yunnan, who has contributed hundreds of papers for the world of bamboo taxonomy and biology. S. Sungkaew is regarded as a young-blood bamboo taxonomist who has been currently nominated to be a member of BPG (Bamboo Phylogeny Group, http://www.eeob.iastate.edu/research/bamboo/).

Cyanogenic Glycosides in Bamboo Plants Grown in Manipur, India

Kananbala Sarangthem, Hoikhokim, Th.Nabakumar Singh and G.A.Shantibala
Department of Life Sciences, Manipur University, Canchipur, Manipur,India

Abstract

Bamboo cultivation is practiced in many tropical countries. In Manipur ,India the fresh succulent bamboo shoot slices, locally called '*Soibum*' is a highly prized vegetable item. Cyanogenic glycosides are phytotoxins which occur as secondary plant metabolites found in nature. . The cyanogenic glycosides present in bamboo shoots are Taxiphyllin. Taxiphyllin is hydrolysed to glucose and hydroxybenzaldehyde cyanohydrin. This benzaldehyde cyanohydrin then decomposes to hydroxy benzaldehyde and Hydrogen cyanide (HCN). By adequate processing like peeling ,slicing, fermenting, repeated washing, boiling,cooking ,roasting and canning, the cyanogenic glycosides and HCN can be reduced prior to consumption ,thus significantly reducing the potential health risk.

Keywords: Cyanogenic glycosides, Bamboo, Manipur

Introduction

Bamboo is a group of woody perennial evergreen plants in the grass family Poaceae, subfamily Bambusoideae, tribe Bambuseae. Some of its members are giant bamboo , forming by far the largest members of the grass family. Bamboo is the fastest growing woody plant in the world. Their growth rate (4.7inches/day)) is due to a unique rhizome-dependent system, but is highly dependent on local soil and climate conditions.

They are of economic and high cultural significance in East Asia and South East Asia where they are used extensively as a building material, in gardens, and as a food source. The shoots (new bamboo culms that come out of the ground) of bamboo (fig.1) are edible. They are used in numerous Asian dishes, and are available in markets in various sliced forms, both fresh and canned version. In Manipur, the fresh succulent bamboo shoots and the fermented preparation of bamboo shoot slices (fig.2), locally called "soibum" is a highly prized vegetable item. The "soibum" (fig.3) is manufactured traditionally by storing thin slices of fresh succulent and soft bamboo shoots in specialised containers/chambers for 2-3 months. The fermented chambers are either made of bamboo planks or roasted earthern pots. The inner surface of bamboo chambers are lined with banana leaves and a thin polythene sheets. There are different localities in Manipur where traditional fermentation of bamboo shoots is in progress (Khongkhang,Bishnupur, Andro,Noneh, Tengnoupal,Churachandpur,Kotha etc.). Bamboo shoots of many species like *Bambusa tulda,B. balcooa, Dendrocalamus hamiltonii, Melocanna bambusoides, Arundanaria callosa* were used for fermentation purpose.

Bamboo shoots are traditional component of Asian cuisine. Its consumption increase world wide expanding from oriental to western world and a health warning is appropriate as bamboo shoots contain cyanogenic glycosides that break down to produce hydrogen cyanide(HCN) , which can cause both acute and chronic toxicity in humans(Food Standards Australia, New Zealand,2005) . However, the cyanide content is reported to decrease substantially following harvesting. By adequate processing like peeling ,slicing, fermenting and cooking , the cyanogenic glycosides can be reduced prior to consumption ,thus significantly reducing the potential health risk.

Cyanogenic glycosides are phytotoxins which occur as secondary plant metabolites in at least 2000 plant species, of which a number of species are used as food in some areas of the world. Cassava and sorghum are especially important staple foods containing cyanogenic glycosides (Conn1979; Nartey 1980; Rosling 1994). There are approximately 25 cyanogenic glycosides known. The major cyanogenic glycosides found in the edible parts of plants being; amygdalin (almonds); dhurrin(sorghum); linamarin& lotaustralin (cassava,lima beans); prunasin(stone fruit);and taxiphyllin(bamboo shoots). The potential toxicity of a cyanogenic plant depends primarily on the potential that its consumption will produce a concentration of HCN that is toxic to exposed animals or humans . Several factors are important in this toxicity: The first aspect is the processing of plant products containing cyanogenic glycosides. When the edible parts of the plants are macerated, the catabolic intracellular enzyme ß-glucosidase can be released, coming into contact with the glycosides. This enzyme hydrolyzes the cyanogenic glycosides to produce hydrogen cyanide and glucose and ketones or benzaldehyde(Harborne1972,1993). The hydrogen cyanide is the major toxic compound causing the toxic effects. The cyanogenic glycosides present in bamboo shoots is Taxiphyllin. Taxiphyllin is hydrolysed to glucose and hydroxybenzaldehyde cyanohydrin. This benzaldehyde cyanohydrin then decomposes to hydroxy benzaldehyde and HCN (Schwarzmair 1997).

Plant products , if not adequately detoxified during the processing or preparation of the food, are toxic because of the release of this preformed hydrogen cyanide. The second aspect is the direct consumption of the cyanogenic plant. Maceration of edible parts of the plants as they are eaten can release ß-glucosidase. The ß-glucosidase is then active until the low pH in the stomach deactivates the enzyme. Additionally, it is possible that part of the enzyme fraction can become reactivated in the alkaline environment of the gut. At least part of the potential hydrogen cyanide is released, and may be responsible for all or part of the toxic effect of cyanogenic glycosides in the cases of some foods (WHO,1993).

In the intact plant, the enzyme and the glycosides remain separated , but if the plant tissue is damaged both are put in contact and cyanohydric acid is released (Bell 1981;Grunert et al.,1994). Cyanohydric acid is extremely toxic to a wide spectrum of organism, due to its ability of linking with metals(Fe++, Mn++ andCu++) that are functional groups of many enzymes inhibiting the reduction of oxygen in the cytochrome respiratory chain, electron transport in the photosynthesis and the activities of enzymes such as catalase ,oxidaes(Cheeke 1995). The level of cyanogenic glycosides produced is dependent upon the age and variety of the plant, as well as environmental factors(Cooper-Driver & Swain1976;Woodhead&Bernays, 1977). Although there are reports elsewhere of bamboo species containing significant potentially very toxic amounts of cyanogenic glycosides in their shoots, however the available materials does not confirm that some bamboo species do indeed contain very

high level of cyanogenic glycosides in their shoots .There are no clear differences between species and sufficient information to generalised.

The present work is undertaken to assess cyanogenic glycosides in fresh and fermented succulent bamboo shoots to stimulate new uses of bamboo shoots in existing markets and to assist developing foods security in food poor areas.

Materials and Methods

The emerging young fresh succulent bamboo shoots (about 20cm in diameter and 15cm in height) of the species of *Bambusa balcooa, B. tulda, Dendrocalamus halmiltonii, Arundinaria callosa, , Bambusa pallida* etc., were collected during the growing season(month of May–September 2008) from different districts/localities of Manipur(Churachandpur, Khongkang, Tengnoupal,Phalbung, Kangpokpi and Bashikhong) . Different portions of the fresh succulent bamboo shoots (outer hard sheath, inner soft shoots and other parts of the bamboo plants) were assessed for cyanide content

The traditionally fermented samples were collected from different districts/localities in Manipur where traditional fermentation of bamboo shoots is done in large scales (Khongkhang , Andro,Noneh, Tengnoupal,Churachandpur,Kotha etc.). Bamboo shoots of many species like *Bambusa tulda, B. balcoa, Dendrocalamus hamiltonii, Melocanna bambusoides, Arundanaria callosa* were used for fermentation

Estimation of Cyanogenic Glycosides:

Cyanogenic glycosides estimation was done using the technique of the picrate-impregnated paper according to Bradbury et al., 1999. Fresh plant material (bamboo shoots) was cut into small thin slices and placed into a small flat bottomed vial. Phosphate buffer (0.5ml of 0.1M at pH7) was added followed by brief crushing the materials with a glass rod. A picrate paper (fig.4) attached to a plastic backing strip was added and the vial immediately closed with a screw stopper. After about 16h at 30^{o} C, the picrate paper was removed and immersed in 5.0ml water for not less than 30 min. The absorbance was measured at510nm and the total cyanide content was determined

Results and Discussions

The results in table. 1 give the total cyanide content of tip, middle, and base of the outer hard sheath (discarded portion) covering the soft inner tissues and the inner soft bamboo shoots samples taken for consumption as food determined by the picrate method. The results showed an average of 0.02 to 0.17mg/g of HCN in the outer hard sheath and 0.03 to 1.7 mg/g of HCN in the soft portion of the bamboo shoots. The total cyanide levels are highest at the tip and lowest at the base of the soft inner shoot but just the reverse for the hard cover sheath. Table 2 represents the total cyanide content in different portion of the bamboo plants(*Melocanna bambusoides and Bambusa pallida).* The fleshy fruits of muli- *Melocanna bambusoides (fig.5)a*re eaten raw or cooked –its seeds are also eaten by the people as a substitute for rice.It also content low concentration of HCN(0.01mg/g) which renders it toxic free for consumption. The rhizome ,which is not utilized contain high content of

HCN(0.14mg/g). The acute lethal dose of HCN for human beings is 0.5-3.5 mg/kg body weight, animals is 0.66 to 15mg/kg body weight .Cyanide inhibits the action of cytochrome oxidase, carbonic anhydrase & other enzyme system. It blocks the final step of oxidative phosphorylation and prevents the formation of ATP and its use as energy source.It reduce the oxygen carrying capacity of the blood by combining with the ferric iron atom (Harborne1972,1993) .

Bamboo shoots may contain significantly higher levels of HCN, however ,the HCN content is reduced substantially during fermentation processing prior to consumption as in Table 3 . Since HCN are highly volatile ,the loss of HCN during the fermentation processes like peeling,slicing,cutting ,repeated washing(3-4 times) is quite rapid. During cooking/parboiling ,roasting and canning reduces the HCN below the toxic level . Boiling bamboo shoots for 20 min. at 98^0 C removed nearly 70% of the total HCN content but higher temperature and longer intervals removed progressively up to 96%(Ferreira et al., 1995). Thus it may perhaps not present a problem for consumers. However, due care in preparation remain necessary.

References

Bell, E.A.1981.The biochemistry of plants.Academic Press,New York

Bradbury,M.G., Egan,S.V.,and Bradbury,J.H.1999.Determination of all forms of cyanogens in cassava roots and cassava products using picrate paper kits. Journal of the Science of food and agriculture, 49, 93-99

Cheeke,P.r.1995.Endogenous toxins and mycotoxins in forage and their effects on livestock.J.Ani.Sci.,73,909-918

Conn , E.E. 1979. Cyanogenic glycosides. International review of biochemistry. In Biochemistry and Nutrition 1A, Neuberger, A., & Jukes, T.H. (eds), University Park Press, Baltimore, 27 ,21-43.

Cooper-Driver,G.A.;Swain,T.1976. Cyanogenic polymorphism in bracken in relation to hervivore predation .Nature, 260 ,604

Ferriera,V.L.P.; Yotsuyanagi ,K.; and Carvalho, C.R.L.1995. Elimination of cyanogenic compounds from bamboo shoots *Dendrocalamus giganteus* Munro.Trop.Sci.,35,342-346

Gruhnert,C.:Biehl,B.;Selmar,D.1994.compartmentation of cyanogenic glucosides and their degrading enzymes.Planta,195,36-42

Harborne, J.B. 1972 .Cyanogenic glucosides and their function. In: Phytochemical ecology.Academic Press, London,104-123

Harborne,J.B.1993. Plant toxins and their effects on animals. In: introduction to Ecological Biochemistry. Academic Press,London,71-103

Nartey, F. 1980. Toxicological aspects of cyanogenesis in tropical foodstuffs in Toxicology in the Tropics. Editors R.L. Smith and E.A. Bababumni, Taylor & Francis Ltd, London, 53-73.

Rosling,H.1994. Measuring effects in humans of dietary cyanide exposure from cassava.Acta Horticulture,375,271-283

Schwarzmaier,U.1977.Cyanogenesis of *Dendrocalamus*: taxiphyllin. Phytochemistry .16,1599-1600

Woodhead,S.; Bernays,E.1977.Change in release rates of cyanide in relation to palatability of *Sorghum* to insects.Nature,270,235-236

World Health Organization (WHO) 1993. Toxicological evaluation of certain food additives and naturally occurring toxicants. WHO Food Additive Series;30. World Health Organization, Geneva.

Table.1: Total cyanide content in bamboo plants determined by Picrate method

Name of the Species	Portion of the fresh bamboo shoots	Conc. of HCN (mg/g) in outer hard sheath covering the soft shoots	Conc. of HCN (mg/g) in inner soft bamboo shoots
Bambusa balcooa	Tip	0.02	2.15
	Middle	0.036	1.38
	Base	0.086	0.62
Dedrocalamus hamiltonii	Tip	0.043	2.42
	Middle	0.063	0.86
	Base	0.104	0.15
Arundanaria callosa	Tip	0.02	0.14
	Middle	0.01	0.05
	Base	0.07	0.03
Bambusa tulda	Tip	0.08	0.17
	Middle	0.10	0.83
	Base	0.13	0.28
Melocanna bambusoides	Tip	0.06	1.81
	Middle	0.10	0.68
	Base	0.17	0.35
Bambusa palli	Tip	0.04	0.27
	Middle	0.08	0.17
	Base	0.12	0.13

Table.2: Total cyanide content in different parts of the bamboo plants determined by Picrate method

Name of the species	Portion of the Plant	Conc. of HCN (mg/g)
Melocanna bambusoides	Fruit	0.01
	Leaves	0.09
	Inflorescence	0.07
	Rhizome	0.14
Bambusa pallida	Leaves	0.09
	Inflorescence	0.07
	Rhizome	0.04

Table.3: Total cyanide content in fermented bamboo shoot slices (soibum)

Sl.No	Fermented bamboo shoots collected from different districts of Manipur	Conc. of HCN (mg/g)
1	B. Salvaphai -CCpur District	0.21
2	Khongkang- CDL District	0.28
3	Tengnoupal -CDL District	0.29
4	Exudate (khongkang fermentation)	13.22

Fig.1 : Emmerging Young Succulent Bamboo Shoots

Fig.2 : Bamboo Shoot Slices for Fermentation process

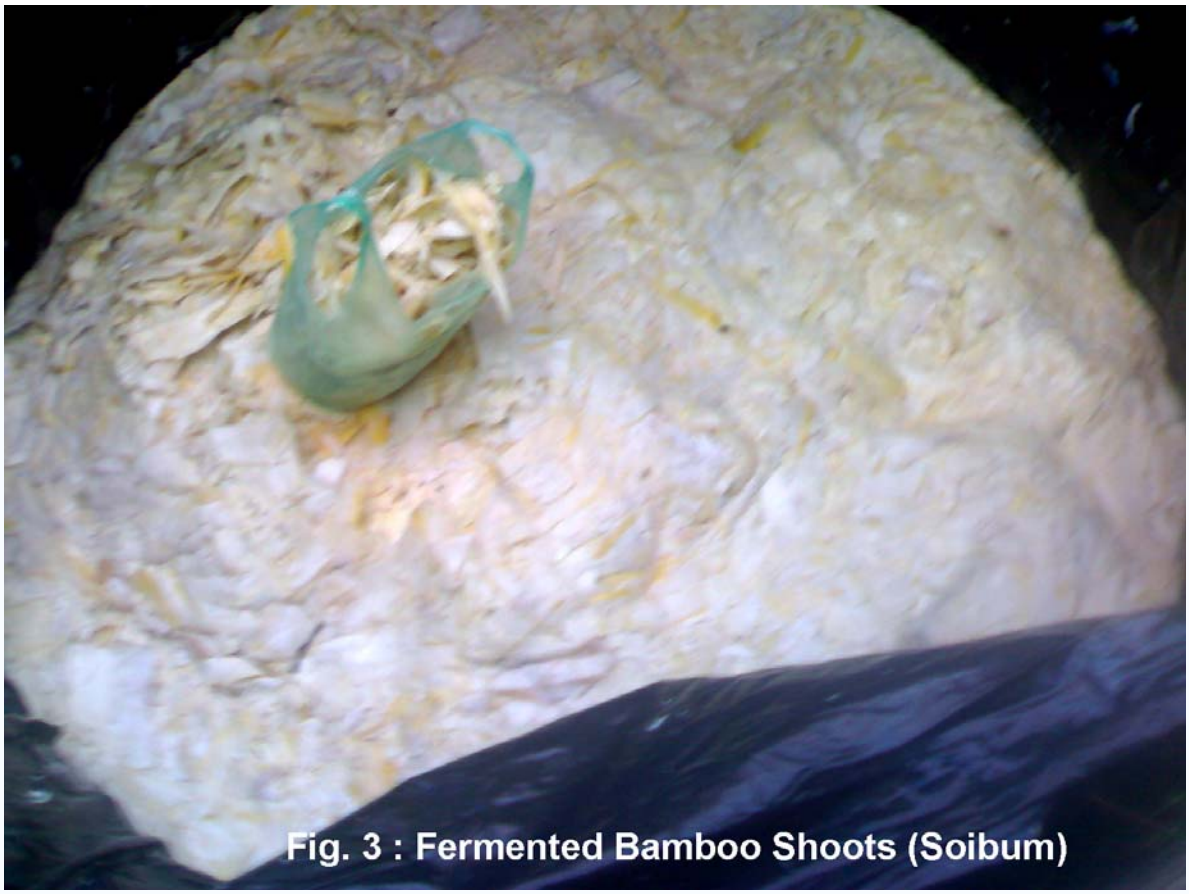


Fig. 3 : Fermented Bamboo Shoots (Soibum)

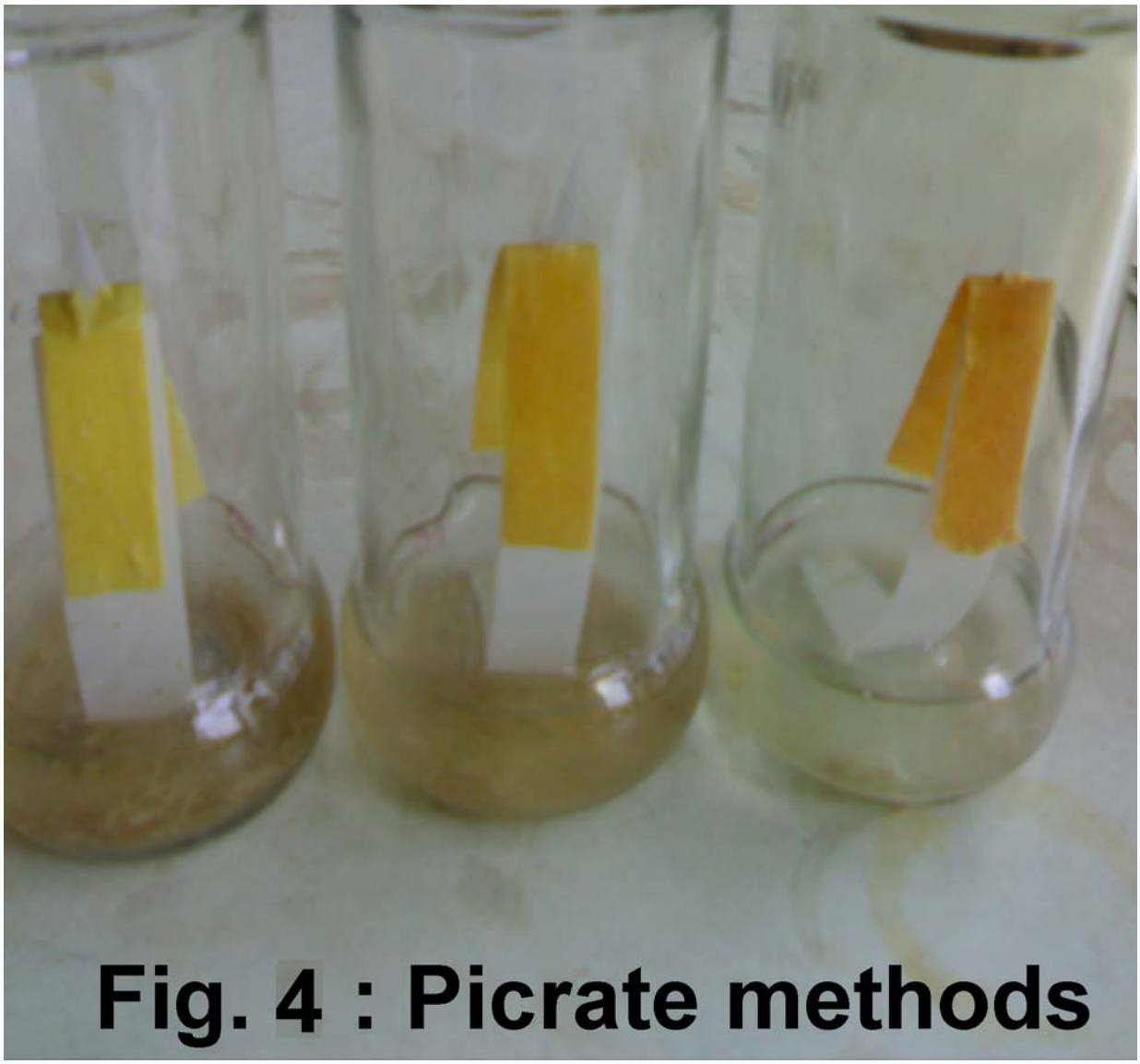


Fig. 4 : Picrate methods

Fig. 5 : Bamboo Seeds of Melocanna bambusoides

The First Report of Flowering and Fruiting Phenomenon of Melocanna baccifera in Nepal

Keshab Shrestha
Natural History Museum, Swayambhu, Kathmandu, Nepal

Abstract

Melocanna baccifera is a bamboo species found in eastern, central and western part of Nepal. Least information is available about this bamboo in Nepal so far. This bamboo showed sporadic flowering for the first time in Nepal in 2007-2008. Flower showed dimorphism, upper part being sterile where as the lower part is fertile bearing numerous pear shaped fruits. This paper deals with the flowering, fruiting phenomenon and ethnobotanical use of this bamboo species in Nepal.

Introduction

Nepal is a small country in the world occupying about 0.09 percent of the earth's surface with the area of 147,181 sq.km. Due to topographical variation within a short range starting from 64 m of elevation to the highest altitude of 8,848 m, Nepal is regarded as a high biodiversity zone and possesses 6500 species of higher plants including Bamboo species. Nepal possesses 81 species of bamboo out of 1,573 species worldwide. It comes to be about 24 percent in world's ratio.

In Nepal, bamboo occupies about 62,890 hectares of land. The natural forest hosts 38,000 hectares and rest is agricultural land. The total standing stock has been estimated at 15 million cubic meters with biomass value of 1,060 metric tons. The annual production of bamboo is estimated at 3.01 million cubic of which 2.64 million culms are consumed locally and 0.64 million culms are exported to India (Kesari, 2005).

Nepal has 5 genera and 27 species under large bamboo species which are commonly called *Bans* in local language and come in Bambusae tribe. Small bamboos include 15 genera and 35 species. On the other hand 3 genera and 4 species fall under dwarf bamboo species. Of them, 45 species are indigenous and rest is exotic. *Melocanna baccifera* is a large bamboo species found in Nepal.

Methodology

Eastern and Central Nepal were visited in course of regular plant survey from Natural History Museum, likewise a private visit was also arranged to study the fruiting phenomenon. Informations were gathered from the local people. Interviewing with local inhabitants and collection of the samples were done during the flowering and non-flowering seasons. Relevant literatures (Poudyal, 2006, Keshari (2005), Shrestha 1998, 200; Stapleton

1994, Gamble 1896 and Shibata personal communication 2008) were consulted. Help from sketching and photographs were also taken. Other associated plants were also recorded with their local names

Result

There are altogether five species of *Melocanna*, but Nepal represents only one described species so far. The author for the first time found this species from east Nepal in 1996. This species is very interesting in terms of culms, rhizomes and fruits, but there is no report of fruiting of this species till 2008.

The author found the species in Bahundangi and Sanischare village of Jhapa district in east Nepal in 1996. The culms were found growing along the edge of the paddy field where it formed a line of culms surrounding the agricultural land. The bamboo was erect, smooth without any branches and was cylindrical (Fig. 1). The erect shoot has uniform culms whose diameter was almost 5.0 cm and culm height ranges from 15.0 to 18.3 cm, the culms were green and spiny, occasionally with yellowish-green internodes and white cuticles below the nodes. The culm-wall was thin and non durable. Culm-sheaths were persistent and brittle. Sheath blade was very long and narrow. Half of the culms were without branches. Almost similar sized branches arose from every node. The leaves were large.

Flowers: For the first time in Nepal flowers appeared in 2007. The flower started to appear in 2007 and lasted till the summer of 2008. Thereafter large sized fruiting resembling a pear appeared in early 2008. Its fruiting attracted many peoples of that area.

Inflorescence: The inflorescence was large compound panicle. Spikelets were acuminate fasciculate and one sided (Fig. 2). There were two types of flowers; one was in fertile stage and the next on sterile nodes in the same culm. The fertile flowers were at the lower nodes whereas sterile were at the upper nodes of the culms. There were several sterile and fertile flowers arising from the same nodes and were hanging down from the nodes (Fig. 3,4).

Empty sterile glumes were indefinite, acuminate, and striate. Flowering glumes similar to empty glumes, palea also similar, not keeled.

Lodules two and narrow
Stamens five to seven
Filaments free or irregularly joined
Ovary glabrous
Style elongated
Stigma two to four, short and hairy
Fruit caryopsis, very large and pear shaped (Fig 5) with long beaked pericarp very thick, Greenish-yellowish-white skin externally. Small whitish ovules were embedded in a cavity filled with liquid (Fig.6)

Ethnobotany: *Melocanna baccifera* are reported in many parts of Nepal except the far west region. In the eastern Jhapa, Central in Rautahat and Chitwan and Pokhara, Syangja and Palpa districts in the west.

Locally the species is known as *Philinge Bans* in the eastern and *Lahure Bans* in the western Nepal. In central Nepal, it does not have any common name.

Since the fruiting was not observed but most of the peoples believe that the bamboo has never a big fruit like *Melocanna* and the fruiting is due to some misfortune. Due to this ignorancy, villagers cut all the culms and throw them away (Fig. 7).

The fruits are used as game ball for children. Hundreds of people visited this place to see this unique body of fruit (Fig. 8). Children generally cut the fruit and taste the sap inside which they liked most due to its sweet test like that of coconut-fluid. People do not have idea that the shoots are edible, but villagers before fruiting used the culm for basketry, mat, house wall, roof gum or fluid and leaves as fodder. The bamboos were planted nearby their houses or huts and kitchen garden. Other species of bamboo like *Dendrocalamus strictus, Bambusa nutans, and Dendrocalamus gigantean* were also noticed in central and east Nepal. They are used as hedge to boarder paddy fields and consider ornamental due to its beautiful poles and amphimorph or metamorph nature. Due to its more publicity, media were also attracted to the village and made interesting telecast in television also. Popular newspapers are looking for more information about this bamboo species. The author made clear of the rumors that such phenomena with this bamboo occur once in 7-51 years in other countries like India, Bangladesh, China, Indonesia, Myanmar and Sri Lanka. This was the first observation in Nepal; this bamboo has many values and should be conserved effectively.

Table 1. Associate Species around *Melocanna baccifera* Grove in Pourai Village, Rautahat District

1. *Ficus semicordata* (Kanyu)
2. *Morus macroura* (Kimbu)
3. *Zingiber*
4. *Anthocephalus chinensis* (Kadam)
5. *Zizyphus mauritiana* (Bayer)
6. *Bauhinia variegata* (Koiralo)
7. *Syzygium cumini* (Jamun)
8. *Piper longum* (Pipla)
9. *Solanum surrattense* (Kantakari)
10. *Cissampelos pareira* (Jaluko)
11. *Musa paradisiaca* (Kera)
12. *Ageratum conyzoides* (Gande)
13. *Amaranthus spinosus* (Lunde kanda)
14. *Shorea robusta* (Sal)
15. *Dioscorea bulbifera*
16. *Colebrookea oppositifolia* (Gittha)
17. *Thysanolaena maxima* (Amliso)
18. *Ficus racemosa* (Dumri)
19. *Tinospora sinensis* (Gurjo)
20. *Annona squamata* (Saripha)
21. *Dalbergia sisso* (Sisau)
22. *Eupatorium odoratum* (Tite hawi)
23. *Moringa oleifera* (Sahijan)
24. *Bombax ceiba*
25. *Caesalpinia dicapeta* (Arile kanda)
26. *Mimosa pudica* (Lajwanti)
27. *Ricinus communis* (Ander)
28. *Cynodon dactylon* (Dubo)
29. *Stellaria monosperma* (Jethi madhu)
30. *Bambusa nutans* (Mal bans)
31. *Dendrocalamus strictus* (Lathi bans)
32. *Dendrocalamus hookeri* (Bhalu bans)
33. *Bambusa multiplex*
34. *Schleichera oleosa* (Kusum)
35. *Prunus persica* (Aru)
36. *Anogeissus latifolia* (Bhanjhi)
37. *Litchi chinensis* (Litchi)
38. *Perilla frutescens* (Silam)
39. *Lagerstoemia parviflora* (Botdhaiyaro)
40. *Cannabis sativa* (Bhang)
41. *Persicaria pentagyna* (Pire)
42. *Polygonum hidropiper*
43. *Diplazium esculentum* (Pani nyuro)

Ethnic people of the area in Pourai Village: Tamang, Thing, Bhote, Bamjang (Fig. 9), Jimba, Pakhin, Gurung, Magar, Yonzon, Majhi, Gongaba, Thokar, Syanba, Kami, Damai, Danuwar, Rai, Shrestha

Others: Mainali, Nyoupane (Brahmins) and Chhetri.

Occupation: Agriculture and forestry.

Conclusion

Flowering in *Melocanna beccifera* occurs after a period of 30-40 years, but the propagation of this bamboo is as easy as other species. Seeds if available propagates easily and from propagates this species can easily be proliferated even in Kathmandu. The fruits very easily fall down on ground even by a gentle breeze or wind and germinate quickly.

Large pear shaped fruiting makes the bamboo very attractive, the villagers in Nepal have no idea about the importance of this species and believe on misfortune when the plant blossom. They also destroyed all the calms after flowering and fruiting due to ignorancy. Fruits and young shoots are eaten in Bangladesh and India. Culms are strongly used to different purposes including paper making and scaffolding.

This plant if used under sustainable way may help to reduce poverty to some extent. This plant adds beautifying orchard, control erosion and help to bring prosperity in the society. Conservation education has been felt essential so to conserve bamboos like *Melocanna* species in Nepal.

Acknowledgement

The author is thankful to the Natural History Museum, Tribhuvan University for all the necessary permission for this study. Thanks are due to the local and media peoples of that area for encouragement and remarks on the conservation of the bamboo. Last but not the least thank is due to Mr. Bhaiya Khanal, Associate Professor for his constant help in the field and reading this manuscript.

Reference

Anonymous 2008, Samacharpatra daily newspaper. May 10

----------1998. Distribution of Bamboos in India. Bamboo and Rattan Genetic Resources in Certain Asian Countries. Edited by Vivekananda, K., A.N. Rao and V. Ramanatha Rao. IPGRI, INBAR, Malaysia.Pp 46-61.

Gamble, J. S. 1896: The Bambusae of British India, Annals of the Royal Botanic Garden, Calcutta, Micro Methods Limited, Johnson Reprint Corporation.

Keshari, V.P. 2005 Bamboo: From Poor Man's Timber to Green Gold. Hamro Kalpana Brikshya (15) (164): 10-14, Department of Forest, Kathmandu, Nepal.

Poudyal, P.P. (2006): Bamboos of Sikkim (India), Bhutan and Nepal. New Hira Books Enterprises, Nepal.

Seethalakshi, K.K and M.S. Muktesh Kumar1998Bamboos of India a compendium. Bamboo Information centre-India, Kerala Forest Research Institute, Peechi and International Network for Bamboo and Rattan, Beijing, Endogen and New Delhi

Shibata S. 2008. Personnel communication by email.

Shrestha, K. 1998. Distribution and Status of Bamboos in Nepal. Bamboo Workshop and Conference, Integrated Plant Genetic Research International, Beijing, RP. China www2.bioversityinternational.org/publications/Web_version/572/ch29.htm

Shrestha, K. (2008): Bamboo and Environment (In Nepali); Hamro Sampada Ed. S.K. Basnet. P.p. 175-177.

Stapleton, C. (1994): Bamboos of Nepal. Royal Botanic Garden, Kew, Richmond, Survey. TW9 3AE, England, U.K.

Fig.1.Melocanna baccifera in the paddy field

Fig.2. Flowering spikelet

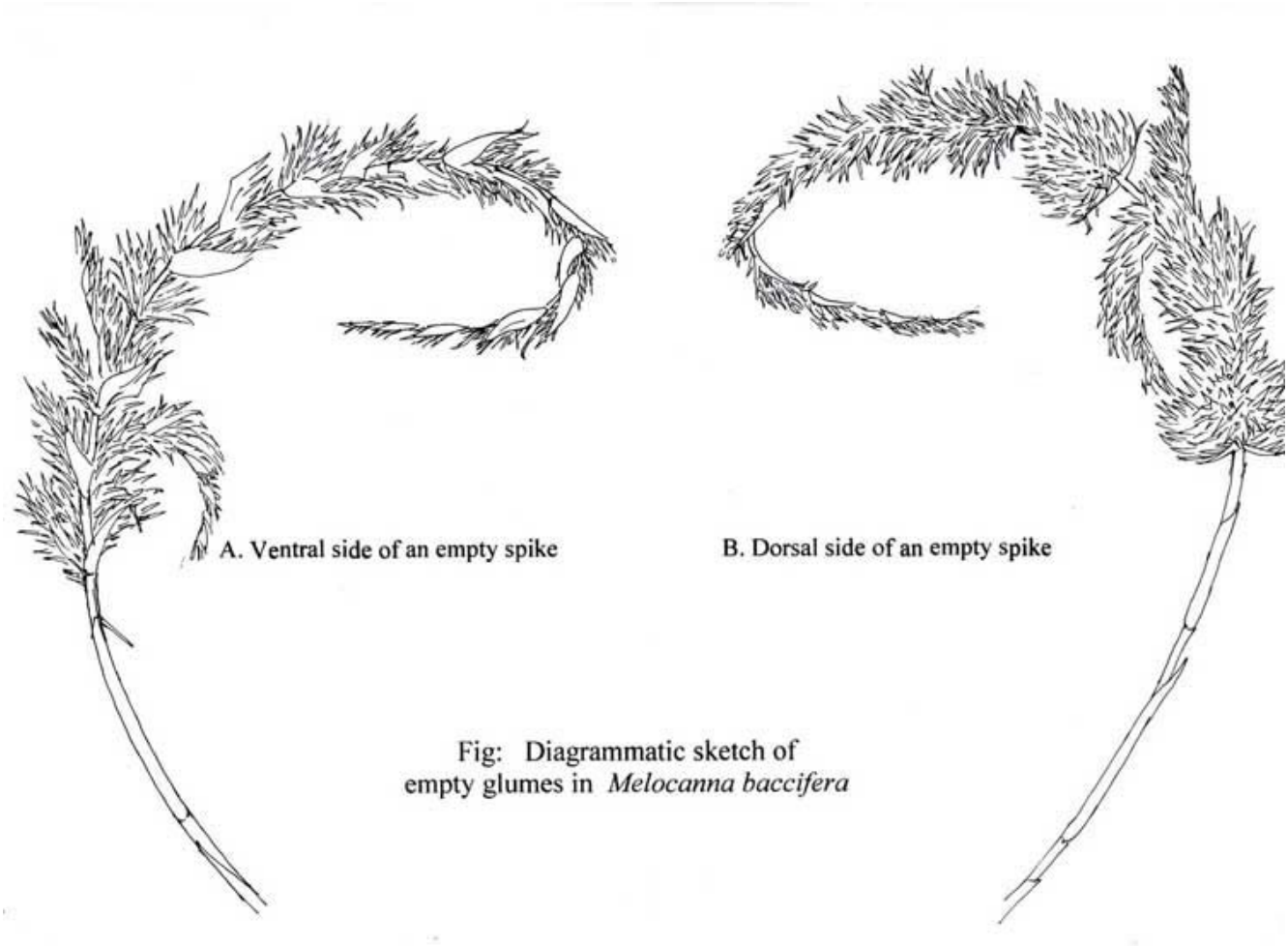


Fig: Diagrammatic sketch of empty glumes in *Melocanna baccifera*

Fig.3 Empty glume

Fig. 4 Sterile flowers

Fig.5. Fruits and flowers together

Fig. 6. Ovule in the cavity

Fig.7. Removing culm with fruits by the villagers

Fig.8. Villagers play with the fruits and flowers

Fig. 9. Melocanna culm in basketary and cage in the village

Species Relationships in *Dendrocalamus* Inferred from AFLP Fingerprints

S. Pattanaik* and J.B. Hall**
*Scientist, Rain Forest Research Institute (ICFRE), Jorhat, India.
**Director, Postgraduate Studies, School of the Environment & Natural Resources, Bangor University, United Kingdom.

Abstract

Species of *Dendrocalamus* are characterized by their sympodial rhizomes and large sized dense clumps. The genus contains over fifty species from tropical and subtropical region of the old world, many of which are economically exploited by rural communities in south and southeast Asia. The original description of the genus was based on the type species *Dendrocalamus strictus*, which was subsequently expanded to include pericarp characters that were used to distinguish between *Dendrocalamus* and *Bambusa* (Munro 1868; Bentham 1883; Gamble 1896). While at present it is taxonomically convenient for *Dendrocalamus* to be recognized in a broad sense (its species being distinguished by the presence of single-keeled prophylls throughout the inflorescence - Stapleton 1991), the limits between *Bambusa* and *Dendrocalamus* are not satisfactorily defined. In the present study amplified fragment length polymorphism markers (AFLPs) were used to investigate phylogenetic relationships among ten included *Dendrocalamus* and five out group species. Neighbour-Joining and Maximum parsimony analyses of AFLP dataset suggested the current circumscription of *Dendrocalamus* to be polyphyletic. Further, the analyses did not find support for the various earlier infrageneric classifications within *Dendrocalamus*. The implications of the findings are discussed.

Introduction

Dendrocalamus is a woody bamboo genus placed in the subtribe Bambusinae and tribe Bambuseae (Ohrnberger 1999). Species referred to the genus are characterized by their sympodial rhizomes and large sized dense clumps. The genus contains over fifty species, naturally distributed in the tropical and subtropical region of the old world, many of which are economically exploited by the communities in south and southeast Asia. The original description of the genus was based on the type species *D. strictus*. The description was expanded subsequently to include pericarp characters, which were used to distinguish between *Dendrocalamus* and *Bambusa* (Munro 1868, Bentham 1883, Gamble 1896). While at present it is taxonomically convenient for *Dendrocalamus* to be recognized in a broad sense, the limits between *Bambusa* and *Dendrocalamus* are not satisfactorily defined thus creating confusion in their systematic classification. And lack of sound taxonomy is acting as hindrance in the scientific conservation and management of the woody bamboos belonging to this genus.

Since *Dendrocalamus* was separated from *Bambusa* by Nees von Esenbeck in 1834, over 70 species names have been assigned to the genus although Ohrnberger (1999) retains only 51 of these. Most of the species that have not been maintained by Ohrnberger have been reduced to synonymy or to infraspecific rank. A few are transferred to, or sunk into, other genera: *Ampelocalamus* (subtribe Thamnocalaminae); *Gigantochloa* and *Pseudoxytenanthera* (Bambusinae).

Various infrageneric classifications of *Dendrocalamus* have been adopted by Chinese botanists. Hsueh and Li (1988) proposed the first infrageneric classification of *Dendrocalamus* by recognizing two subgenera and five sections, limiting the assignments to only those species reported from China. Ohrnberger (1999) assigned species only to sections *Dendrocalamus*, *Bambusoidetes*, *Sinocalamus* and *Draconicalamus*. Out of the 51 taxa recognized by Ohrnberger, 22 were assigned to particular sections while 29 taxa were unplaced. A more recent taxonomic revision of Chinese *Dendrocalamus* (Li and Stapleton 2006) retains the subgenera proposed by Hsueh and Li (1988) but disregards sectional assignments, merging sections *Dendrocalamus* and *Bambusoidetes* as subgenus *Dendrocalamus,* and sections *Sinocalamus* and *Draconicalamus* as subgenus *Sinocalamus*. Li and Stapleton (2006) transferred 11 taxa previously referred to subgenus *Sinocalamus* to subgenus *Dendrocalamus*. The major problem faced in the infrageneric classification of *Dendrocalamus* is the paucity of published morphological character information for many of the species. Twenty seven species do not appear to have been referred to any subgenus or section under any of the proposed schemes.

Bamboos have always been a taxonomically challenging group of plants because, while the classification of flowering plants depends largely on the characteristics of reproductive organs, flowering is rare in many bamboo species. Some bamboo species flower at intervals as long as 120 years and for some there is no report of flowering to date. Because of apparent paucity of morphological characters in bamboos, taxonomists have long sought different sources of taxonomically informative data. The availability of molecular data in the final decade of the twentieth century enabled taxonomists to review phylogenetic concepts of the Poaceae more objectively. Initially DNA products *viz.*, isozymes and secondary compounds like phenolics were used in exploring relationships among taxa (Chou and Hwang 1985), species identification (Alam *et al.* 1997) and assessment of infraspecific polymorphism (Biswas 1998). In a study involving five *Dendrocalamus* taxa, *Arthrostylidium naibunensis* W.C. Lin and *Chimonobambusa quadrangularis* Makino, a *Dendrocalamus* cluster could be differentiated from the other two genera using phenolic compounds and isozyme patterns of esterase and peroxidase. Within *Dendrocalamus* two clusters were recognized: *Dendrocalamus asper* associated with *D. giganteus,* while *D. latiflorus* associated with its variety *D. latiflorus* var. *mei-nung*. However, *Dendrocalamus strictus* was distant from these two clusters. Later on, variation in DNA itself was the subject of investigations. The more pertinent studies involving named *Dendrocalamus* taxa are those of Loh *et al.* (2000) and Sun *et al*. (2005) but these studies entailed only limited sampling of the genus. In the first, two *Dendrocalamus* taxa were sampled, with *D. brandisii* clustering with taxa from *Bambusa,* and *D. giganteus* appearing genetically distant from all other taxa included. In the second study three *Dendrocalamus* taxa were sampled. These three taxa did not form a separate clade but clustered within *Bambusa,* which was split into two distinct clades. *D. membranaceus* showed close affinity to *D. strictus* and both were placed within one *Bambusa* clade, whereas *D. latiflorus* was associated with the other *Bambusa* clade. The study reported wide genetic variation within *Dendrocalamus* and raised questions about its monophyly.

These earlier molecular studies included limited samples from *Dendrocalamus* and did not throw much light on the infrageneric relationships within the genus. In the present investigation there was wider provision within *Dendrocalamus* with ten putative taxa, and five outgroup taxa from the subtribe Bambusinae. Amplified fragment length polymorphism (AFLP) markers were used to (i) test the monophyly of *Dendrocalamus,* and (ii) assess the molecular support for various infrageneric assignments proposed in *Dendrocalamus.*

Materials and Methods

Site Description

Genetic material was collected from the bambuseta of five botanical gardens in India: Forest Research Institute, Dehra Dun; National Botanical Garden of Botanical Survey of India, Howrah; State Forest Research Institute, Chessa; ICAR research complex for northeastern hill region, Basar; Rain Forest Research Institute, Jorhat. The genetic material of the monotypic African bamboo *Oxytenanthera abyssinica* was available as the result of previous research work in Bangor, UK (Inada 2004).

Genetic Material

Leaves were collected from ten *Dendrocalamus* and five outgroup taxa from subtribe Bambusinae. The leaves were dried using silica gel as per the procedure of Chase and Hills (1991) and then transported from the field sites in India to the laboratory at CAZS - Natural Resources, Bangor University, for DNA extraction and analysis. Six of the *Dendrocalamus* taxa (*D. strictus, D. hamiltonii, D. membranaceus, D. brandisii, D. sikkimensis, D. asper*) represented subgenus *Dendrocalamus* and two other taxa (*D. giganteus* and *D. calostachyus*) represented subgenus *Sinocalamus*, in the infrageneric classification of *Dendrocalamus* by Li and Stapleton (2006). No information was available regarding the infrageneric assignment of *D. sahnii* and *D. somdevai.* The outgroup taxa were from the genera *Bambusa, Melocalamus, Oxytenanthera, Dinochloa* and *Thyrsostachys*, all of which belong, like *Dendrocalamus,* to subtribe Bambusinae as recognized by Ohrnberger (1999).

DNA Extraction

DNA was extracted from 50 mg of dried leaf tissue using a modified CTAB protocol of Doyle and Doyle (Doyle and Doyle 1990). The DNA extractions were checked for quality by running a 1% agarose mini-gel (run at 50 V for 30 minutes) in TBE buffer (1 X) containing 0.5 μg/ml ethidium bromide. The genomic DNA was visualized and photographed under a ultra-violet light source. Quantitation of genomic DNA was done using the fluorescent dye Pico green in the Fluostar Galaxy Fluorometer.

Generation of AFLP Markers

The AFLP assay was performed following the protocol of Vos *et al*. (1995), adapted for the Beckman Coulter Sequencer. The process was carried out in four steps. In the first step, two restriction enzymes *Eco*RI and *Mse*I (*Tru*9I) were used to digest the genomic DNA of the samples. In the second step, double stranded adapters

complementary to the cut ends (overhangs) produced by enzymes *Eco*RI and *Mse*I were ligated to the cut DNA fragments. In the third step, a PCR (pre-selective PCR) was performed with universal primers E00 and M00. The thermocycler conditions included 30 cycles consisting of 30 seconds denaturation at 94°C, 60 seconds annealing at 56°C, 60 seconds extension at 72°C and finally 600 seconds extension at 72°C. In the fourth step, a second PCR (selective PCR) was done with selective primers, each with three nucleotide extensions (E00+3; M00+3). The selective primer E00+3 was end-labelled with fluorescent dye D4 instead of the radioactive labelling described in the original protocol of Vos *et al.* (1995). The thermocycler conditions included 13 touchdown cycles to avoid amplifying non-specific sequences (30 seconds denaturation at 94°C, 30 seconds annealing at 65°C which was then reduced by 0.7°C per cycle, 60 seconds extension at 72°C), 23 normal cycles (30 seconds denaturation at 94°C, 30 seconds annealing at 56°C, 60 seconds extension at 72°C) and 420 seconds final extension at 72°C.

A primer screening experiment was done to select five primer sets, which were then used to amplify AFLP markers from the fifteen taxa included in the present investigation. A negative control (without template DNA) was run in each batch of PCRs to confirm that no contamination had occurred. The reproducibility of AFLP peaks was checked by repeating the whole process a number of times.

Separation and Scoring of AFLP Markers

The selective PCR products were separated through capillary gel electrophoresis in the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc.) and analysed with the fragment analysis software. During fragment analysis the separated fragments were sized with the use of internal size standards (PA400). Following this, the sized fragments were subjected to an AFLP binning analysis that converted the AFLP peak profiles into binary matrix. The presence of a peak was scored 1 and its absence scored 0. Peaks of size ranging from 60 bp to 400 bp were scored.

Data Analysis

Phenetic Analysis

The binary matrix (470 X 15) of multilocus peak patterns generated by the scoring software in CEQ 8000 was converted to a matrix of pairwise distances between OTUs expressed as Jaccard's (Jaccard 1908) distance coefficient using the software package NTSYSpc 2.11X (Rohlf 2000).

Jaccard's distance coefficient was derived as
$D_J = 1 - [a / (n - d)]$,

where:
n, total sample size (a + b + c + d).
a, number of peaks common to both taxa
b, number of peaks for the first taxon
c, number of peaks for the second taxon
d, number of peaks absent from both taxa

Cluster analysis was carried out using the neighbour-joining (NJ) algorithm of Saitou and Nei (Saitou and Nei 1987) in NTSYSpc 2.11X (Rohlf 2000). The generated tree was rooted using the outgroup option, taking *Oxytenanthera abyssinica* as the outgroup taxon. The statistical support for the internal branches was assessed by performing a bootstrap analysis with 1000 replicates in the software package FREETREE (Pavlicek *et al.* 1999). A Mantel test was performed to test how well the phenogram represented the inter-OTU distances, following the procedure described by Koopman *et al.* (2001).

Phylogenetic analysis

Cladistic analysis of the AFLP dataset (470 X 15) was performed under maximum parsimony criterion with PAUP 4.0b10 (Swofford 2002). The large number of includedtaxa (>12) ruled out an exhaustive search. So, heuristic search was used to identify the most parsimonious tree. Heuristic search was performed with the following criteria -1000 replicates, random additions of sequence, tree-bisection-reconnection (TBR) branch swapping, character optimizations using accelerated transformation (Perrie and Brownsey 2005). Output trees were rooted using the outgroup option with *Oxytenanthera abyssinica* as the outgroup taxon. Statistical support for internal branches was assessed using the bootstrap analysis (Felsenstein 1985) in PAUP 4.0b10 with following criteria – 1000 replicates, heuristic search and a 50% confidence level.

Results

The five AFLP primer sets used in the present investigation generated 609 marker loci, out of which 99.2 % (604) loci were polymorphic and only 0.8 % (5) loci were monomorphic across the 15 OTUs. The dataset also contained 134 (22.0 %) loci where only one peak was detected. The number of AFLP marker loci generated by the individual primer sets varied from 101 to 133 with a mean of 121.8.

The genetic distance estimates based on Jaccard's measure varied from 0.47 to 0.92 (Table 1). Although referred to the Bambusinae, the monotypic African bamboo *Oxytenanthera abyssinica* shared very few peaks with other taxa included in the study and was found genetically distant from them (distance ranged from 0.77 to 0.92). However, even within *Dendrocalamus* there was wide genetic variation. *Dendrocalamus strictus* appeared isolated with a minimum distance of 0.77 (from *D. somdevai*) and a maximum distance of 0.85 (from *D. asper*). Among the outgroups included in the present investigation *Bambusa balcooa* was found to be the closest to *Dendrocalamus sensu lato* with a mean distance of 0.60.

Cluster analysis with the neighbour-joining algorithm resulted in a single tree (Figure 1) with high co-phenetic correlation coefficient (r = 0.971). A well-supported (85% bootstrap support) major cluster was recovered containing all *Dendrocalamus* taxa (except *D. strictus*) with *Melocalamus compactiflorus* as sister lineage. *D. strictus* was recovered near the root of the tree distant from the major cluster containing other *Dendrocalamus.* Three clusters could be recognized within the major cluster that had varying degree (above 50%) of bootstrap support. Cluster 1 (partially supported with bootstrap support of 93%) consisted of *Dendrocalamus membranaceus, D. somdevai* and *D. brandisii.* Cluster 2 (89% bootstrap support) consisted of *Dendrocalamus sikkimensis, Bambusa balcooa* and *D. hamiltonii.* Cluster 3 consisted of *D. giganteus* and *D. asper*.

Table 1 Half-matrix of pairwise Jaccard's distance coefficients between 15 operational taxonomic units. Rows are labelled with taxon name (D. = *Dendrocalamus*). Columns are labelled with accession code only.

Operational taxonomic units	S3	S4	S9	S10	S11	S15	S30	S13	S14	S23	S27	S52	S6	31	S32
(S3) *D. membranaceus*	0														
(S4) *D. somdevai*	**0.51**	0													
(S9) *D. brandisii*	**0.55**	**0.55**	0												
(S10) *D. giganteus*	0.66	0.65	0.60	0											
(S11) *D. sikkimensis*	0.68	**0.59**	**0.59**	0.64	0										
(S15) *D. sahnii*	0.61	**0.56**	**0.57**	**0.58**	**0.56**	0									
(S30) *D. calostachyus*	0.63	0.60	**0.53**	**0.57**	**0.57**	**0.55**	0								
(S13) *Dinochloa*	0.66	0.63	0.61	0.69	0.64	0.62	**0.58**	0							
(S14) *Thyrsostachys*	0.69	0.68	0.70	0.75	0.74	0.73	0.71	0.73	0						
(S23) *Bambusa*	0.65	**0.59**	**0.57**	0.61	**0.51**	**0.54**	**0.54**	0.63	0.74	0					
(S27) *Melocalamus*	0.69	0.67	0.68	0.66	0.67	0.63	0.63	0.70	0.73	0.66	0				
(S52) *D. strictus*	0.79	0.77	0.84	0.84	0.85	0.84	0.82	0.82	0.83	0.87	0.85	0			
(S6) *D. asper*	0.70	0.69	**0.59**	0.62	0.65	0.64	0.63	0.73	0.76	0.66	0.71	0.85	0		
(31) *D. hamiltonii*	0.65	**0.54**	**0.58**	0.62	**0.52**	**0.53**	**0.55**	0.66	0.72	**0.47**	0.68	0.83	0.60	0	
(S32) *Oxytenanthera*	0.83	0.83	0.82	0.83	0.82	0.81	0.80	0.84	0.84	0.83	0.83	0.92	0.82	0.77	0

However, the relative relationship among these three clusters was not clear due to polytomy. Further, the affinity of *D. sahnii, D. calostachyus* and *Dinochloa macclellandii* remained unresolved.

Maximum parsimony analysis of the AFLP dataset (consisting of 470 characters out of which 460 were parsimony informative) with heuristic search yielded two equally parsimonious trees [Figure 2(a) and (b)], giving two alternate but equally likely hypotheses of evolutionary relationships among the OTUs. Each tree was 1468 steps (character state changes) long with consistency index (CI) = 0.320, retention index (RI) = 0.298 and rescaled consistency index (RC) = 0.095. The two most parsimonious trees were congruent for most part of the trees but differed with the placement of *Dendrocalamus calostachyus*. Strict consensus of the parsimonious trees resulted in a polytomy (Figure 3). Three clades were recovered that were identical to the clusters in Neighbour-Joining tree. Because of polytomy the relationship among these three clades was not clear. Bootstrap analysis with 1000 bootstrap replicates showed partial support for the three clades. Only part of clade 1, part of clade 2 and clade 3 had above 50% bootstrap support.

Discussion

Neither the phenetic nor the phylogenetic approach adopted in the present investigation supported the monophyly of *Dendrocalamus* as currently circumscribed. The placement of *Bambusa balcooa* and *Dendrocalamus strictus* in the cladogram (Figure 3) and the placement of *Bambusa balcooa, Dendrocalamus strictus* and *Dinochloa macclellandii* in the phenogram (Figure 1), suggested otherwise. *Bambusa balcooa,* instead of forming a separate lineage, was recovered in a clade/cluster shared with *Dendrocalamus hamiltonii* and *D. sikkimensis*. This supports the findings of Stapleton (1994a) who had reported the closeness of *Bambusa balcooa* to *Dendrocalamus* species on morphological grounds, stressing similarity in the profuse aerial roots at the culm nodes, the large rhizomatous branch bases and the culm wax. Further similarities between *Bambusa balcooa* and *Dendrocalamus hamiltonii* can be found in the reproductive parts with both species having 3 stigmas each. *Dinochloa macclellandii* whose affinity was unresolved in the neighbour-joining phenogram (Figure 1), was recovered as a sister lineage to the clades containing *Dendrocalamus* in the most parsimonious trees (Figures 2a and b). The placement of *Dendrocalamus strictus* near the root of the tree away from rest of the *Dendrocalamus sensu lato* was not entirely unexpected considering the findings of Chou and Hwang (1985), who had reported the isolation of *D. strictus* from other *Dendrocalamus* taxa based on studies involving isozymes and phenolics.

Morphologically, the isolation of *D. strictus* could be explained by presence of inflorescence comprising of fascicular pseudospikelets (2.2–2.5 cm in diam.) on each node of flowering branches, distinguishing them from other species included in the study (Yang *et al.* 2008). Ecologically also, *D. strictus* is very distinct from the other *Dendrocalamus* taxa included in the present investigation. *Dendrocalamus strictus* naturally occurs in dry deciduous open forests, receiving as little as 750 mm annual rainfall and exposed to low relative humidity. The other *Dendrocalamus* taxa included in the present investigation are confined to moister areas (moist deciduous to wet evergreen forests), with an annual rainfall in excess of 1500 mm.

There was congruence between neighbour-joining cluster analysis and the maximum parsimony analysis as far as three monophyletic clusters/clades were concerned. The first of congruent groups consisted of

Dendrocalamus membranaceus, D. somdevai and *D. brandisii.* The second congruent group consisted of *Bambusa balcooa, Dendrocalamus hamiltonii* and *D. sikkimensis*. The third congruent group consisted of *Dendrocalamus giganteus* and *D. asper.* The placement of *Dinochloa macclellandii* differed in the two analyses. The affinity of *Dendrocalamus sahnii* and *D. calostachyus* were inconclusive in both the analyses. The three monophyletic clusters/clades did not agree to the sectional assignments within *Dendrocalamus sensu lato* circumscribed by Hsueh and Li (1988), Ohrnberger (1999), and Li & Stapleton (2006).

Bambusinae as circumscribed by Ohrnberger (1999) is an Old World tropical subtribe with its centre of diversity in southeast Asia. It contains seventeen genera, the relationships among which are not fully understood. In the present investigation *Bambusa balcooa* was placed within *Dendrocalamus sensu lato* supporting the closeness, or even inseparability of these two genera. *Melocalamus* and *Thyrsostachys* were recovered as sister lineages to *Dendrocalamus* and *Bambusa.* Watanabe *et al.* (1994), the first to study phylogenetic relationships among Asian bamboos using restriction fragment length polymorphism of chloroplast DNA, recovered a clade representing subtribe Bambusinae *sensu* Ohrnberger (1999), containing *Bambusa, Dendrocalamus, Gigantochloa* and *Thyrsostachys*. Internally, however, Watanabe's clade was poorly resolved in terms of relationships among *Bambusa, Gigantochloa* and *Dendrocalamus,* suggesting close relationships among these genera. *Thyrsostachys* had emerged as a sister lineage to the other genera included in Watanabe's study. The study of Loh *et al.* (2000) and Ramanayake *et al.* (2007), using AFLPs and RAPDs respectively, also indicated a close relationship between *Bambusa* and *Gigantochloa.* The combined evidence from these earlier molecular studies and the present investigation suggest that taxa belonging to *Bambusa*, *Dendrocalamus* and *Gigantochloa* form a close complex but are relatively distant from *Melocalamus, Thyrsostachys* and *Oxytenanthera.*

The phylogenetic trees generated in the present study are plausible hypotheses for relationships within *Dendrocalamus*, but need validation from other evidences. The low statistical support for some of the clusters/clades might improve with inclusion of more informative characters, which could be generated by using more selective primer sets. The study confirms that the current taxonomic treatment of *Dendrocalamus* is unsatisfactory and needs revision. A broader study encompassing a wider selection of taxa from *Bambusa, Dendrocalamus* and *Gigantochloa*, and inclusion of evidence from multiple data source (including AFLP and sequencing of fast evolving genes) might be expected to produce a robust phylogenetic tree for this suite of closely related taxa.

Acknowledgements

The first author acknowledges the financial help in the form of Commonwealth Scholarship from the Commonwealth Scholarships Commission, United Kingdom. Also, the help received from friends and colleagues at FRI, Dehra Dun; RFRI, Jorhat; SFRI, Itanagar; ICAR Research Complex for NEH region, Basar;.Central National Herbarium, BSI, Kolkata is gratefully acknowledged.

References

Alam, M.K.; Sarker, R.H.; Hassan, M.A. 1997. Chemotaxonomic studies in peroxidase isozyme of bamboos from Bangladesh. Bangladesh Journal of Botany, 26, 99-105.

Bentham, G. 1883. Gramineae. In Bentham, G.; Hooker, J.D. ed., Genera Plantarum, III, Reeve, London. pp. 1094-1215.

Biswas, S. 1998. Contribution to the isozyme studies on Indian bamboo, *Dendrocalamus strictus* (Roxb.) Nees with emphasis on diversity evaluation. Annals of Forestry, 5, 168-172.

Chase, M.W.; Hills, H.H. 1991. Silica gel: an ideal material for field preservation of leaf samples for DNA studies. Taxon, 40, 215-220.

Chou, C.H.; Hwang, Y.H. 1985. A biochemical aspect of phylogenetic study of Bambusaceae in Taiwan. III. The genera *Arthrostylidium*, *Chimonobambusa* and *Dendrocalamus*. Botanical Bulletin of Academia Sinica, 26, 155-170.

Doyle, J.J.; Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. Focus, 12, 13-15.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39, 783-791.

Gamble, J.S. 1896. The Bambuseae of British India. Annals of the Royal Botanic Garden, Calcutta, 7, 1-133.

Hsueh, C.; Li, D.Z. 1988. A study on the genus *Dendrocalamus* Nees from China. Journal of Bamboo Research, 7, 1-19.

Inada, T. 2004. African bamboos: an appraisal with special reference to *Oxytenanthera abyssinica*, the savanna bamboo. Ph.D. thesis, School of Agricultural and Forest Sciences, University of Wales, Bangor. 274 pp.

Jaccard, P. 1908. Nouvelles recherches sur la distribution florale. Bulletin de la Société Vaudense des Sciences Naturelles, 44, 223-270.

Koopman, W.J.M.; Zevenberger, M.J.; Van Den Berg, R.G. 2001. Species relationships in *Lactuca* s.l. (Lactuceae, Asteraceae) inferred from AFLP fingerprints. American Journal of Botany, 88, 1881–1887.

Li, D.Z.; Stapleton, C. 2006. *Dendrocalamus*. In Flora of China, 22, 39-46. http://www.efloras.org

Loh, J.P.; Kiew, R.; Set, O.; Gan, L.H.; Gan, Y.Y. 2000. A study of genetic variation and relationships within the bamboo subtribe Bambusinae using amplified fragment length polymorphism. Annals of Botany, 85, 607-612.

Munro, W. 1868. A monograph of Bambusaceae, including descriptions of all the species. Transactions of the Linnean Society of London, 26, 1-157.

Ohrnberger, D. 1999. The bamboos of the World: annotated nomenclature and literature of the species and the higher and lower taxa. Elsevier, Amsterdam. 585 pp.

Pavlicek, A.; Hrda, S.; Flegr, J. 1999. FreeTree: freeware programme for construction of phylogenetic trees on the basis of distance data and bootstrap/jackknife analysis of tree robustness. Application in the RAPD analysis of the genus *Frenkelia.* Folia Biologica, 45, 97-99.

Perrie, L.R. and Brownsey. 2005. Genetic variation is not concordant with morphological variation in the fern *Asplenium hookerianum sensu lato* (Aspleniaceae). American Journal of Botany, 92, 1559-1564.

Rohlf, F.J. 2000. NTSYSpc: numerical taxonomy and multivariate analysis system, version 2.11X. Applied Biostatistics, New York.

Saitou, N.; Nei, M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4, 406-425.

Stapleton, C.M.A. 1991. A morphological investigation of some Himalayan bamboos with an enumeration of taxa in Nepal and Bhutan. Ph.D. thesis, University of Aberdeen.

Stapleton, C.M.A. 1994. The bamboos of Nepal and Bhutan. Part I. Edinburgh Journal of Botany, 51, 1-32.
Sun, Y.; Xia, N.; Lin, R. 2005. Phylogenetic analysis of *Bambusa* (Poaceae: Bambusoideae) based on internal transcribed spacer sequences of nuclear ribosomal DNA. Biochemical Genetics, 43, 603-512.
Swofford, D.L. 2002. PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sinauer, Sunderland.
Vos, P. ; Hogers, R. ; Bleeker, M. ; Reijans, M. ; Van de Lee T. ; Hornes, M. ; Frijters, A. ; Pot, J.; Peleman, J. ; Kupier, M. ; Zabeau, M. 1995. AFLP: a new technique for DNA finger printing. Nucleic Acids Research, 23, 4407-4414.
Watanabe, M.; Ito, M.; Kurita, S. 1994. Chloroplast DNA phylogeny of Asian bamboos (Bambusoideae, Poaceae) and its systematic implication. Journal of Plant Research, 107, 253-261.
Yang, H.Q.; Yang, J.B.; Peng, Z.H.; Gao, J.; Yang, Y.M.; Peng, S.; and Li, D.Z. 2008. A molecular phylogenetic and fruit evolution analysis of the major groups of the paleotropical woody bamboos (Gramineae: Bambusoideae) based on nuclear ITS, GBSSI and plastid trnL-F DNA sequences. Molecular Phylogenetics and Evolution, 48, 809-824.

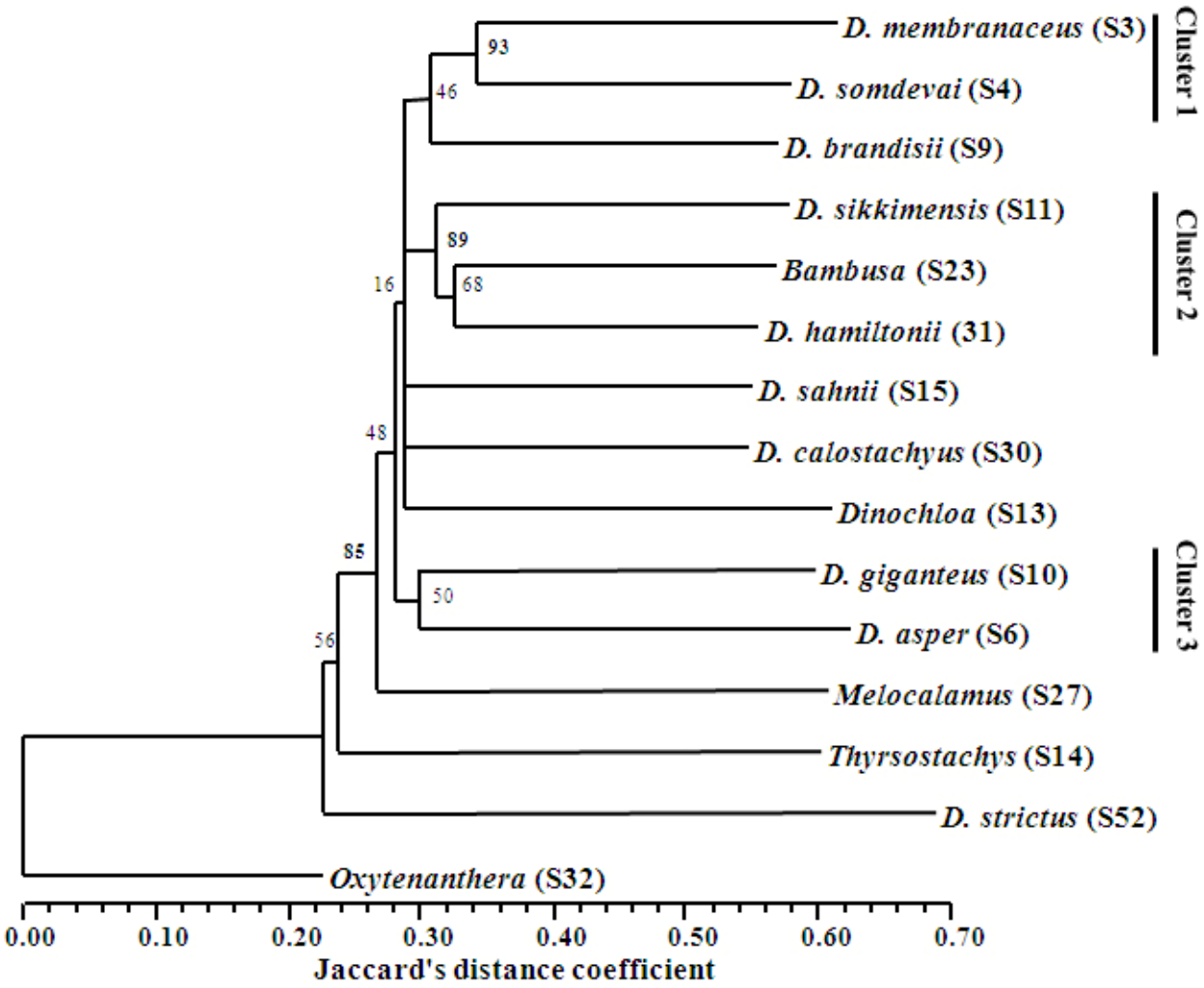
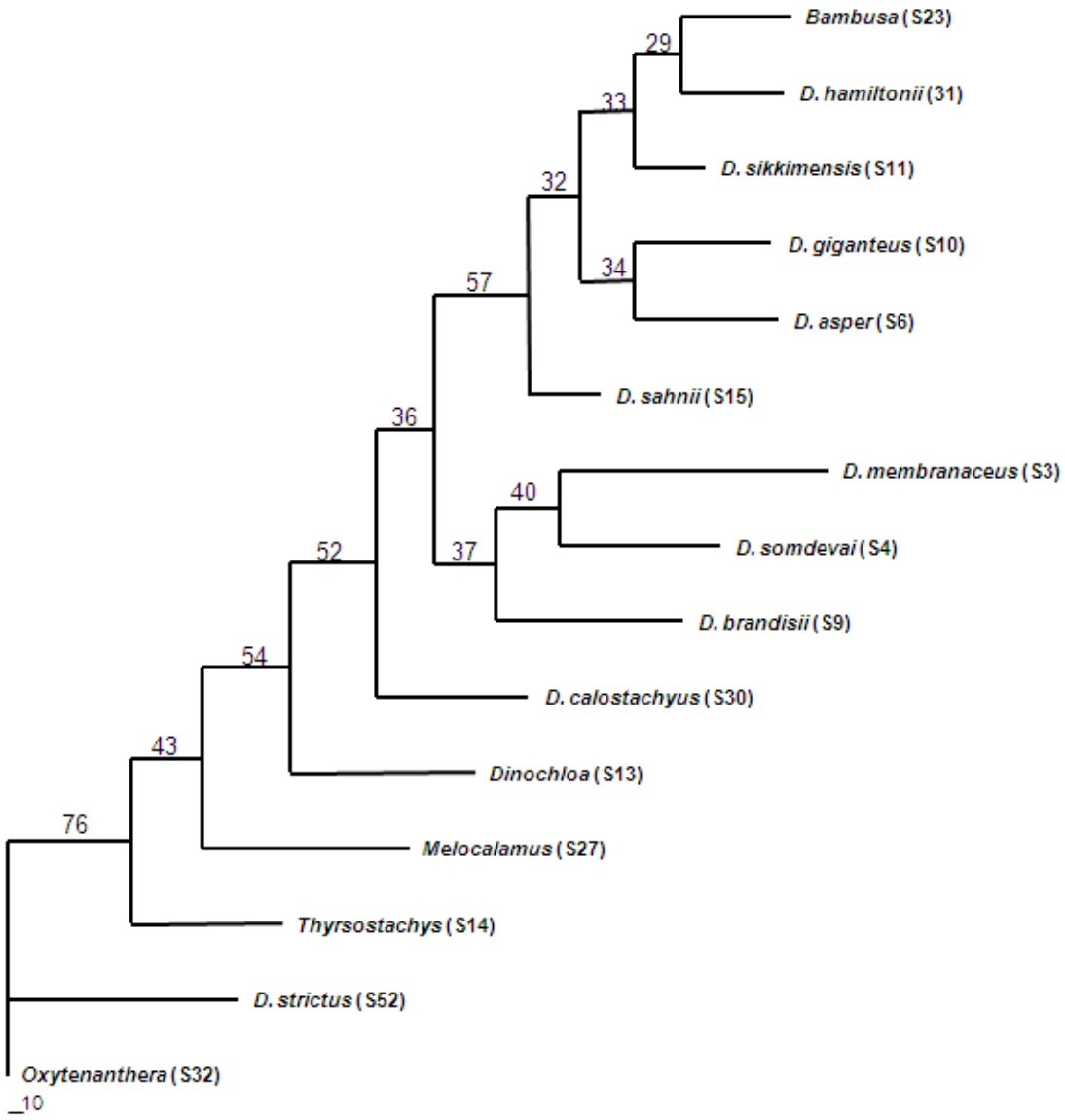
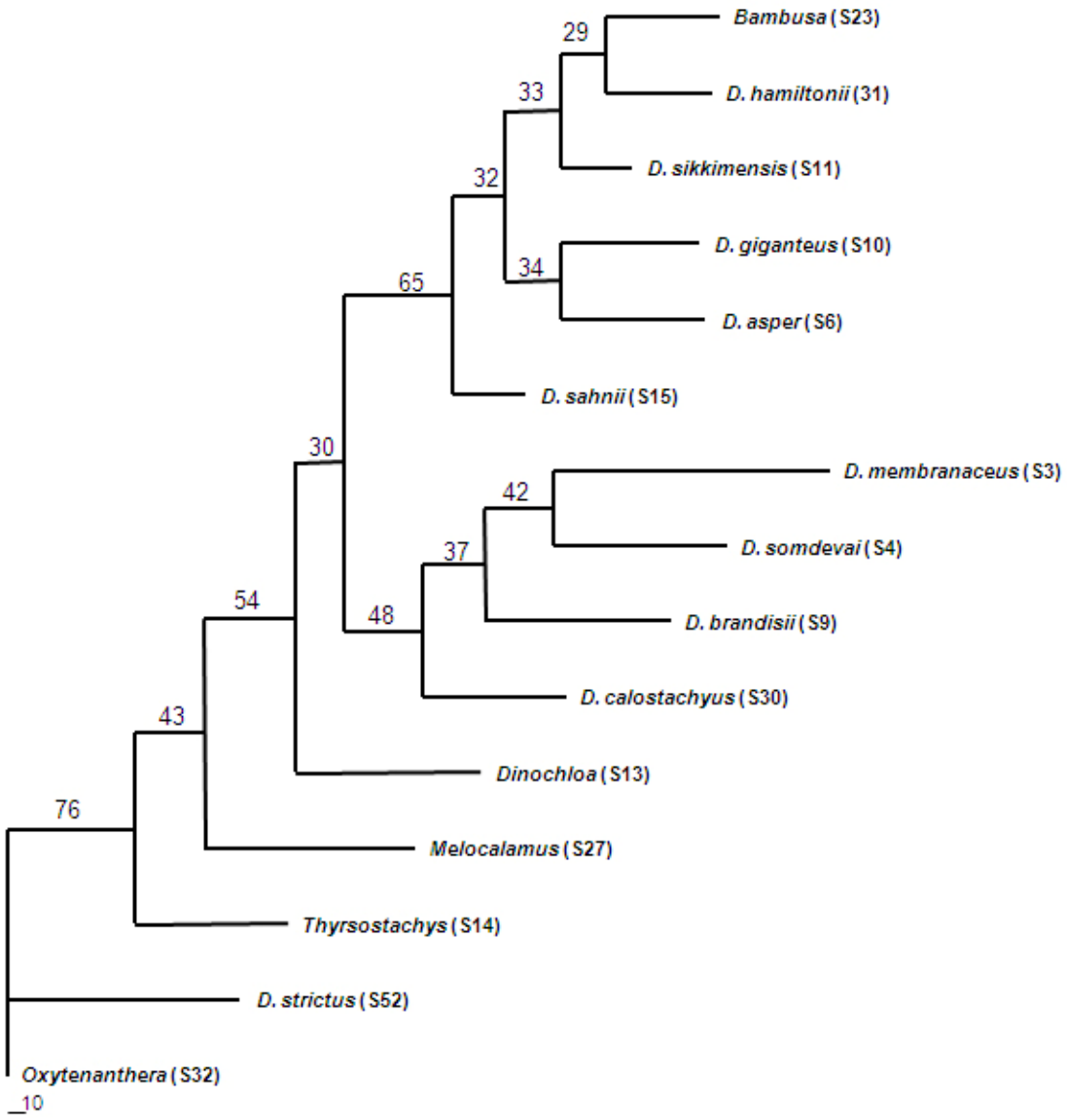


Figure 1. Neighbour-joining phenogram showing phenetic relationships among the 15 sampled taxa from *Dendrocalamus* and the subtribe Bambusinae.

Numbers at the nodes indicate bootstrap (%) support for the respective clusters. Bar scale indicates additive distance between pairs of taxa.


Bambusa (S23)
29
D. hamiltonii (31)
33
D. sikkimensis (S11)
32
D. giganteus (S10)
34
57
D. asper (S6)
D. sahnii (S15)
36
D. membranaceus (S3)
40
D. somdevai (S4)
52
37
D. brandisii (S9)
54
D. calostachyus (S30)
43
Dinochloa (S13)
76
Melocalamus (S27)
Thyrsostachys (S14)
D. strictus (S52)
Oxytenanthera (S32)
_10

(a)



(b)

Figure 2a & b. Phylograms depicting two equally parsimonious trees resulting from the maximum parsimony analysis of a character matrix of 470 AFLP markers.

Each tree has a length of 1468 steps, CI = 0.320, RI = 0.298, RC = 0.095. Values above segments indicate character state changes (gains/losses of AFLP bands) supporting respective nodes. Accession codes are indicated within brackets. The horizontal bars below trees represent 10 character state changes.

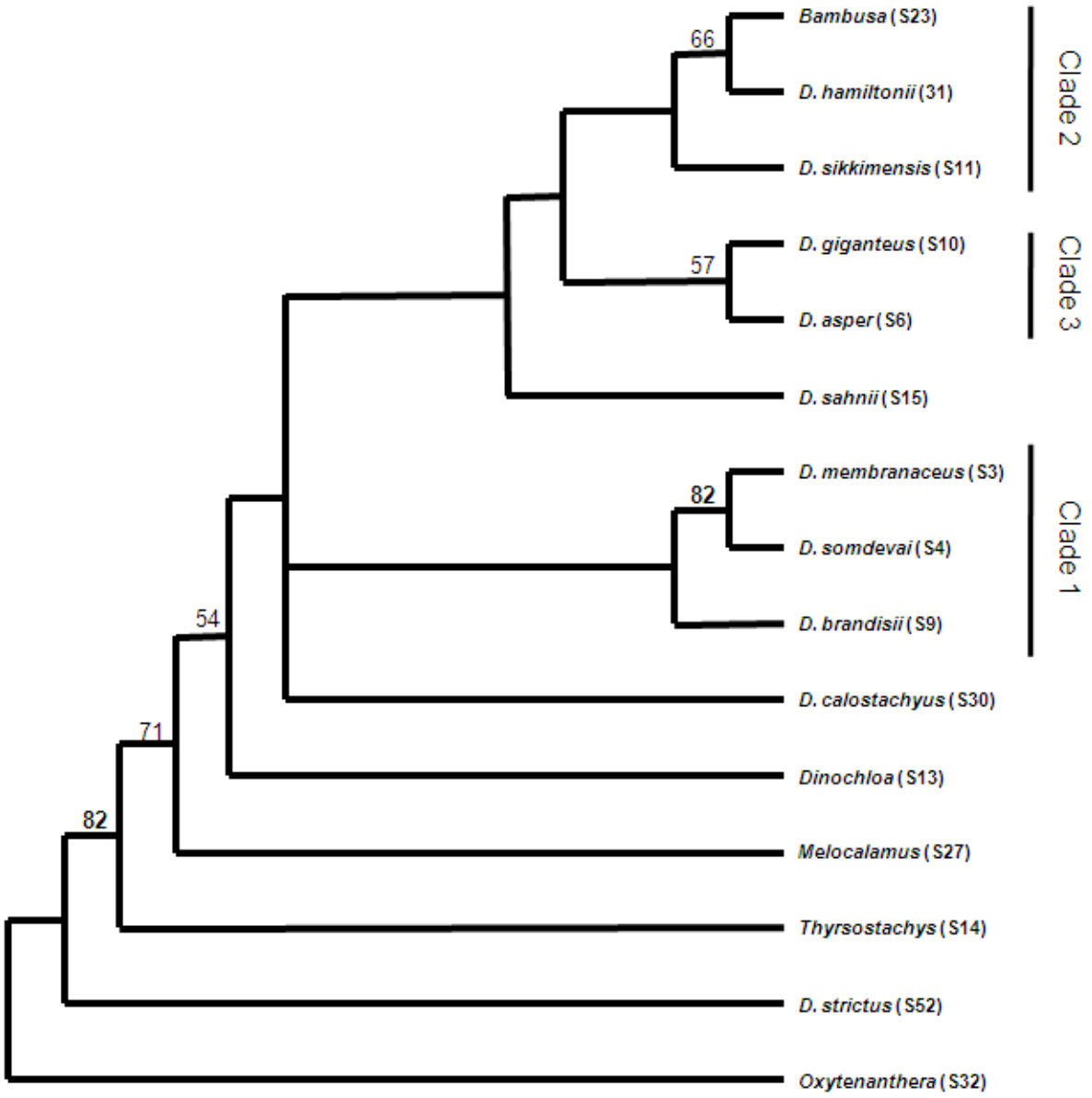


Figure 3. Cladogram depicting strict consensus of the two parsimonious trees obtained in the maximum parsimony analysis of a character matrix of 470 AFLP markers.

Length =1480 steps (character state changes), CI = 0.317, RI = 0.289, RC = 0.092. Values above segments indicate bootstrap support for the respective nodes. Bootstrap support for nodes with less than 50% support and which collapse under the 50% majority rule tree is not shown. Accession codes are indicated within brackets. Clades conforming to the clusters of neighbour-joining analysis are indicated.

Flowering gene expression in the life history of two mass-flowered bamboos, *Phyllostachys meyeri* and *Shibataea chinensis* (Poaceae: Bambusoideae)

Yoko Hisamoto [a, b] , and Mikio Kobayashi [a, b]
[a] United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan
[b] Department of Forest Science, Faculty of Agriculture, Utsunomiya University, Mine-machi, Utsunomiya, Japan

Abstract

A total of 4 copies of the flowering promoting gene *FLOWERING LOCUS T* (*FT*) homolog *PmFT* were cloned and sequenced, and 2 fragment copies of the flowering repressing gene *CENTRORADIALIS* (*CEN*) homolog *PmCEN* were amplified. The average identities of amino acid sequences among the copies of *PmFT* and *PmCEN* were 97% and 95%, respectively. The orthologous regions were used with a real-time RT PCR method for gene expression analyses in stages of the life history of *Phyllostachys meyeri* McClure and *Shibataea chinensis* Nakai, with emphasis on their mass flowering behaviors. Both genes were expressed during the reproductive phase and in sterile leaves in the vegetative phase, whereas *PmCEN* alone was expressed in seedlings and juvenile clones. *PmFT* expression was strongest in leaves of the flowering culm. Relatively weak expression of both gene homologs in *S. chinensis*—*ScFT* and *ScCEN*— was detected during the reproductive phase; the expression of *ScFT* was highest in mature leaves. Only *ScFT* was detected at a low level in the vegetative phase after flowering. The expression of *FT* homologs in the vegetative phase in both bamboo species suggested that sporadic flowering would occur in the following year(s). The highest expression level of *FT* homologs were detected in the flowering culms in both bamboo species, suggesting that the same molecular mechanism of flowering promoting genes discovered in model plants might underlie the mass flowering process of the bamboo plants.

Introduction

Many bamboos have a life history trait of monocarpic mass flowering and death (Janzen 1976), suggesting that cross-breeding to produce a new genetic cultivar would be difficult in bamboos. If the molecular mechanism of bamboo flowering could be clarified and genetic modification made feasible, bamboo propagation technology might be fundamentally reformed.

A number of genes that control flowering time have been isolated and characterized in *Arabidopsis* (Komeda 2004). Corbesier et al. (2007) discovered that the *FLOWERING LOCUS T* (*FT*) gene is a candidate for encoding florigen and that the *FT* protein moves from an induced leaf to the shoot apex and causes flowering. On the other hand, *TERMINAL FLOWER 1* (*TFL1*)/*CENTRORADIALIS* (*CEN*) acts as a flowering repressing gene to

delay the flowering time and alters the inflorescence architecture in *Arabidopsis* (Alvares et al. 1992) and in *Antirrhinum* (Bradley et al. 1996). Rice *TFL1/CEN* orthologs, *RCN1* and *RCN2*, delay transition into flowering and alter panicle morphology (Nakagawa et al. 2002).

We have investigated the relationships between various flowering behaviors in the genus *Phyllostachys* and have examined the nucleotide sequence variation in the *FT* homolog *PmFT* (Hisamoto and Kobayashi 2007). In the present study, we first cloned 4 copies of *PmFT* and amplified 2 fragment copies of the *CEN* homolog *PmCEN* from *Phyllostachys meyeri* McClure. Then, we analyzed their expression patterns in the life history, including mass flowering and death and the recovery of a grove of *P. meyeri*, as well as in mass-flowering *Shibataea chinensis* Nakai.

Materials and Methods

Plant Materials

Phyllostachys meyeri and *Shibataea chinensis* were cultivated in the Fuji Bamboo Garden, Japan. The life histories of *P. meyeri* and *S. chinensis* are summarized in Figure 1. *P. meyeri* bloomed in high synchrony with determinate inflorescences of the capitate type (Figure 1; LF, IF), and then the culms died. Two months after the mass flowering, slender, short regenerated culms emerged and bear indeterminate inflorescences of the lax type (Figure 1; LR, IR), whereas the flowered culms died. Caryopses matured in June and seedlings emerged in July 2004 (Figure 1; SS). One year after the mass flowering, several slender vegetative culms emerged (Figure 1; LS). The juvenile clumps formed a clone with monopodial rhizomes in 2007 (Figure 1; LJ).

S. chinensis bore young inflorescences in February 2008 (Figure 1; LY, IY). The grove was in full bloom with green leaves in March (Figure 1; LM, IM). This stage was considered to correspond with the mass-flowering stage in *P. meyeri*. Flowering terminated around April 20. All the inflorescences withered, but did not bear any caryopses. Even after flowering, the flowered culms remained verdant with green foliage leaves (Figure 1; LW), and bore new leaf buds on the axils.

We collected samples of *P. meyeri* and *S. chinensis* in various stages of flowering as follows: in *P. meyeri*, leaves (LF) and inflorescences (IF) of the mass-flowered culms, inflorescences (IR) and leaves (LR) of the regenerated culms in flower, leaves (LS) of the regenerated sterile culms, leaves of the juvenile clumps (LJ), and young stems of the seedlings (SS); in *S. chinensis*, leaves (LY) and inflorescences (IY) in the young stage, leaves (LM) and inflorescences (IM) in the mature stage, and leaves (LW) remaining after flowering.

Isolation and Sequencing of FT and CEN Homologs from Phyllostachys meyeri

Genomic DNA was isolated from the leaves of *Phyllostachys meyeri* by the modified CTAB method (Hasebe and Iwatsuki 1990). Full-length *PmFT* and partial *PmCEN* sequences were amplified using the primer pairs shown in Table 1.

Table 1 Primer pairs used for PCR amplification

Primer name	Derivation	Position	Sequence of primer (5□-3□)	Designed for
PmFT_5end	*PmFT* (AB240578)	1□21	ATGGTCGGCGGGGACAGGGAT	cloning and sequencing
PmFT_3end		897□919	TCACCAGGGGTACATCCTTCTTC	
PmCEN_F	rice *FDR2* (AF159882)	211□231	GGTCATGAGCTCTACCCATCA	
PmCEN_R		592□610	GCCTCCTGGCTGCAGTCTC	
FT_F	*PmFT1* 4 (AB240578; AB498760□ AB498762)	185□371 (intron: 202□ 346)	GGACATTTTACACACTCGTGAT	real-time RT-PCR
FT_R		468□562 (intron: 429□ 551)	CAGTGACCAGCCAGTGTAGATA	
CEN_F	*PmCEN1* 2 (AB498763; AB498764)	71□193 (intron: 89□189)	CGGTCTTTCTTCACATTGGTTA	
CEN_R		371□391	AGCATCTGTTGTCCCAGGTAT	
GAPDH_F	rice *GAPDH* mRNA (AF546879)	244□261	GCTACCCAGAAGACTGTT	
GAPDH_R		371□391	GTGCTGCTAGGAATGATGTTGA	

Each PCR reaction was performed in a volume of 25 µl containing 1 ng of the template DNA, 1.25 U Takara LA *Taq*, 20 pmol of each primer, 2 × GC buffer II, and 0.25 mM dNTP (Takara). Amplification was performed in a GeneAmp PCR system 9700 programmed for the following sequence of steps: (A) initial denaturation of 1 s at 95°C; (B) 14 cycles of 15 s at 95°C and 12 min at 68.5°C; (C) 8 cycles of 15 s at 95°C followed by 12 min at 68.5°C, wherein the time for each successive cycle increased by 5 s; and (D) post-elongation for 10 min at 72°C. The PCR products were subcloned into pSTBlue-1 (Novagen) and sequenced using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems).

Analysis of Gene Expression

Total RNA was extracted from the samples using the RNA Plant Mini Kit (Qiagen) and treated with DNase according to the manufacturer's protocol. First-strand cDNA was synthesized from 3 µg of the total RNA by SuperScript III reverse transcriptase (Invitrogen) with an oligo $(dT)_{20}$ primer and dNTP mixture according to the manufacturer's instructions.

We performed real-time PCR for a volume of 25 µl containing 1×, 10^{-1}×, or 10^{-2}× cDNA, 10 pmol of each primer, and 2×QuantiTect SYBR Green PCR Master Mix using the QuantiTect SYBR Green RT-PCR Kit (Qiagen). We used the same gene-specific primers for both *Phyllostachys meyeri* and *Shibataea chinensis*, and we employed rice *GAPDH* gene primers as the control (Table 1). Amplification was performed in a 7500 Real Time PCR System (Applied Biosystems) programmed for an initial denaturation of 15 min at 95°C, followed by 41 cycles of 15 s at 94°C, 30 s at 56°C, and 35 s at 72°C. The final cycle was 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C for a dissociating stage to check the specificity of the PCR amplification. All experiments were repeated at least 3 times.

Results

Cloning of FT and CEN Homologs in Phyllostachys meyeri

In a preliminary study, we identified full-length *PmFT* based on the rice *RFT1* gene (AB240578; Hisamoto et al. 2008), and we detected 4 copies of *PmFT* by a Southern blot analysis and determined complete sequences of 2 copies. In the present study, we cloned a total of 4 genomic copies among 28 clones of PCR products amplified using primer pairs designed from the 5′- and 3′-end sequences of *PmFT* (Figure 2a). The 4 copies, *PmFT1* to *PmFT4*, were composed of 4 exons and 3 introns. Exon 1 of *PmFT2* was 204 bp in length including a 3 bp-insertion, whereas those of the other copies were 201 bp.In all the copies, the lengths of exons 2, 3, and 4 were 61 bp, 41 bp, and 233 bp, respectively. The lengths of the introns were 165–169 bp, 123–162 bp, and 91 bp in introns 1, 2, and 3, respectively. Four-bp and 36-bp insertions were found in introns 1 and 2 of *PmFT4*. The nucleotide sequence identities among the 4 copies were 92–98%.

We obtained two partial *PmCEN* sequences, *PmCEN1* and *PmCEN2*, which were amplified using primer pairs designed from the rice *FDR2* gene (Figure 2b). The sequences started from the nucleotide corresponding to the 232nd nucleotide of *FDR2* mRNA and were composed of 4 exons and 3 introns. Exon 1 of *PmCEN1* was 88 bp in length including a 3-bp insertion, whereas exon 1 of *PmCEN2* was 85 bp in length. In both copies, the lengths of exons 2, 3, and 4 was 62 bp, 42 bp, and 213 bp, respectively. The lengths of the introns were 101 or 105 bp, 106 or 190 bp, and 95 bp in introns 1, 2, and 3, respectively. A total of 84 bp of insertions was found in intron 2 of *PmCEN1*. The nucleotide sequence identity between the 2 copies was 80%.

We aligned the putative amino acid sequences of the 4 copies of *PmFT* and the 2 copies of *PmCEN* with *FT* and *TFL1* in *Arabidopsis*, and the homologs in rice and poplar (Figure 3). Amino acid sequence identity among the 4 copies was 96–98%, whereas the identities between *PmFT* and the other *FT* proteins were low: 71% in *Arabidopsis*, 82% in poplar, and 88% in rice. The amino acid sequence identity between the 2 copies of *PmCEN* was 95%, but the amino acid identities between *PmCEN* and the other *TFL1/CEN* proteins were low: 75% in *Arabidopsis*, 81% in poplar, and 86% in rice.

Expression Patterns of FT and CEN Homologs in Two Bamboo Species

As shown above, the nucleotide and amino acid sequences were highly conserved among the 4 copies of *PmFT*, as well as the 2 copies of *PmCEN*. We carried out gene expression analyses of all the copies of *PmFT* and *PmCEN* in the life history of *Phyllostachys meyeri* and the flowering process of *Shibataea chinensis* by real-time RT-PCR using primers specific for each gene and normalized by reference to the *GAPDH* gene (Figure 4).

In *P. meyeri*, *PmFT* was strongly expressed in the leaves of mass-flowered culms and regenerated culms in flower, while it was weakly expressed in their inflorescences (Figure 4a). In particular, the level of expression was almost 45 times higher in the leaves than in the inflorescence of mass-flowered culms. Expression of *PmFT* was not detected in seedlings or juvenile plants, but it was detected in regenerated sterile culms even though they did not flower. On the other hand, the expression of *PmCEN* was stronger in the inflorescences than in the leaves. The expression level in seedlings was 15 times as high as that in the inflorescences of regenerated flowered culms. Expression was also detected in regenerated sterile culms and juvenile plants. For a detailed

investigation of the mass-flowered stage, we analyzed the expression levels in mass-flowered *S. chinensis* from young to withered stages. Similar to *PmFT*, the expression level of the *FT* homolog, *ScFT* was higher in leaves than in inflorescences (Figure 4b), in which the expression level increased as the inflorescences matured. *ScFT* was weakly expressed in the withered leaves remaining after flowering. On the other hand, expression of the *TFL1/CEN* homolog *ScCEN* was highest in mature inflorescences. Its second highest expression was in leaves of the young stage, followed by leaves in the mature stage. *ScCEN* expression was not detected in the leaves remaining after flowering.

Discussion

We amplified *PmFT* and *PmCEN* using primers designed for rice *FT* and *CEN* homologs (Table 1). The *FT* and *TFL1* genes belong to the same gene family, and exert opposing effects on flowering time. These effects have been related to the presence of critical amino acid residues: tyrosine at position 85 and glutamine at position 140 in *FT*; and histidine at position 88 and aspartic acid at position 144 in *TFL1* (Hanzawa et al. 2005, Ahn et al. 2006). The amino acid residues in *PmFT* and *PmCEN* copies match these amino acid residues, except that *PmFT4* has glutamic acid at position 140 (Figure 3). High homology was detected in amino acid sequences rather than in nucleotide sequences among the 4 copies of *PmFT* and between the 2 copies of *PmCEN* (Figures 2 and 3), suggesting that these copies were functionally homologous in *Phyllostachys meyeri*. Therefore, we designed primers for expression analysis at positions conserved in the 4 copies of *PmFT* as well as between the 2 copies of *PmCEN* (Table 1; Figure 2, arrows). In addition, the position of the critical tyrosine at 85 was included in the forward primer of *PmFT* to avoid confusion between *PmFT* and *PmCEN*.

In *Arabidopsis*, activation of *FT* transcription in leaf vascular tissue induces flowering (Corbesier et al. 2007). They provided evidence that *FT* does not activate an intermediate messenger in leaves and concluded that the *FT* protein acts as a long-distance signal that induces *Arabidopsis* flowering. Tamaki et al. (2007) also reported that a protein encoded by a rice *FT* ortholog, Hd3a, moves from the leaf to the shoot apical meristem and induces flowering, and suggested that the Hd3a protein may be the rice florigen. In the present study, the highest expressions of *PmFT* and *ScFT* were detected in leaves rather than inflorescences, suggesting that these two bamboo *FT* homologs have roles similar to their genes in *Arabidopsis* and rice. However, expression of *PmFT* continued in regenerated sterile culms, and *ScFT* expression continued in withered leaves remaining after their full-bloom stage. We have investigated the regeneration process of bamboo clumps every year, and confirmed that *P. meyeri* exhibited sporadic flowering for 4 years from 2004 to 2008 and that *S. chinensis* extensively flowered this year and the culms did not die (data not shown). Thus, we suggest that the *PmFT* expression in regenerated culms indicates sporadic flowering after monocarpic mass-flowering in *P. meyeri*, while the *ScFT* expression in withered leaves shows not monocarpic but polycarpic mass flowering in *S. chinensis*. From this result, if the expression of *FT* homolog is analyzed in a sterile clump, it might be possible to predict whether the clump will bloom or not.

The recessive mutants of *TFL1* produced determinate rather than indeterminate inflorescences in *Arabidopsis* (Alvarez et al. 1992) and *Antirrhinum* (Bradley et al. 1996); it was proposed that the *TFL1/CEN* gene product supports the activity of an inhibitor of flower initiation. Overexpression of the rice *TFL1/CEN* homologs, *RCN1*and *RCN2*, caused a delay in the flowering transition and altered the panicle morphology (Nakagawa et al.

2002). *RCN1* expression was observed in all the tissues of leaves, roots, flowers, vegetative meristems, and reproductive meristems. *RCN2* expression was also detected in all the tissues, but its level was higher in vegetative and reproductive meristems than in other tissues. In the present study, expression of *PmCEN* and *ScCEN* was detected in all tissues, except for withered leaves of *S. chinensis* (Figure 4). In the reproductive phase, *PmCEN* expression was strongest in the inflorescences of regenerated flowering culms, followed by inflorescences of mass-flowered culms in *P. meyeri*. The *ScCEN* expression level was highest in mature inflorescences, and it was lower in young inflorescences in *Shibataea chinensis*. We have investigated the inflorescence architecture of these two bamboos: in *P. meyeri*, determinate inflorescences are borne in mass-flowered culms, whereas indeterminate inflorescences are borne in regenerated culms in flower (data not shown). *S. chinensis* bears indeterminate inflorescences (Hisamoto et al. 2009). The sufficient expected amount of *ScCEN* expression in the inflorescences suggested that this gene promotes indeterminate inflorescence architecture. In the seedlings and juvenile clumps, only *PmCEN* was detected and strongly expressed. This result suggests that not only introduction of *PmFT* but also inhibition of *PmCEN* is necessary in order to force sterile bamboos to flower.

Recently, several flowering genes were isolated from woody plants, such as poplar *FT/TFL1* homologs (Igasaki et al. 2008), grapevine *FT/TFL1* homologs (Carmona et al. 2007), rubber tree *LEAFY* homolog (Dornelas et al. 2005), citrus *FT* homolog (Endo et al. 2005), citrus *LEAFY* homologs (Pillitteri et al. 2004), and apple *LEAFY* homologs (Wada et al. 2002). The apple *FT* homolog was strongly expressed and forced flowers to occur ectopically (Wada's personal communication). These studies aim to develop new breeding technologies for the acceleration of flowering by genetic modification, because woody plants have a very long juvenile phase that is an obstacle in their breeding. Woody bamboos constitute important resources that are used as foods and materials for building construction or crafts without any emissions. They also have a very long vegetative phase and exhibit monocarpic mass flowering and death (Janzen 1976). Therefore, it is important to elucidate the molecular mechanisms of flowering in woody bamboos to develop a new technology for controlling their sexual reproduction. Thus, we now intend to exploit a new vector system to induce such a *FT* gene.

Acknowledgments

We greatly appreciate Mr. Harutsugu Kashiwagi of the Fuji Bamboo Garden for providing us various information on bamboo flowering and the materials. This work was partly supported by Grant-in-Aid for Exploratory Research no.18658062, Scientific Research (B) no.21380089 from the Ministry of Education, Culture, Sports, Science and Technology and Specific Research Assistance B from the Asahi Glass Foundation. Hisamoto, Y. was partly supported by a Grant-in-Aid for JSPS Fellows no. 20.7324.

References

Ahn, J.H.; Miller, D.; Winter, V.J.; Banfield, M.J.; Lee, J.H.; Yoo, S.Y.; Henz, S.R.; Brady, R.L.; Weigel, D. 2006. A divergent external loop confers antagonistic activity on floral regulators FT and TFL1. The EMBO Journal, 25, 605-614.

Alvares, J.; Guli, C.L.; Yu, X, -H.; Smyth, D.R. 1992. Terminal flower: A gene affecting inflorescence development in *Arabidopsis thaliana*. Plant Journal, 2, 103-116.

Bradley, D.; Carpenter, R.; Copsey, L.; Vincent, C.; Rothstein, S.; Coen, E. 1996. Control of inflorescence architecture in *Antirrhinum*. Nature, 379, 791-797.

Carmona, M.J.; Calonje, M.; Martínez-Zapater, J.M. 2007. The *FT/TFL1* gene family in grapevine. Plant Molecular Biology, 63, 637-650.

Corbesier, L.; Vincent, C.; Jang, S.; Fornara, F.; Fan, Q.; Searle, I.; Giakountis, A.; Farrona, S.; Gissot, L.; Turnbull, C.; Coupland, G. 2007. FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. Science, 316, 1030-1033.

Dornelas, M.C.; Rodriguez, A.P.M. 2005. The rubber tree (*Hevea brasiliensis* Muell. Arg.) homologue of the *LEAFY/FLORICAULA* gene in preferentially expressed in both male and female floral meristems. Journal of Experimental Botany, 56, 1965-1974.

Endo, T.; Shimada, T.; Fujii, H.; Kobayashi, Y.; Araki, T.; Omura, M. 2005. Ectopic expression of an *FT* homolog from *Citrus* confers an early flowering phenotype on trifoliate orange (*Poncirus trifoliata* L. Raf.). Transgenic Research, 14, 703-712.

Hanzawa, Y.; Money, T.; Bradley, D. 2005. A single amino acid converts a repressor to an activator of flowering. Proceedings of the National Academy of Sciences of the United States of America, 102, 7748-7753.

Hasebe, M.; Iwatsuki, K. 1990. Adiantum capillus-veneris chloroplast DNA clone bank as useful heterologous probes in the systematics of the leptosporangiate ferns. American Fern Journal, 80, 20-25.

Hisamoto, Y.; Kashiwagi, H.; Kobayashi, M. 2008. Use of flowering gene *FLOWERING LOCUS T* (*FT*) homologs in the phylogenetic analysis of bambusoid and early diverging grasses. Journal of Plant Research, 121, 451-461.

Hisamoto, Y.; Kashiwagi, H.; Kobayashi, M. 2009. Mass flowering and flower morphology of *Shibataea chinensis* Nakai (Poaceae: Bambusoideae) cultivated in the Fuji Bamboo Garden, Japan. Journal of Japanese Botany, 84, in press.

Hisamoto, Y.; Kobayashi, M. 2007. Comparison of nucleotide sequences of fragments from rice *FLOWERING LOCUS T* (*RFT1*) homologs in *Phyllostachys* (Bambusoideae, Poaceae) with particular reference to flowering behaviour. Kew Bulletin, 62, 463-473.

Igasaki, T.; Watanabe, Y.; Nishiguchi, M.; Kotoda, N. 2008. The *FLOWERING LOCUS T/TERMINAL FLOWER 1* family in Lombardy poplar. Plant Cell Physiology, 49, 291-300.

Janzen, D.H. 1976. Why bamboos wait so long to flower. Annual Review of Ecology and Systematics, 7, 347-391.

Komeda, Y. 2004. Genetic regulation of time to flower in *Arabidopsis thaliana*. Annual Review of Plant Biology, 55, 521-535.

Nakagawa, M.; Shimamoto, K.; Kyozuka, J. 2002. Overexpression of *RCN1* and *RCN2*, rice *TERMINAL FLOWER 1/CENTRORADIALIS* homologs, confers delay of phase transition and altered panicle morphology in rice. The Plant Journal, 29, 743-750.

Pillitteri, L.J.; Lovatt, C.J.; Walling, L.L. 2004. Isolation and characterization of *LEAFY* and *APETALA1* homologues from *Citrus sinensis* L. Osbeck 'Washington'. Journal of the American Society for Horticultural Science, 129, 846-856.

Tamaki, S.; Matsuo, S.; Wong, H.L.; Yokoi, S.; Shimamoto, K. 2007. Hd3a protein is a mobile flowering signal in rice. Science, 316, 1033-1036.
Wada, M.; Cao, Q.; Kotoda, N.; Soejime, J.; Masuda, T. 2002. Apple has two orthologues of *FLORICAULA/LEAFY* involved in flowering. Plant Molecular Biology, 49, 567-577.

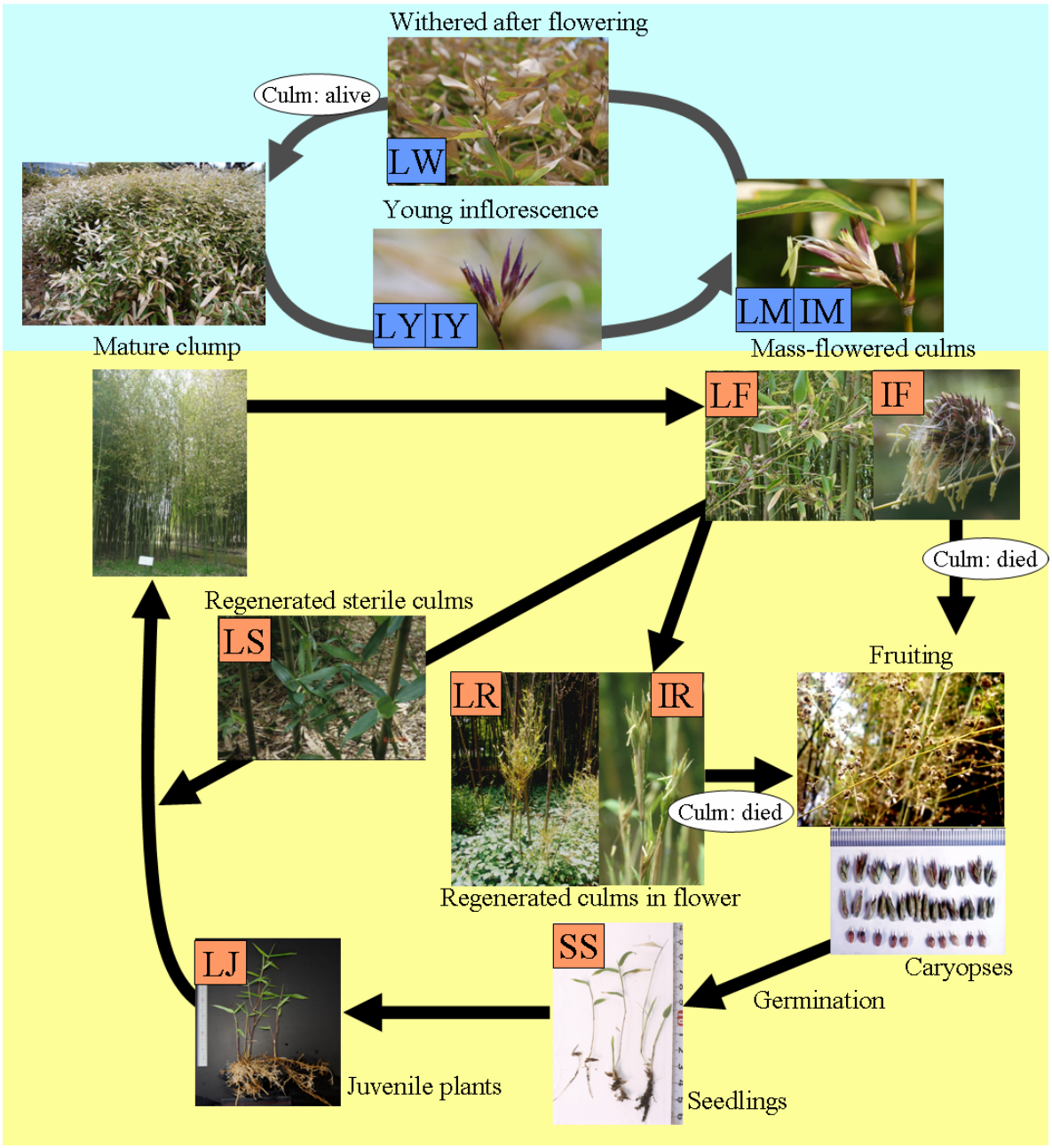
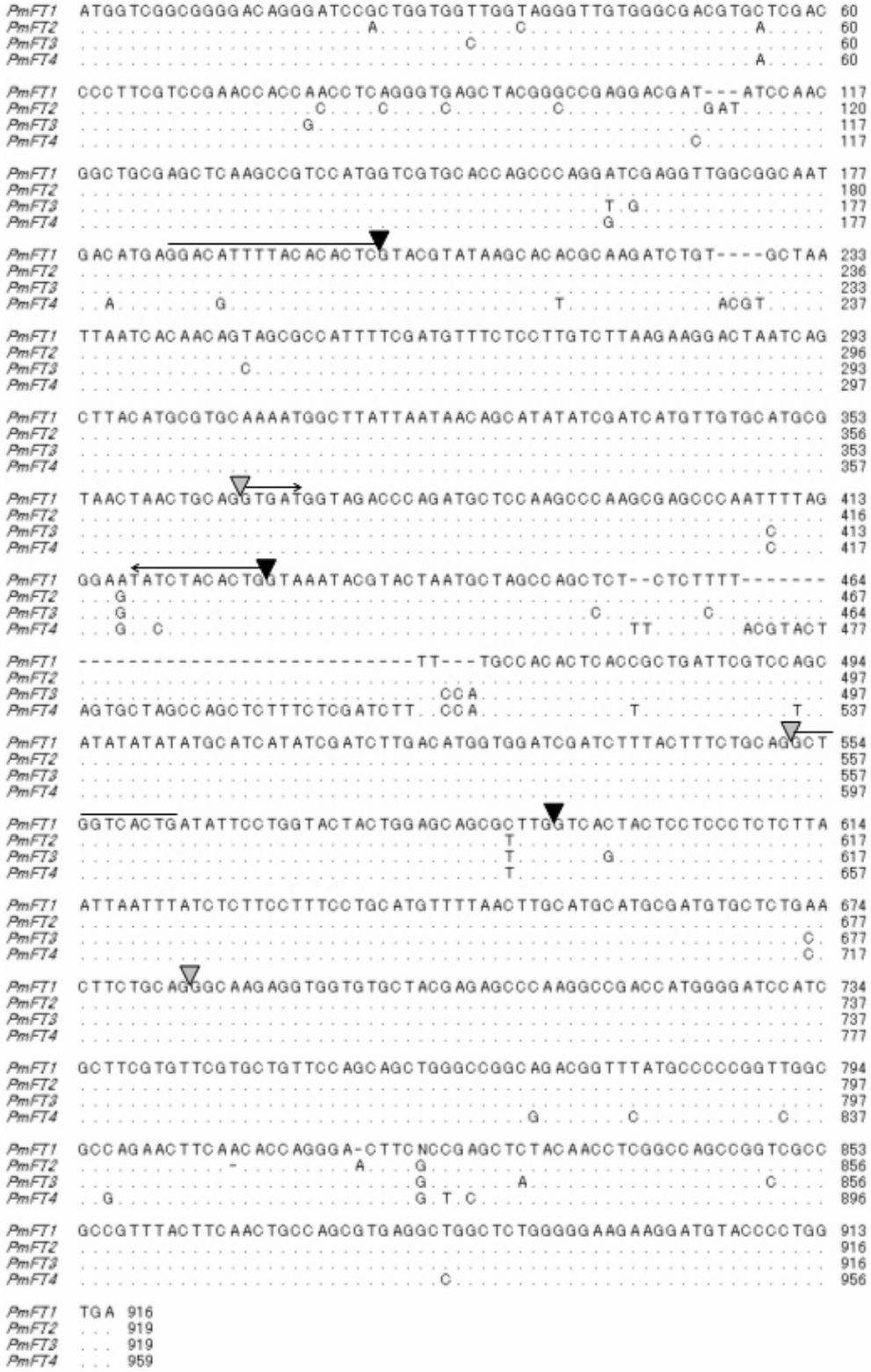


Figure 1 Combined diagram of the life histories of *Phyllostachys meyeri* (yellow region) and *Shibataea chinensis* (blue region). Each symbol shown on the photograph corresponds to Figure 4: in *P. meyeri*, leaves (LF) and inflorescences (IF) of mass-flowered culms, leaves (LR) and inflorescences (IR) of regenerated culms in flower, leaves (LS) of sterile regenerated culms, leaves of juvenile clumps (LJ), and young stems of the seedlings (SS); in *S. chinensis*, leaves (LY) and inflorescences (IY) in the young stage, leaves (LM) and inflorescences (IM) in the mature stage, and leaves (LW) remaining after flowering.

a


PmFT1 ATGGTCGGCGGGGACAGGGATCCGCTGGTGGTTGGTAGGGTTGTGGGCGACGTGCTCGAC 60
PmFT2 A C A 60
PmFT3 C 60
PmFT4 A 60
PmFT1 CCCTTCGTCCGAACCACCAACCTCAGGGTGAGCTACGGGCCGAGGACGAT---ATCCAAC 117
PmFT2 C C C C GAT 120
PmFT3 G 117
PmFT4 C 117
PmFT1 GGCTGCGAGCTCAAGCCGTCCATGGTCGTGCACCAGCCCAGGATCGAGGTTGGCGGCAAT 177
PmFT2 180
PmFT3 T G 177
PmFT4 G 177
PmFT1 GACATGAGGACATTTTACACACTCGTACGTATAAGCACACGCAAGATCTGT----GCTAA 233
PmFT2 236
PmFT3 233
PmFT4 A G T ACGT 237
PmFT1 TTAATCACAACAGTAGCGCCATTTTCGATGTTTCTCCTTGTCTTAAGAAGGACTAATCAG 293
PmFT2 296
PmFT3 C 293
PmFT4 297
PmFT1 CTTACATGCGTGCAAAATGGCTTATTAATAACAGCATATATCGATCATGTTGTGCATGCG 353
PmFT2 356
PmFT3 353
PmFT4 357
PmFT1 TAACTAACTGCAGGTGATGGTAGACCCAGATGCTCCAAGCCCAAGCGAGCCCAATTTTAG 413
PmFT2 416
PmFT3 C 413
PmFT4 C 417
PmFT1 GGAATATCTACACTGGTAAATACGTACTAATGCTAGCCAGCTCT--CTCTTTT------- 464
PmFT2 G 467
PmFT3 G C C 464
PmFT4 G C TT ACGTACT 477
PmFT1 ----------TT---TGCCACACTCACCGCTGATTCGTCCAGC 494
PmFT2 497
PmFT3 CCA 497
PmFT4 AGTGCTAGCCAGCTCTTTCTCGATCTT CCA T T 537
PmFT1 ATATATATATGCATCATATCGATCTTGACATGGTGGATCGATCTTTACTTTCTGCAGGCT 554
PmFT2 557
PmFT3 557
PmFT4 597
PmFT1 GGTCACTGATATTCCTGGTACTACTGGAGCAGCGCTTGGTCACTACTCCTCCCTCTCTTA 614
PmFT2 T 617
PmFT3 T G 617
PmFT4 T 657
PmFT1 ATTAATTTATCTCTTCCTTTCCTGCATGTTTTAACTTGCATGCATGCGATGTGCTCTGAA 674
PmFT2 677
PmFT3 C 677
PmFT4 C 717
PmFT1 CTTCTGCAGGGCAAGAGGTGGTGTGCTACGAGAGCCCAAGGCCGACCATGGGGATCCATC 734
PmFT2 737
PmFT3 737
PmFT4 777
PmFT1 GCTTCGTGTTCGTGCTGTTCCAGCAGCTGGGCCGGCAGACGGTTTATGCCCCCGGTTGGC 794
PmFT2 797
PmFT3 797
PmFT4 G C C 837
PmFT1 GCCAGAACTTCAACACCAGGGA-CTTCNCCGAGCTCTACAACCTCGGCCAGCCGGTCGCC 853
PmFT2 - A G 856
PmFT3 G A C 856
PmFT4 G G T C 896
PmFT1 GCCGTTTACTTCAACTGCCAGCGTGAGGCTGGCTCTGGGGGAAGAAGGATGTACCCCTGG 913
PmFT2 916
PmFT3 916
PmFT4 C 956
PmFT1 TGA 916
PmFT2 919
PmFT3 919
PmFT4 959

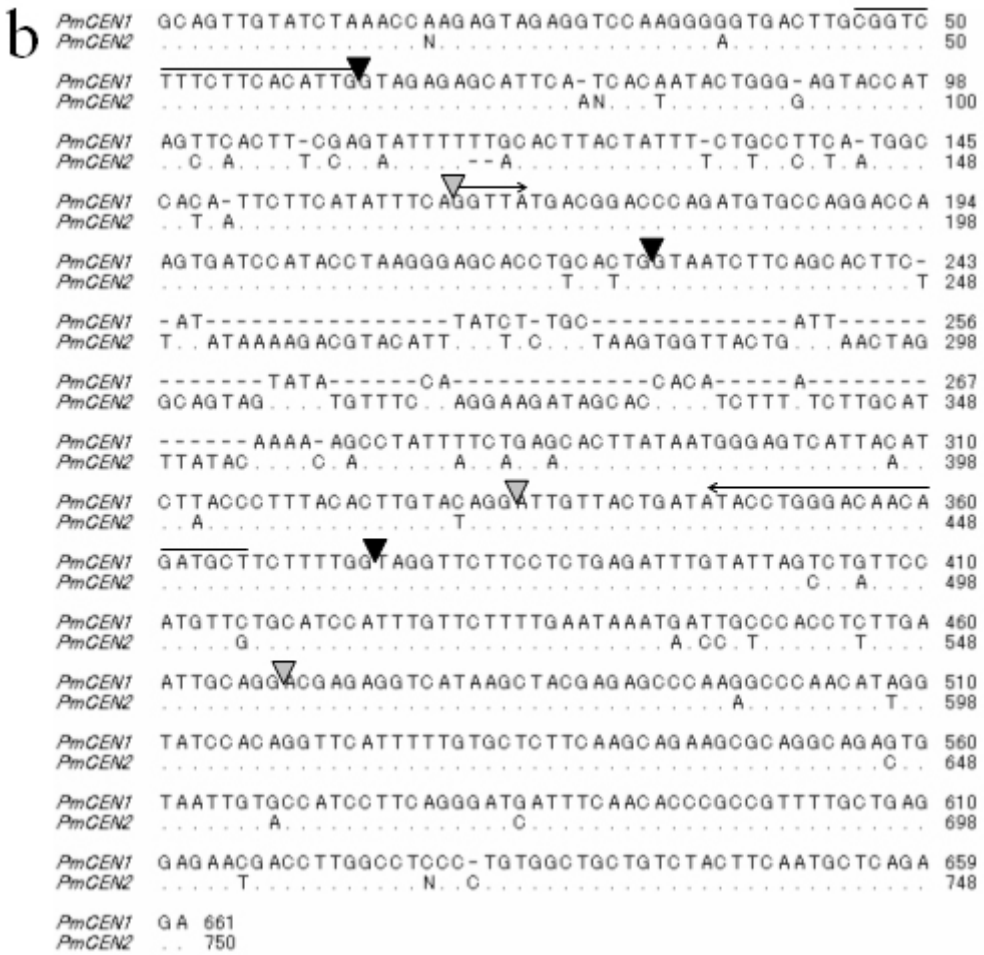


Figure 2 Alignment of nucleotide sequences between (a) 4 copies of *PmFT* and (b) 2 copies of *PmCEN*. A dot indicates a nucleotide identical to the *PmFT1* sequence. An insertion/deletion is shown with a dash and a stop codon with an asterisk. Black and gray arrowheads indicate the start and end of introns, respectively. Arrows show the positions of primers designed for gene expression analysis; the positions correspond with Table 1.

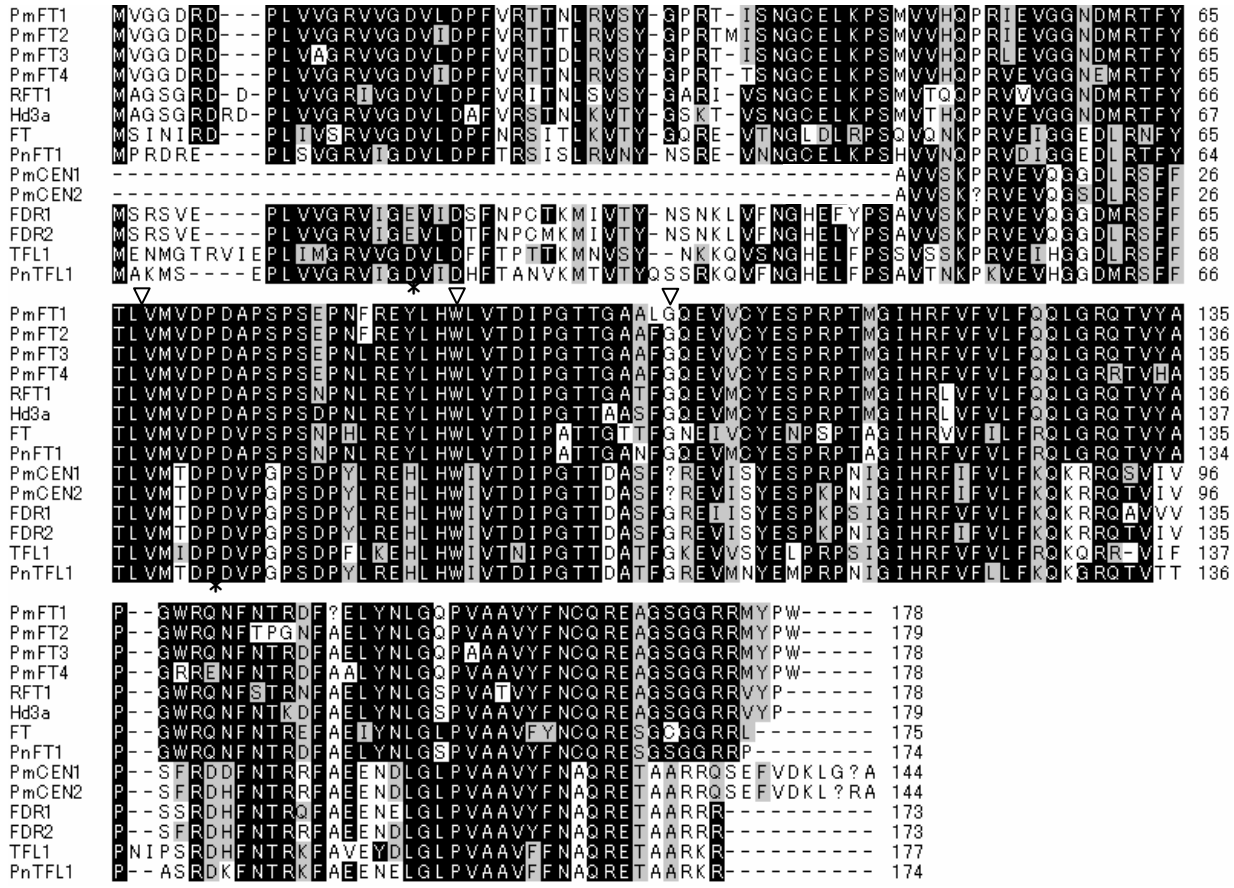
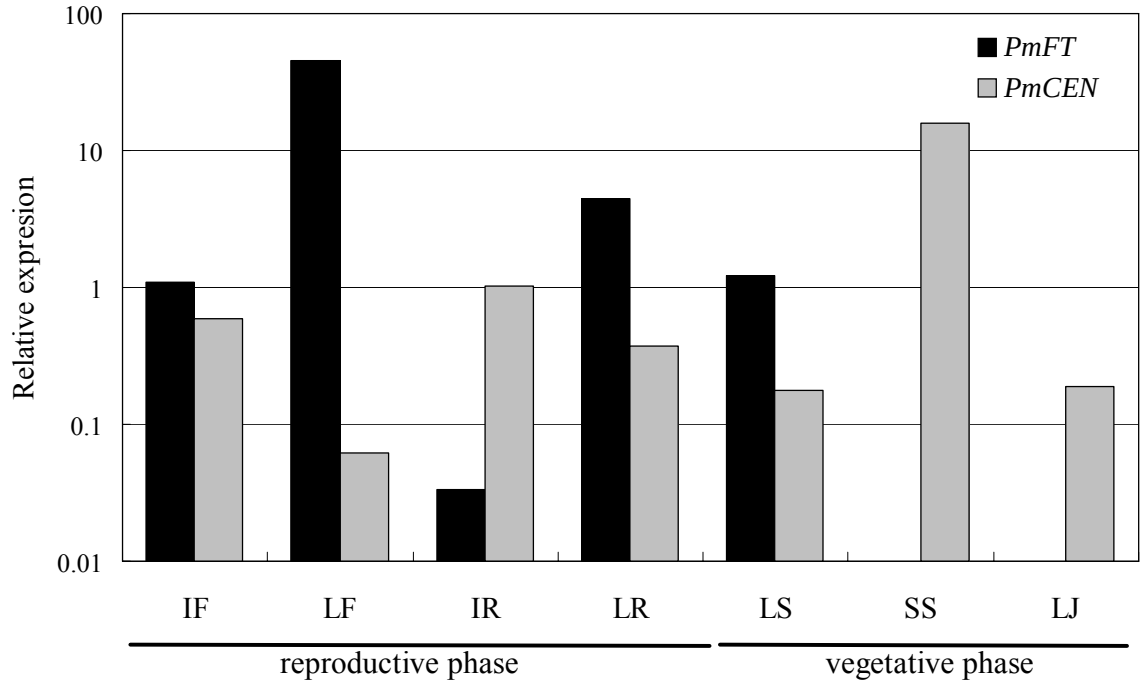


Figure 3 Alignment of the putative amino acid sequences of 4 copies of *PmFT* and partial sequences of 2 copies of *PmCEN* with *FT* (AB027504) and *TFL1* (U77674) in *Arabidopsis*; *RFT1* (AB062676) and *Hd3a* (AB433508) for *FT* homologs and *FDR1* (AF159883) and *FDR2* (AF159882) for *CEN* homologs in *Oryza sativa*; and *PnFT1* (AB369069) and *PnTFL1* (AB369067) in poplar. Amino acids in black and gray are identical and similar, respectively. A dash indicates gaps introduced to maximize the alignment among sequences. In *PmCEN1* and *2*, amino acid residues from 1 to 51 have not been determined yet. Arrowheads indicate the positions of introns. Asterisks indicate amino acids that are critical to the definition of proteins in the *FT* and *TFL1* families.

a


100
10
1
0.1
0.01
Relative expresion
PmFT
PmCEN
IF
LF
IR
LR
LS
SS
LJ
reproductive phase
vegetative phase

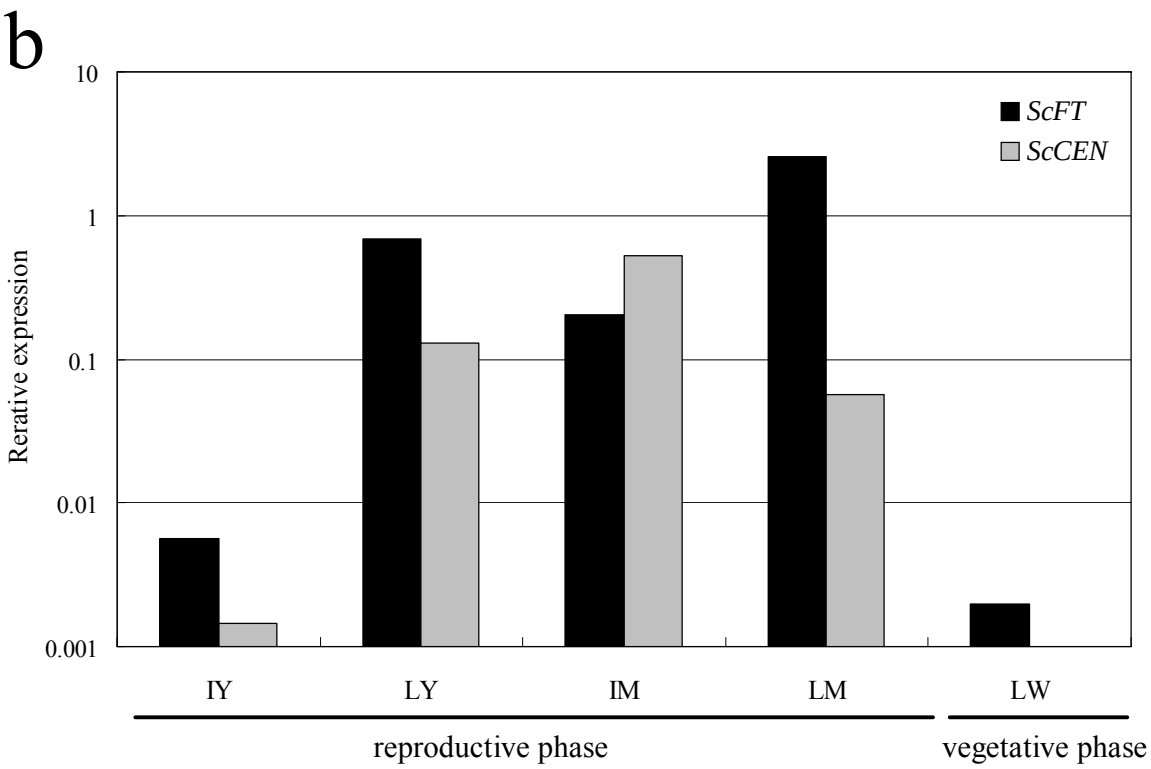


Figure 4 Expression of *FT* and *CEN* homologs in various organs of (a) *Phyllostachys meyeri* and (b) *Shibataea chinensis* in different stages of flowering. Expression levels were measured by real-time RT-PCR and normalized by reference to the *GAPDH* gene. a: IF, LF, inflorescences and leaves of mass flowered culms; IR, LR, inflorescences and leaves of regenerated culms in flower; LS, leaves of sterile regenerated culms; SS, young stems of seedlings; LJ, leaves of juvenile plants in *P. meyeri*. b: IY, LY, young inflorescences and leaves accompanying them; IM, LM, mature inflorescences and leaves; LW, leaves remaining after flowering in *S. chinensis*.

Relationships between *Phuphanochloa* (Bambuseae, Bambusoideae, Poaceae) and its related genera

Sarawood Sungkaew[1], Atchara Teerawatananon[2,3], and Trevor Hodkinson[3]

[1] Department of Forest Biology, Faculty of Forestry, Kasetsart University, Bangkok, Thailand.
[2] Thailand Natural History Museum, National Science Museum, Techno polis, Pathum Thani, Thailand.
[3] Department of Botany, School of Natural Sciences, Trinity College Dublin, University of Dublin, Ireland.

Abstract

Morphological and molecular relationships between a newly established bamboo genus *Phuphanochloa* and it related genera are discussed. Morphologically, *Phuphanochloa* is superficially similar to several bamboo genera e.g. *Bambusa*, *Bonia*, *Dendrocalamus*, *Gigantochloa*, and *Thyrsostachys*. It is, however, somewhat vegetatively similar to either *Gigantochloa* or *Thyrsosstachys*, particularly on the basis of the culm-sheath. In contrast, it is more reproductively similar to *Bambusa* in having distinct and disarticulating rachilla of the spikelet. However, the peculiar syndrome in breaking up of the spikelets at maturity is the best character to set *Phuphanochloa* apart from *Bambusa*. Phylogenetic analysis based on combined five plastid DNA regions; *trnL intron*, *trnL-F* intergenic spacer, *atpB-rbcL* intergenic spacer, *rps16* intron, and *matK*, showed that *Phuphanochloa* is, with high support, sister to the group consisting of eight *Bambusa* species representing all its four subgenera; subg. *Bambusa* (*B. malingensis*), subg. *Dendrocalamopsis* (*B. oldhamii* and *B. beecheyana*), subg. Leleba (*B. tuldoides*, *B. pachinensis*, and *B. dolichomerithalla*), and subg. *Lingnania* (*B. emeiensis* and *B. chungii*). According to morphology and molecular results, *Phuphanochloa* can not be included in any of these subgenera. These eight species of *Bambusa* can not be treated as members of *Phuphanochloa* either. This is because there are some conflicts between morphology and molecular on *Bambusa*. And also, the generic delimitation of such large genus is systematically problematic. There is therefore, *Phuphanochloa* is best regarded as a distinct genus being closely related to *Bambusa* sensu lato.

Keywords: Bambuseae, Morphological and molecular relationships, *Phuphanochloa*

Introduction

Phuphanochloa Sungkaew & Teerawat. is a bamboo genus newly established (Sungkaew et al. 2008). It is apparently a monotypic genus, consisted of a single species, *P. speciosa* Sungkaew & Teerawat. The type locality of this genus is Phu Phan National Park, in Sakon Nakhon Province, north-eastern Thailand where the generic name was named after. Formerly, *Phuphanochloa* was only known from its type locality. After more investigations were carried out, it is found that *Phuphanochloa* also occurs in Loei Province, north-eastern Thailand, especially in Phu Kradung National Park. Sungkaew et al. 2008 reported that *Phuphanochloa* looked

morphologically similar to other four bamboo genera namely *Bambusa*, *Bonia*, *Dendrocalamus*, and *Gigantochloa*.

This study is a step-forward for a better understanding on *Phuphanochloa*. More information from another ally, *Thyrsostachys*, which is also superficially similar to *Phuphanochloa*, was added. The greater sample size of related genera, especially *Bambusa*, *Dendrocalamus* and *Gigantochloa*, for molecular analysis was conducted. The aim of this study is primarily to study morphology and molecular relationships between *Phuphanochloa* and its allies. It also aims to discuss the status of this genus.

Materials and Methods

Morphological relationship

A comparison on morphological characters between *Phuphanochloa* and its allies based on former study (Sungkaew et al. 2008) and more information from this study were compiled. Herbarium specimens of some species of these allies from the Forest Herbarium (BKF) and the Faculty of Forestry, Kasetsart University Herbarium were examined.

Molecular relationship

Using DNA sequences, the relationships of *Phuphanochloa* in comparison with its related genera was investigated. Single and combined genes of five plastid DNA regions, *trnL intron*, *trnL-F* intergenic spacer, *atpB-rbcL* intergenic spacer, *rps16* intron, and *matK*, were phylogenetically analyzed. Combined analysis of plastid DNA regions are often useful for improving phylogenetic resolution and support (Reeves et al. 2001). These five regions have shown to be useful for phylogenetic study of grasses and bamboos for both lower and higher taxonomic ranks (Sungkaew et al. 2009). Twenty-nine bamboo species of the subtribe Bambusinae sensu Soderstrom and Ellis (1987) and Sungkaew (2008) were sampled (Table 1) as the ingroup. Three species of the subtribe Melocanninae according to Ohrnberger (1999) were selected to be the outgroup because they lie outside the ingroup species which are the members of the core Bambusinae (Sungkaew et al. 2009). DNA extractions and relevant processes, including DNA sequencing which performed on an ABI Prism^{TM} 310 Genetic Analyzer (Applied Biosystems), were carried out in Trevor's Molecular Laboratory in the Department of Botany, School of Natural Sciences, Trinity College Dublin, University of Dublin, Dublin 2, Ireland (all molecular protocols see Sungkaew et al. 2009). Successful DNA sequences were edited and assembled using AutoAssembler Software, version 2.1. The sequences were then imported to PAUP 4.0* Beta 2 (Swofford 1998) for alignment. Sequences were aligned by eye. Gaps were scored as additional binary characters (scoring gaps of identical size and position only). The resulting sequences were subjected to maximum parsimony analysis using the heuristic search options in PAUP 4.0* Beta 2 (Swofford 1998). Searches included 1,000 replicates of random stepwise addition saving no more than 100 trees for tree bisection reconstruction (TBR) branch swapping per replicate. Bootstrapping included 1,000 replicates and the same heuristic search settings as the individual searches except that simple addition sequence was used instead of random stepwise addition.

Table 1 Taxa and vouchers of all sequences

Taxon	Voucher/Herbarium	Source
Bambusinae		
Bambusa bambos (L.) Voss	SS&AT 030704-16/THNHM&KUFF	Thailand
Bambusa beecheyana Munro	Stapleton 1313/KEW	USA[1], cultivated
Bambusa chungii McClure	Stapleton 1320/KEW	USA[1], cultivated
Bambusa dolichomerithalla Hayata	Stapleton 1343/KEW	USA[1], cultivated
Bambusa malingensis McClure	Stapleton 1332/KEW	USA[1], cultivated
Bambusa oldhamii Munro	SS&AT 111/THNHM&KUFF	Thailand, cultivated
Bambusa oliveriana Gamble	Stapleton 1321/KEW	USA[1], cultivated
Bambusa pachinensis Hayata	Stapleton 1333/KEW	USA[1], cultivated
Bambusa tulda Roxburgh	Stapleton 1328/KEW	USA[1], cultivated
Bambusa tuldoides Munro	Stapleton 1327/KEW	USA[1], cultivated
Dendrocalamus asper (J.H. Schultes) Backer ex K.Heyne	BAM1[2]	Malaysia, cultivated
Dendrocalamus asper (J.H. Schultes) Backer ex K.Heyne	SS&AT 110704-1/THNHM&KUFF	Thailand, cultivated
Dendrocalamus brandisii (Munro) Kurz	SS&AT 260903-8/THNHM&KUFF	Thailand
Dendrocalamus copelandii (Gamble ex Brandis) N.H.Xia & Stapleton	SS&AT 20/THNHM&KUFF&TCD	Thailand
Dendrocalamus dumosus (Ridley) Holttum	SS&AT 389/THNHM&KUFF&TCD	Thailand
Dendrocalamus giganteus Munro	BAM45[2]	Malaysia, cultivated
Dendrocalamus hamiltonii Nees & Arnott ex Munro	SS&AT 787/THNHM&KUFF	Thailand
Dendrocalamus khoonmengii Sungkaew, A. Teerawatananon & Hodk.	SS&AT 257/THNHM&KUFF&TCD	Thailand
Dendrocalamus latiflorus Munro	SS&AT 113/THNHM&KUFF	Thailand, cultivated
Dendrocalamus membranaceus Munro	SS&AT 020704-4/THNHM&KUFF	Thailand
Dendrocalamus pendulus Ridley	SS&AT 231/THNHM&KUFF	Thailand
Dendrocalamus sinicus Chia & J.L. Sun	SS&AT 127/THNHM&KUFF	Thailand, cultivated
Dendrocalamus strictus (Roxburgh) Nees	SS&AT 718/THNHM&KUFF	Thailand
Gigantochloa albociliata Munro	SD 1436/KEW	Thailand
Gigantochloa atroviolacea Widjaja	Stapleton 1311/KEW	USA[1], cultivated
Gigantochloa ligulata Gamble	SS&AT 090704-4/THNHM&KUFF	Thailand
Gigantochloa scortechinii Gamble	SS&AT 309/THNHM&KUFF	Singapore, cultivated
Bambusa emeiensis L.C.Chia & H.L.Fung	SS&AT 624/THNHM&KUFF	China, cultivated
Phuphanochloa speciosa Sungkaew & Teerawat.	SS&AT 191/THNHM&KUFF&TCD	Thailand
Thyrsostachys siamensis Gamble	SS&AT 020704-3/THNHM&KUFF	Thailand
Melocanninae		
Cephalostachyum pergracile Munro	SD 1435/KEW	Thailand
Pseudostachyum polymorphum Munro	SS&AT 176/THNHM&KUFF	Thailand
Schizostachyum zollingeri Steudel	SS&AT 090704-1/THNHM&KUFF	Thailand

Remarks; Abbreviations are as follows; KEW, Kew herbarium, England; KUFF, Herbarium of Faculty of Forestry, Kasetsart University, Bangkok, Thailand; THNHM, Thailand Natural History Museum, National Science Museum, Techno Polis, Pathum Thani, Thailand; TCD, Herbium, School of Botany, Trinity College, Dublin, Ireland; SS, S. Sungkaew; AT, A. Teerawatananon; SD, S. Dransfield.

[1] California, United States of America

[2] Bambusetum, Rimba Ilmu Botanic Garden, University of Malaya, Kuala Lumpur, Malaysia; specimen collected by K.M. Wong

Results

Morphological relationship

A morphological character of *Phuphanochloa* in comparison to its allies based on former study (Sungkaew et al. 2008) together with more information from this study were compiled and is presented in Table 2.

Molecular relationship

The justification to combine datasets in the analyses in this study was based on an examination of groupings (and support for these) found in the single-gene analyses (data not shown). No major and well supported incongruences were found between the results from single gene region analyses and it was deemed appropriate to combine datasets.

The aligned metrix of the combined five plastid DNA regions (*trnL intron*, *trnL-F* intergenic spacer, *atpB-rbcL* intergenic spacer, *rps16* intron, and *matK*) was 4,243 bp long. 13 characters were excluded and of the remaining 4,230 characters, 4,153 were constant, 29 were variable but parsimony-uninformative, and 48 were parsimony informative. The tree search using maximum parsimony found three equally most parsimonious trees, of 81 steps. CI and RI were 0.97 and 0.98 respectively. One of the three equally most parsimonious trees is shown as a phylogram with bootstrap values and strict consensus information in Figure 1. Bootstrap (BS) percentages (≥50%BS) are described as low (50–74%), moderate (75–84%), and high (85–100%).

Phuphanochloa is sister to the group consisting of *Bambusa* species; *B. emeiensis*, *B. oldhamii*, *B. malingensis*, *B. tuldoides*, *B. pachinensis*, *B. dolichomerithalla*, *B. chungii*, and *B. beecheyana* with high bootstrap support (85%BS, Figure 1). These eight *Bambusa* species is highly supported as a monophyletic group (83%BS). Within this *Bambusa* group, more groupings are found. *B. pachinensis* is sister to *B. dolichomerithalla* and *B. chungii* is sister to *B. beecheyana*, both with low bootstrap support of 63% and 65% respectively. A subgroup comprising five species; *B. tuldoides*, *B. pachinensis*, *B. dolichomerithalla*, *B. chungii*, and *B. beecheyana*, was formed but collapsed in the strict consensus analysis.

Thyrsostachys groups with some species of other two genera with high support (85%BS); *Gigantochloa* (*G. ligulata* and *G. albociliata*) and *Bambusa* (*B. tulda* and *B. bambos*).

Dendrocalamus species bunch together with some representatives from other two genera with high support (100%BS); *Bambusa* (*B. oliveriana*) and *Gigantochloa* (*G. scortechinii* and *G. atroviolacea*). Two representatives of *D. asper* group with two species of *Gigantochloa*, *G. scortechinii* and *G. atroviolacea*, with low support (63%BS). In addition, these two *Gigantochloa* species are grouped together with low support (64%BS).

Table 2 Comparative table of habit and morphological characters between *Bambusa, Bonia, Dendrocalamus, Gigantochloa* and *Phuphanochloa*

Characters	*Bambusa*	*Bonia*	*Dendrocalamus*	*Gigantochloa*	*Phuphanochloa*	*Thyrsostachys**
Habit	usually erect	scrambling	Usually erect	usually erect	usually erect	usually erect
Branch number at mid-culm branch complement	several	Single	Several	several	several	several
Culm-sheath auricles; oral setae/ Culm-sheath blade	usually conspicuous; always present/ usually erect	usually conspicuous, occasionally inconspicuous or small; usually present, occasionally absent/ erect to deflexed	conspicuous, but often small to absent; present or absent/ erect to deflexed	usually absent or small; usually absent, occasionally present/ erect to deflexed	usually absent or small; always absent/ spreading to deflexed, never erect	usually absent or small; usually absent/ erect, occasionally deflexed
Number of glumes per spikelet	0–3	0–2	(1–)2–4(–9)	1–5	1–4	1–3(–4)
Number of fertile florets per spikelet	2–13	3–9	1–8	(1–)2–5	7–9	1–3
Rachilla internodes	distinct and disarticulating	distinct and disarticulating	obscure and not disarticulating	obscure and not disarticulating	distinct and disarticulating	obscure and not disarticulating
Stigma	typically (1–)3, plumose	typically 3, plumose	typically 1(–3), plumose	typically 1, plumose	typically 3, slightly plumose	typically 1–3, plumose
Filaments	typically free	typically free	typically free	always fused into a firm tube	typically free	typically free
Breaking up at maturity of spikelets	either break up above the glume(s) or between the florets	unknown	usually break up above the glume(s)	usually break up above the glume(s)	usually break up in one of two ways (both of which are usually present on any single individual); either above the glume(s) or above the lowest floret	usually break up above the glume(s)

* results from this study, otherwise from Sungkaew et al. 2008

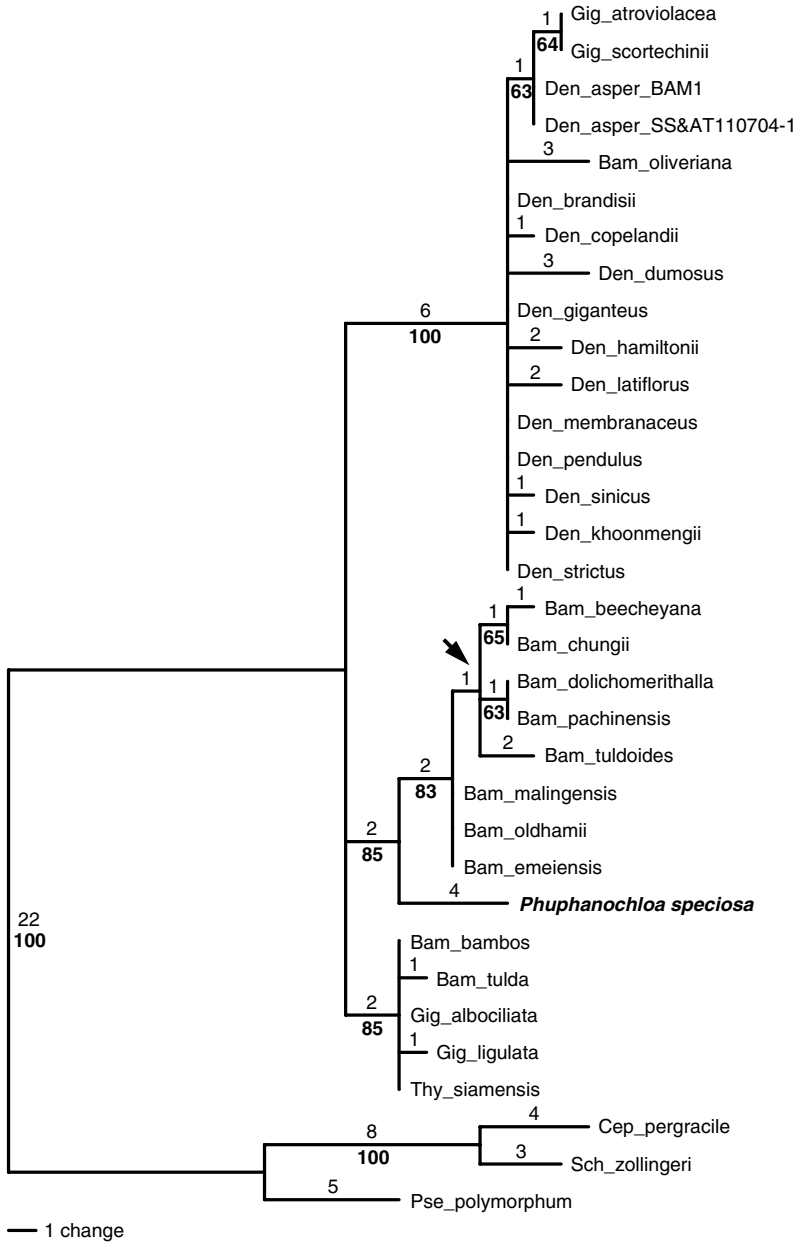


Figure 1. One of three equally most parsimonious trees shown as a phylogram obtained from comparative sequence analysis of combined *trnL-F, atpB-rbcL, rps16* and *matK* sequence data. Values above branches represent the number of steps supporting each branch. Values below branches represent the percentages of bootstrap supporting each branch. Arrow head represents node not supported by strict consensus.

Bam=Bambusa; Cep=Cephalostachyum; Den=Dendrocalamus; Gig=Gigantochloa; Pse=Pseudostachyum; Sch=Schizostachyum; Thy=Thyrsostachys.

Discussion

Morphological relationship

On the basis of morphology, *Phuphanochloa* may superficially be similar to several bamboo genera namely *Bambusa*, *Bonia*, *Dendrocalamus*, *Gigantochloa*, and *Thyrsostachys*. Vegetatively, it looks somewhat similar to either *Gigantochloa* or *Thyrsosstachys*, especially on the basis of the culm-sheath detail (see Table 2). Contrarily, it is more reproductively similar to *Bambusa* in having distinct and disarticulating rachilla of the spikelet. However, *Phuphanochloa* has the spikelets that usually break up at maturity in one of two ways (both of which are usually present on any single individual); either the spikelet totally breaks up above the glume(s) leaving only the elongated rachilla internode (0.5–2 cm long), or it breaks up above the glume(s) and above the lowest floret leaving the upper part of the elongated rachilla internode (to 0.5 cm long) along with the glume(s) and intact lowest floret. This syndrome is not the case in *Bambusa* (Sungkaew et al. 2008). In addition, while *Bambusa* has 1–3 distinctly plumose stigmas but there are usually three, and they are only slightly plumose in *Phuphanochloa*.

Molecular relationship

The results from the combined analysis of five plastid DNA regions (Figure 1) showed that the sister relationship between *Phuphanochloa* and a group comprising eight *Bambusa* species (*B. emeiensis*, *B. oldhamii*, *B. malingensis*, *B. tuldoides*, *B. pachinensis*, *B. dolichomerithalla*, *B. chungii*, and *B. beecheyana*) is highly supported (85%BS). This is congruent with the previous molecular study using a multi-gene region phylogenetic analysis (also using five plastid DNA regions, *trnL* intron, *trnL-F* intergenic spacer, *atpB-rbcL* intergenic spacer, *rps16* intron, and *matK* gene region; Sungkaew et al. 2009). These eight species of *Bambusa* group together with high bootstrap support of 83%. They represent all the four subgenera of *Bambusa* according to Xia et al. (2006). *Bambusa malingensis* represents subg. *Bambusa*; *B. oldhamii* and *B. beecheyana* represent subg. *Dendrocalamopsis*; *B. tuldoides*, *B. pachinensis*, and *B. dolichomerithalla* (treated under *B. multiplex* (Loureiro) Raeuschel ex Schultes & J. H. Schultes var. *multiplex* by Xia et al. (2006)) represent subg. Leleba; and *B. emeiensis* and *B. chungii* represent subg. *Lingnania.* This would suggest that *Phuphanochloa* can not be included in any of these four subgenera of *Bambusa* because of the high support of their sister relationship (85%BS) and the high support of a clade consisting of these eight *Bambusa* species (83%BS). Xia et al. (2006) divided *Bambusa* into four subgenera relying greatly on the culm-sheath. Base on this manner, *Phuphanochloa* would look similar to those of subg. *Lingnania* as they share a common character in having narrow culm-sheath blade. However, there is no evident from our molecular results to support this hypothesis.

There are some conflicts between morphology and molecular information. Some representatives from these four subgenera did not group together systematically. Some of them from different subgenera mis-grouped together, even though with low support, e.g. *B. beecheyana* of subg. *Dendrocalamopsis* groups with *B. chungii* of subg. *Lingnania*. Moreover, *B. bambos*, the type species of *Bambusa* which must taxonomically be regarded as a member of subg. *Bambusa*, did not group with *Bambusa malingensis*. It groups with *B. tulda*, a member of subg. Leleba, and some species of other genera, *Gigantochloa* and *Thyrsostachys* (85%BS). This would suggest that the generic delimitation of *Bambusa* is still unclear. The taxonomy of *Bambusa* is in a state of flux, it is a

large genus with over 100 poorly understood species (Ohrnberger 1999; Xia et al. 2006). Incongruence between the morphological classification and phylogenetic study on this genus has been previously revealed by Sun et al. (2005).

If ones think that the status of *Phuphanochloa* may be uncertain. There are two possible scenarios to cope with this problem. Firstly, *Phuphanochloa* may be simply regarded as a new subgenus of *Bambusa*. Secondly, some species of *Bambusa* particularly those eight species (Figure 1) should be transferred to be new members of the distinct genus *Phuphanochloa*. However, these two ways will not be systematically reasonable until the generic delimitation of *Bambusa* would be clarified. Thus, the best way to do now is to keep *Phuphanochloa* as a distinct genus being closely related to *Bambusa* sensu lato. This idea is now excepted by the BPG (Bamboo Phylogeny Group; *personal communication*).

Generic delimitations of other two genera, *Dendrocalamus* and *Gigantochloa*, are also unclear. *Dendrocalamus* is not strictly monophyletic because a species of *Bambusa* represented by *B. oliveriana*, and two species of *Gigantochloa, G. scortechinii* and *G. atroviolacea*, were embedded in the strongly supported *Dendrocalamus* group (100%BS). The misplacement of these taxa was greatly discussed in Sungkaew *et al.*, (*submitted*) and it requires further investigations.

Acknowledgements

We thank several people who helped or provided us with the plant material used in this study: Drs. Soejatmi Dransfield, Wang Hong, Wong Khoon Meng, Ruth Kiew, Duangchai Sookchaloem, and Chris M.A. Stapleton. This work was supported by: the TRF/BIOTEC Special Program for Biodiversity Research and Training grant T_147003; a Trinity College Dublin, Eire, Postgraduate Studentship and the Trinity College Postgraduate Travel Reimbursement Fund; and the Faculty of Forestry, Kasetsart University, Bangkok, Thailand.

References

Ohrnberger, D. 1999. The bamboos of the world: annotated nomenclature and literature of the species and the higher and lower taxa. Elsevier Science, Amsterdam.

Reeves, G.; Chase, M.W.; Goldblatt, P.; Rudall, P.; Fay, M.F.; Cox, A.V.; Lejeune, B.; Sousa-Chies, T. 2001. Molecular systematics of Iridaceae: evidence from four plastid DNA regions. American Journal of Botany, 88, 2074–2087.

Soderstrom, T.R.; Ellis, R.P. 1987. The position of bamboo genera and allies in a system of grass classification. In: Soderstrom, T. R.; Hilu, K.W.; Campbell, C.S.; Barkworth, M.E. ed, Grass systematics and evolution. Smithsonian Institution Press, Washington, D.C. pp. 225–238.

Sun, Y.; Xia, N.H.; Lin, R. 2005. Phylogenetic analysis of *Bambusa* (Poaceae: Bambusoideae) based on Internal Transcribed Spacer sequences of nuclear ribosomal DNA. Biochem Genet, 43, 603–612.

Sungkaew, S. 2008. Taxonomy and systematics of *Dendrocalamus* (Bambuseae; Poaceae). Ph.D. thesis, University of Dublin, Trinity College, Dublin.

Sungkaew, S.; Stapleton, C.M.A.; Salamin, N.; Hodkinson, T.R. 2009. Non-monophyly of the woody bamboos (Bambuseae; Poaceae): a multi-gene region phylogenetic analysis of Bambusoideae s.s. Journal of Plant Research, 122, 95–108.

Sungkaew, S.; Teerawatananon, A.;. Parnell, J.A.N; Stapleton, C.M.A.; Hodkinson, T.R. 2008. *Phuphanochloa*, a new bamboo genus (Poaceae: Bambusoideae) from Thailand. Kew Bulletin, 63, 669–673.

Swofford, D.L. 1998. Phylogenetic analysis using Parsimony (PAUP) version 4.0. Sinauer Associates, Sunderland.

Xia, N.H.; Chia, L.C.; Li, D.Z.; Stapleton, C.M.A. 2006. *Bambusa* Schreber. In Wu, Z-Y.; Raven, P.H. ed, Flora of China, Vol. 22. Science Press, Beijing & Missouri Botanical Garden, St. Louis. pp. 9–38.

Evaluation of the Polymorphic of Microsatellites Markers in *Guadua angustifolia* (Poaceae: Bambusoideae)

Lorena Torres[1], Diana Carolina Lopez[2], Juan Diego Palacio[2], Myriam Cristina Duque[4], Carlos Andrés Pérez Galindo[3], Iván Andrés González Vargas[1], Heiber Cárdenas Henao[1]

1. Universidad del Valle, Grupo de Eco genética
2. Laboratorio de Biología Molecular Instituto de Investigación de Recursos Biológicos Alexander Von Humboldt
3. Universidad Santiago de Cali
4. Centro Internacional de Agricultura Tropical

Abstract

Guadua angustifolia, one of the world's 20 best bamboos known for their physical and mechanical properties and wide use in the construction industry. It has been used intensively in Colombia reducing native populations to a few hectares. So far, the only strategy for the conservation of genetic and phenotypic variability of the species, although unknown, is the existence of the Germplasm Bank of Bambusoideae located in the Botanical Garden Juan María Céspedes, Tuluá - Valle del Cauca, with accessions from 16 provinces of Colombia. In this study 26 microsatellite markers were designed and evaluated in 46 accessions of *G. angustifolia* to assess the molecular genetic variability of the accessions in the bank and get a new molecular tool enable to conduct population analysis, micro- evolutionary and taxonomic studies.

Amplification of 10 loci was obtained, two showed a pattern of bands with multilocus of genetic origin; in addition, the amplification of 14 loci in Bambusa and between seven or eight loci was reported in other species of Guadua . The eight loci standardized in *G. angustifolia* displayed values of PIC (Polymorphism Index Content) between 0, 3981 and 0, 8517, and probabilities of identity between 0, 0334 and 0, 4134 being medium and highly polymorphic. Therefore, these microsatellites are very good tools to carry out population analyses, taxonomic and microevolutionary studies in *G. angustifolia* and possibly in other species of the genus Guadua and Bambusa, knowledge that will contribute in the creation and implementation of strategies of conservation and sustainable use of the same ones, specially of the Guadua in Colombia.

Introduction

Guadua angustifolia (Karl Sigismund Kunth 1822), American bamboo, is considered one of the 20 best in the world for their excellent physical and mechanical properties, their large size and its wide use in the construction industry (Villegas et al. 2003). In America is distributed from northern Mexico to northeastern Argentina (Young & Judd 1992, Londoño 1991). In Colombia, extends by three mountain ranges from north to south, at

elevations between 500 and 1 500 meters above sea level, dominating the inter-Andean valleys, where they form large associations called "Guaduales" (Londoño 1990).

Due to the continued use of this resource and the extensive colonization of human settlements, few hectares of natural *G. angustifolia* are left in our country (approximately 31 000 ha) (Castaño & Moreno 2004; Villegas et al.2003; Cruz 1994). Therefore it is important the development and implementation of conservation strategies for this species. However, the required prior biological knowledge for its development such as the dynamics of their populations, their genetic diversity, taxonomy and evolution (Frankhan et al.2002), which is lacking today (McNeely 1995; Stapleton & Ramanathan 1995; Bystriakova et al. 2003, 2004).

In 1987 the Bambusoideae Germplasm Bank at the Botanical Garden Juan María Céspedes was established, owned by the Institute for the Research and Preservation of Cultural and Natural Heritage of Valle del Cauca (INCIVA), with the aim of preserving bamboos and deepening their knowledge. This has accessions of *G. angustifolia* from 16 provinces of Colombia (Londoño 1991, Marulanda et al. 2002), thus conserve a high variability of the species, which is convenient topic of study in order to continue conserving *G. angustifolia* using this strategy.

Molecular markers are tools that have allowed to estimate the genetic variability in many species, characterize varieties in germplasm banks, select cultivars, estimated population dynamics and to carry out taxonomic, ecological and evolutionary studies in diverse organisms (Parker et al. 1998, Bachmann 1994, Chasan 1991). In order to obtain basic knowledge for the development of conservation strategies, it is useful to implement them in *G. angustifolia*.

Microsatellites are short DNA sequences of no more than six bases repeated in tandem (Goldstein & Schlötterer 1999), have codominant inheritance, are neutral and highly polymorphic, thus allowing each individual genotype and conduct allocation of parental (Parker et al. 1998, Chambers & MacAvoy 2000), characteristics that facilitate to conduct specific ecological, evolutionary and taxonomy studies, such as estimating the effective size, genetic diversity and structure of populations, allowing to characterize varieties or cultivars identification and selection, identification of breeding systems, gene flow, migration and introgression (Ouborg et al. 1999; Frankhan et al. 2002, Chambers & MacAvoy 2000, Barrera 1996). The use of these molecular markers has not yet been reported for any species of the genus Guadua, so its implementation is relevant.

In this study, the polymorphic information content of 26 microsatellite systems was evaluated in 46 accessions of *G. angustifolia* from the Germplasm Bank of Bambusoideae. Then will be useful in population analysis, and in taxonomic and micro evolutionary studies, knowledge that will help in the creation and implementation of strategies for conservation and sustainable use of Guadua.

Methodology

The study was conducted at the Laboratory of Molecular Biology of the Alexander Von Humboldt (IAvH) Institute located in the facilities of the International Center for tropical Agriculture (CIAT).

The Germ Bank and Sampling

The Germplasm Bank of Bambusoideae is located in the district of Mateguadua, Municipality of Tuluá (Valle del Cauca), approximately 800 meters away from the administrative headquarters of the Botanical Garden Juan María Céspedes owned by the INCIVA. It has an area of about 2 500 m^2. It has climatic conditions characteristic of tropical dry forest with an altitudinal location between 950 to 1 100 meters (Londoño 1990).

Young leaves were collected in good condition from 45 accessions of *G. angustifolia* from different departments of Colombia and one accession from Costa Rica, 14 accessions of different species of the genus Bambusa, and thee of Guadua (Table 1). The leaf tissue was placed in paper bags that were stored immediately in plastic jars filled with silica gel with cobalt indicator. The leaves collected were finely macerated in liquid nitrogen. The mash was kept at -80 ° C until DNA extraction.

DNA Extration and quantification

The DNA was extracted with the Micro extration DNA protocol of Dellaporta (1983) with modifications for rice and with a later mofication for *G. angustifolia* by Potosí et al. (2006).

The evaluation of the DNA was performed by electrophoresis in horizontal agarose gels at 0.8% stained with ethidium bromide, at 48 volts for 30 minutes, adding 2 ul of DNA. It was visualized on a UV transilluminator (Fotodyne Inc). Quantification was carried out in a TECAN spectrophotometer multifunctional Genius, Austria, 260 nm and by agarose gels comparison with DNA Lamda.

Primer microsatellite design

DNA from one individual was used as a source of genomic clones and was used to prepare and enrich GATA tetranucleotide markers in *G. angustifolia*. Genomic DNA was used as starting material- then Psh A1/Hae III double restriction/ligation to linker M28/M29p. M28 5' CTCTTGCTTGAATTCGGACTA M29p 5' pTAGTCCGAATTCAAGCAAGAGCACA), Linker-ligated DNA was denatured and hybridized to biotinylated microsatellite 5' biotin GATA6 (50C), Dynal M270 beads and amplification with M28 primer. Eco RI digestion and ligation into de-phosphorylated Eco RI treated pUC19 followed by electroporation into *E. coli* DH10B. Colony screening was used 5' 32P- GATA6 50C in 5XSSPE washes in 5XSSPE 50C. A set of 24 positive GATA clones were sequenced and 26 primer pairs were tested. Sequences were obtained by amplifying an aliquot of frozen bacterial culture from positive hybridizing 32-P GATA6 colonies using the M13 forward (F) and reverse (R) primers. The amplification reactions were treated with Exonuclease I and alkaline phosphatase to remove excess primer and unincorporated deoxynucleotide triphosphates. After heat deactivation, approximately 10 ng of PCR product was sequenced using M13 forward and/or reverse primers with Applied Biosystems Big Dye V3.1 and ABI3730 .

Table 1. Accessions studied from the Bambusoideae Germplasm Bank of the Botanical Garden Juan María Céspedes.

Accesion Code	Species	Morphological Variants	Provinces
XL 375	Guadua angustifolia	Cebolla	Cauca, Mercaderes
XL 345	Guadua angustifolia	Cebolla	Santander, Floridablanca
XL 542	Guadua superba		Amazonas, Leticia
JA 1006	Guadua amplexifolia		Sucre
XL 343	Guadua angustifolia	Macana	Santander, Puente Nacional
JA 1003	Guadua amplexifolia		Córdoba, Montería
Bicolor 43	Guadua angustifolia	Bicolor	Valle del Cauca, Tuluá
Bambusa vulgaris	Bambusa vulgaris	Bitata	
JA 1004	Guadua amplexifolia		Sucre, Sincelejo
Bambusa vulgaris(M2)	Bambusa vulgaris	Vulgaris	
XL 233	Guadua angustifolia	Macana	Nariño, Ricaute
XL 282	Guadua paniculata		Meta, Serranía de Matupa
XL 124	Guadua angustifolia	Nigra	Valle del Cauca, Tulua
XL 214	Guadua glauca		Putumayo, Mocoa
XL 281	Guadua angustifolia	Cebolla	Meta, Acacia
XL 291	Guadua angustifolia	Macana	Meta, Serranía de la Macarena
Bambusa bambos	Bambusa bambos		
XL 303	Guadua angustifolia	Macana	Meta, Cumaral
JA 1026	Guadua angustifolia	Macana	Huila, Aipe
Guadua weberbaueri	Guadua weberbaueri		
XL 115	Guadua angustifolia	Cebolla	Caquetá, Belén de los Andaquíes
XL 206	Guadua angustifolia	Cebolla	Putumayo, Puerto Caicedo
Bicolor42	Guadua angustifolia	Bicolor	Valle del Cauca, Tuluá
XL 109	Guadua uncinata		Caquetá, Morelia
XL 1012	Guadua angustifolia	Cebolla	Antioquia, Venecia
Guadua paniculata	Guadua paniculata		
Guaduaangus (Costa Rica)	Guadua angustifolia		Costa Rica
JA 1028	Guadua angustifolia	Cebolla	Cundinamarca, Guaduas
XL 91	Guadua angustifolia	Cebolla	Huila, San Agustín
JA 1031	Guadua angustifolia	Cebolla	Caldas, Florencia
XL 235	Guadua angustifolia	Cebolla	Nariño, Ricaute
JA 1023	Guadua angustifolia	Cebolla	Valle del Cauca, El Cairo
XL 344	Guadua angustifolia	Macana	Santander, Curiti
XL 208	Guadua uncinata		Putumayo, Puerto Caicedo
Bambusa vulgaris (M1)	Bambusa vulgaris	Vulgaris	
JA 1042	Guadua angustifolia	Cebolla	Risaralda, Santa Rosa

JA 1049	Guadua angustifolia	Cebolla	Valle del Cauca, Tuluá
JA 1041	Guadua angustifolia	Macana	Risaralda, Pereira
JA 1038	Guadua angustifolia	Macana	Valle del Cauca, Sevilla
JA 1039_2	Guadua angustifolia	Cebolla	Valle del Cauca, Sevilla
JA 1046_1	Guadua angustifolia	Macana	Valle del Cauca, Rio Frio
XL 144	Guadua glauca		Caquetá, Florencia
JA 1038_1	Guadua angustifolia	Macana	Valle del Cauca, Sevilla
JA 1047_1	Guadua angustifolia	Macana	Valle del Cauca, Buga
JA 1048	Guadua angustifolia	Macana	Valle del Cauca, Tulua
JA 1035_ 1	Guadua angustifolia	Castilla	Valle del Cauca, El Cerrito
JA 1045	Guadua angustifolia	Castilla	Quindío, Barcelona
JA 1040	Guadua angustifolia	Cebolla	Valle del Cauca, B/lagrande
JA 1056	Guadua angustifolia	Cebolla	Valle del Cauca, Cali
JA 1055	Guadua angustifolia	Cebolla	Valle del Cauca, Jamundí
JA 1051_2	Guadua angustifolia	Macana	Valle del Cauca, Jamundí
JA 1052_1	Guadua angustifolia	Macana	Valle del Cauca, Jamundí
JA 1056B	Guadua angustifolia	Macana	Valle del Cauca, Cali
JA 1058	Guadua angustifolia	Castilla	Quindío, Quimbaya
JA 1057	Guadua angustifolia	Cebolla	Quindío, La Tebaida
JA 1059_1	Guadua angustifolia	Cebolla	Valle del Cauca, Alcalá
JA 1060_1	Guadua angustifolia	Bicolor	Valle del Cauca, Tuluá
JA 1061	Guadua angustifolia	Cebolla	Valle del Cauca, Restrepo
JA 1062_2	Guadua angustifolia	Bicolor	Valle del Cauca,Vijes
JA 1063	Guadua angustifolia	Cotuda	Valle del Cauca, Restrepo
JA 1064	Guadua angustifolia	Bicolor	Valle del Cauca, Buga

JA= J. Adarve; XL= X. Londoño; ns= whitout number

Amplification of microsatellite regions

Initially, 26 microsatellites designed were tested for *G. angustifolia* by PCR in the accessions of this species. They were subsequently tested in other species of Bambusa and Guadua genus. Each reaction mixture contained 35ng/ul DNA, 10 mM Tris pH 9, 50mM KCl, 2mM MgCl2, 0.1mm of each dNTP, 0.072µM of each primer and 3U Taq polymerase (CIAT Biotechnology Unit) to a final volume of reaction 25 µl. We used a thermal cycler PTC-100 Programmable Thermal Controller, MJ Research, Inc, following the program: 94 ° C for 1 minute (94 ° C for 30 seconds, 54 ° C for 45 seconds, 72 ° C for 45 seconds) 35 times, 72 per 10 minutes, 4 ° C for five minutes, with temperature-specific banding of *G. angustifolia* optimized for each locus, which works for the other species of the genus Bambusa and Guadua (Table 2). The reaction product was assessed on agarose gels 1.5%, stained with ethidium bromide, loading 8 µl of the product together with 2 ml of buffer blue juice.

The identification of each allele per locus was performed by electrophoresis in vertical polyacrylamide gels prepared in 4% TBE 0.5X. A PCR product was added a solution of formamide (0.05% Bromophenol blue and xilencianol, in 95% formamide, 20 mM EDTA) in a 1 ml of solution per 5 ml of PCR product is denatured and a 94 ° C for 5 minutes before serving. The amount of product added to the gel varied with their concentration. The electrophoretic separation was performed at 120W, with an initial flow of 1,800 to 2,000 V with an optimum temperature of 50 ° C. Separation After about an hour apart, the gel was fixed, dyed with silver nitrate and revealed according to the method of Bassam et al. (1991). The reading of each gel was conducted on a white light transilluminator, counting the bands with higher resolution.

Data analysis

From the profiles obtained in the polyacrylamide gels, alleles of each locus were visually based on their molecular size (bp) using as reference the known values of the markers 10bp and 25 bp (Invitrogen Corp., Carlsbad, California). Later, the genotypes were described by generating a matrix of presence / absence (binary) of alleles for each microsatellite. It was counted the number of alleles identified per locus (A) and their frequencies were estimated using the respective SunOS 5.9 platform SAS version 9.1.3. At each locus, we calculated the allelic richness (A-1), the homocigosity (ho) and heterozygosity (Ho) observed, the unbiased expected heterozygosity of Nei (1978), $H_e = n(1-\sum p_i{}^2)/n-1$, the polymorphic information index (PIC) according to Botstein et al.(1980), $PIC = 1-(\sum_{i=1}^{n} p_i{}^2)-\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} 2p_i{}^2 p_j{}^2$ where, p_i was the frequency of the i allele and p_j is the frequency of the j allele. The probability of identity (I) defined as the ability of two individuals at random from the population within a given locus have the same genotype by locus (Paetkau et al. 1995) was estimated as $I = \sum p_i{}^4 + \sum_{i=1}^{n-1}\sum_{j=i+1}^{n}(2p_i p_j)^2$ and the combined probability of identity (Ic) as $I_c = \prod I_k$, where k represents the locus, and indicates, for all microsatellite used, if are good descriptors of the diversity in the germplasm bank.

Table 2.Standardized microsatellites in different species of Guadua and Bambusa genus with temperature of anneling of *G. angustifolia*

Locus	T. anneling in G. angustifolia(°C)	Size (Pb) in G. angustifolia
Bam 1- 14	54	230
Bam 1- 15	54	180
Bam 11- 2	54	120
Bam 1-11	54	280
Bam 1-17	54	250
Bam 1-22	54	280
Bam 1-3	54	150
Bam 1-5	54	180
Bam 1-6	54	250
Bam 16-2	54	200
Bam 17- 2	54	250
Bam 1-8	54	250
Bam 2-1	50	250
Bam 2-11	54	150
Bam 2-13	54	250
Bam 2-2	54	500
Bam 2-3	48	180
Bam 2-5	48	250
Bam 2-6	50	180
Bam 2-7	54	500
Bam 2-8	45	200
Bam 4-2	54	250
Bam 9-2	54	180

Note: The sequences of the primers (5'-3 ') and type of repetition is not included in this table because they have not yet been published and are the property of the entities funding the project.

Results and Discussion

Amplification of the microsatellites regions

Of the 26 microsatellites evaluated were able to standardize in *G.angustifolia* 10 loci in *G. superba, G. amplexifolia* and *G. weberbaueri* 7 loci, in *G. paniculata, G. glauca* and *G. uncinata* 8 loci, in *B. vulgaris* 18 loci and in *B. Bambos* 12 loci (Table 3).
The amplification in agarose gels ranged between 150 and 500 bp and in the acrylamide gels were distinguished alleles between 101 and 500 bp (Figures 1 and 2).

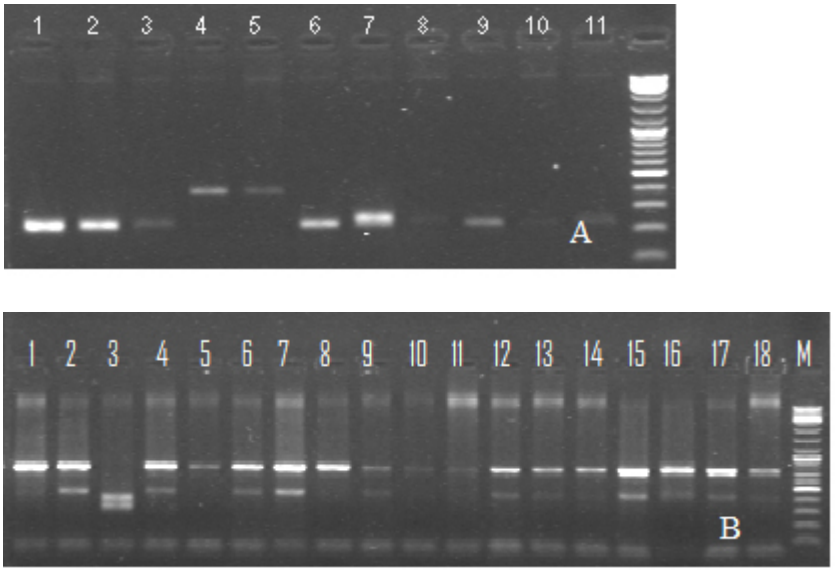


Figure 1. Microsatellites Amplification. A**. Amplification of locus Bam 2-13 in *G. angustifolia* (1-3, 6-11) and *B. vulgaris* (4, 5).** B**. Bam 1-11 in *G. angustifolia* (1-18) and *B. vulgaris* (3).**

Table 3. Optimally standardized microsatellites in each species of Bambusa and Guadua genus.

Especie	G. angustifolia	G. superba	G. amplexifolia	G. paniculata	G. glauca	G. uncinata	G. weberbaueri	B. bambos	B. vulgaris
Bam 2-1	X			X	X	X			
Bam 2-13	X	X	X	X	X	X	X	X	X
Bam 2-5	X	X	X		X	X	X		
Bam 2-2	X	X	X	X	X	X	X	X	X
Bam 2-3	X	X	X	X	X	X	X	X	X
Bam 2-6	X			X					X
Bam 1-11	X	X	X	X	X	X	X	X	X
Bam 2-7	X	X	X	X	X	X	X		
Bam 2-11	X	X	X	X	X	X	X	X	X
Bam 2-8	X								
Bam 1-14									X
Bam 1-15								X	X
Bam 11-2									X
Bam 1-17								X	X
Bam 1-22								X	X
Bam 1-3									X
Bam 1-5									X
Bam 1-6								X	X
Bam 16-2									X
Bam 17-2								X	X
Bam 1-8								X	X
Total	10	7	7	8	8	8	7	12	18

The amplification of microsatellite regions in species of the same genus has been reported in plants such as cassava (Roa et. Al. 2000), in forest trees (Dayananda et al. 1997), and apparently is very common in the grasses. Indeed, within the subfamily Bambusoideae, Nayak & Rout (2005) also succeeded in amplifying 18 microsatellite regions in different species of the genus Bambusa. Marulanda et al. (2007) tested Single Sequences Repeats (SSR) in rice and sugarcane in different species of the Guadua genus, obtaining successful amplification of 37 of these sequences, which indicates the great genetic proximity between the genus in this family, as reported Ishii & McCouch (2000), Kresovich et al. (1995) and Zhao & Kochert (1992), who have identified sequences of rice capable of amplifying in different species of maize and bamboo.

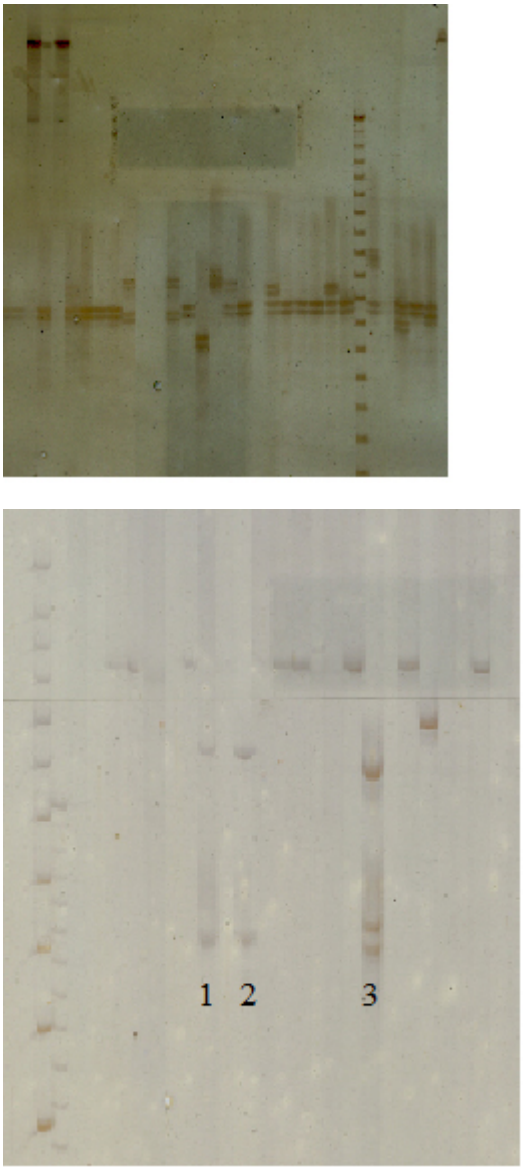


Figure 2. Viewing acrylamide gels at 4% for the identification of alleles of the loci A. Bam 2-13 and B. Bam 2-11 (Bambusa: 1, 2, 3)

This high conservation of microsatellite loci within the family Poaceae promotes the study of genetic variability in species where these markers have not been developed yet, since it would be possible to use these SSR sequences that have been evaluated, such as those obtained in this study , instead of making the whole process of design libraries, saving time and money. In turn, this would permit obtaining the knowledge of the genetic diversity in those species for which this area is unknown, as in the case of most bamboos. As a result, it is recommended to evaluate this new set of microsatellites in other species of the subfamily Bambusoideae.

Regarding the loci Bam 2-8 and Bam 2-11, although amplified in all species of Guadua and Bambusa they presented a profile of bands in acrylamide gels similar to those with a multilocus gene origin (Avise 1994). However, only the locus Bam 2-11 were achieved analyzable, consistent, and reproducible, bands in all the accessions studied (Figure 3). Nevertheless, it is recommended a further analysis of its primers design.

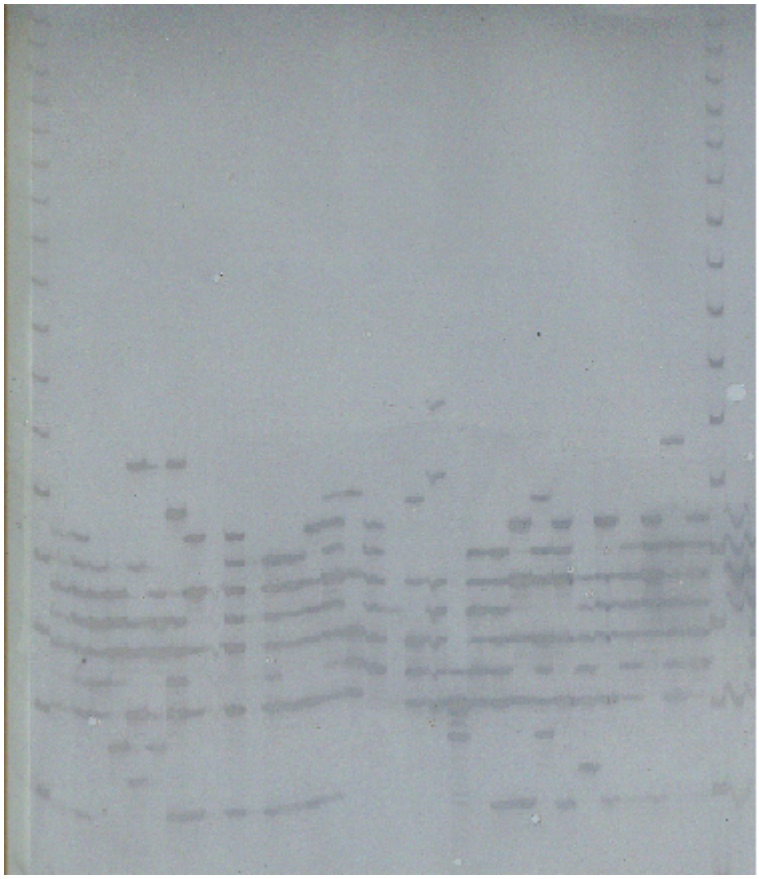


Figure 3. Perfil de bandas obtenidas con el locus Bam 2-11 en geles de acrilamida al 4%.

Evaluation of microsatellites

With the eight loci amplified in 46 accessions of *G. angustifolia*, 69 alleles were found in total, with an average of 8625 ± 3662 alleles per locus, ranging between 5 and 14 alleles and allelic richness averaged 7625 ± 3662 (Table 4). Three null alleles were obtained in the loci Bam 2-6, Bam 2-2 and Bam 2-7, one in each locus, respectively. With the Bam 2-11 locus, profiles were obtained in acrylamide from 5 to 9 bands per individual, with 20 alleles in the 46 accessions of *G. angustifolia*. On average, there were more heterozygotes than homozygotes in the eight loci. In the locus Bam 1-11, heterozygous individuals are not distinguished, while in the locus Bam 2-1, the majorities were it (Table 4).

The polymorphic information content (PIC) has been widely used as a descriptor of the degree of information that provides a site of the genome. PIC values above 0.5 indicate highly polymorphic loci, such as the loci 2-1 Bam, Bam 2-5, Bam 2-7, Bam 2-6 and Bam 1-11; informational medium values between those with 0.25 and 0.5, as were the loci Bam 2-13, Bam 2-2 and Bam 2-3 and informative little lower than the value 0.25. According to this descriptor, no loci were monomorphic for the 46 accessions of *G. angustifolia* and the most informative locus was Bam 2-6 with a PIC value of 0.8517 (Figure 4). However, this index came from studies in human genetics and the purpose of assessing the likelihood of being able to deduct from the genotype of an offspring, of which their parents had received a particular feature. Because of this, when wild populations are being studied in the absence or individuals without knowing their parents, this index is not the most desirable, also implies the absence of recombination.

Table 4. Descriptive estimators of genetic diversity obtained for 8 microsatellite systems evaluated in the accessions of *G. angustifolia*.

Locus	T anneling	High- Frecuency alleles		PIC value	Probability	A	A-1	ho	Ho	he	He
		Allele (bp)	Frecuency		Identity (I)						
Bam 2-1	50	232	0.2283±0.0619	0.8511	0.1082	14	13	0.0435	0.9565	0.1349	0.8843
Bam 2-13	54	228	0.7609±0.0629	0.3981	0.4134	11	10	0.5870	0.4130	0.5896	0.4196
Bam 2-5	48	254	0.2174±0.0608	0.6750	0.1269	13	12	0.4130	0.5870	0.2966	0.7191
Bam 2-2	54	500	0.6848±0.0685	0.4672	0.2871	5	4	0.6957	0.3043	0.5017	0.5094
Bam 2-3	48	166	0.7609±0.0629	0.3860	0.3730	6	5	0.6304	0.3696	0.5951	0.4139
Bam 2-6	50	152	0.1196±0.0478	0.8517	0.0334	9	8	0.8696	0.1304	0.1385	0.8807
Bam 1-11	54	437	0.6304±0.0712	0.5043	0.2480	6	5	10,000	0.0000	0.4518	0.5604
Bam 2-7	54	429	0.3913±0.0720	0.6294	0.1566	5	4	0.6522	0.3478	0.3103	0.7051
Total						69	61				
Average						8.625±3.662	7.625±3.662	0.611±0.290	0.389±0.290	0.377±0.185	0.6356±0.1894

Number of alleles per locus (A), allelic richness (A-1); observed homocigosity (ho), observed heterozygosity (Ho); expected homocigosity (I) Nei unbiased heterozygosity (He), polymorphic information content (PIC); probability of identity (I).

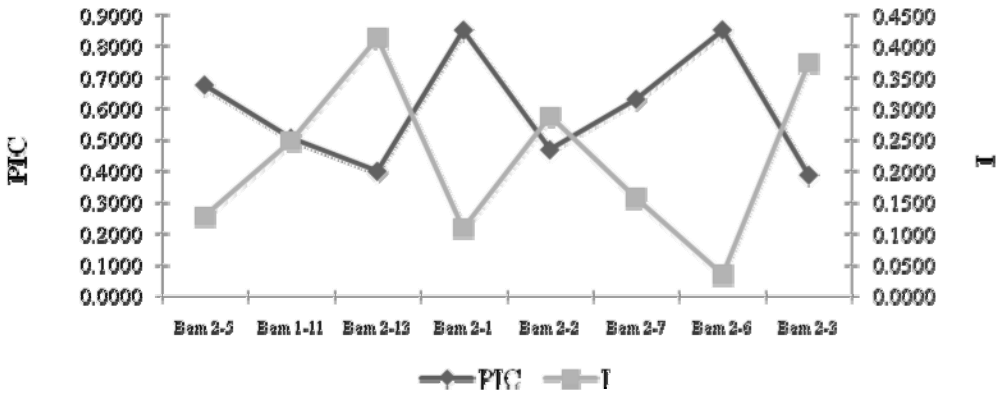


Figure 4. Comparison of the polymorphic information content (PIC) and probability of identity (I) obtained with eight loci in 46 accessions of *G. angustifolia*

The probability of identity (I) may be a better estimate of the degree of information of the genome of a site obtained by the SSR in populations, because it indicates how high is the probability of finding two individuals at random in the same locus in particular, and therefore that is so discriminating that site. Furthermore, it is not based on any postulate, but rather more a probability. Among lower the values of I are the most informative sites studied using microsatellites. According to this index, the locus Bam 2-6 is the most informative (0.0334), followed by Bam 2-1 (0.1082), and the least informative loci is Bam 2-13 (0.4134) (Figure 10). In turn, the combined probability of identity, evaluate how good is the set of all microsatellites for the diversity analysis, in this case, Ic was 7.882x10-07, indicating that the eight loci analyzed for 46 accessions *G. angustifolia* are sufficiently informative to study the genetic diversity in this bank.

Acknowledgments

The Institute for Research and Preservation of Cultural and Natural Heritage of Valle del Cauca, INCIVA, to The University Santiago de Cali and the Center for Research in Basic Sciences, Environmental and Technological Development of the Universidad Santiago de Cali (CICB), by funding this research.
The Laboratory of Molecular Biology and Tissue Collection of the Research Institute of Biological Resources Alexander Von Humbodt IAvH-by funding and technical support during the investigation.
A Miryam Cristina Duque for his selfless assistance in conducting statistical analysis and interpretation of them and Dora Jhoana Rios for his help in the standardization and analysis of microsatellites.

References

Avise, J.C. 1994. Molecular markers, natural history & evolution. New York, Chapman & Hall. 511p.

Bachmann, K. 1994. Tansley Review No. 63: molecular markers in plant ecology. New Phytologist, 126 (3): 403-418.

Barrera, E. 1996.Obtencion de frecuencias microsatélites en frijol (Genero Phaseolus). Tesis de pregrado. Cali-Colombia, Universidad del Valle, Facultad de Ciencias. 103p.

Bassam, B.J.; Caetano. G & Gresshorff , P.M.. 1991. Fast and sensitive silver staining of dna in polyacrilamide gels. Analitical Biochemistry 190: 80-83.

Botstein, D.; White R.L.; Skolnick, M and Davis R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32:314-331.

Bystriakova, N; Kapos, V; Stapleton, C. & Lysenko, I. 2003. Bambú Biodiversity. UNEP-WCMC/INBAR.

Bystriakova, N; Kapos, V & Lysenko, I. 2004. Bambú Biodiversity. UNEP-WCMC/INBAR.

Castaño, F & Moreno R. D. 2004. Guadua para todos, cultivo y aprovechamiento. Bogotá, Panamericana Formas e Impresos S.A. 187p.

Chambers, G. K. &. Macavoy, E.S. 2000. Microsatellites: consensus and controversy. Comparative Biochemistry and Physiology 126: 455–476.

Chasan, R. 1991. Molecular biology and ecology: a marriage of more than convenience. The Plant Cell, 3 (11):1143-1145.

Cruz, I. A. 1994. La Guadua: Nuestro Bambú. Armenia, Fudegraf. 293p

Dayanandan, S, K; Bawa, S & Kesseli, R. 1997. Conservation of Microsatellites Among Tropical Trees (Leguminosae). American Journal of Botany 84 (12):1658-1663

Frankhan, R; Ballou, J.D. & Briscoe, D.A. 2002. Introduction to Conservation Genetics. United Kingdom, Cambridge University Press. 617p

Goldstein, D.B &. Schlötterer, C. 1999. Microsatellites: evolution and applications. Oxford University Press, New York. 350p.

Ishii, T & McCouch, S.R. 2000. Microsatellites and microsynteny in the chloroplast genomes of *Oryza* and eight other Gramineae species. Theor Appl Genet 100:1257-1266

Kresovich, S; Szewc-Mcfadden, A.K; Blick, S & McFerson, J.R. 1995. Abundance and characterization of simple sequence repeats (SSRs) isolated from a size-fractionated genomic library of *Brassica napus* L. (rapseed). Theor. Appl. Genet. 91:206-211.

Londoño, X. 1990. Aspectos sobre la distribución y la ecología de los bambúes de Colombia (Poaceae: Bambuoideae). Caldasia 16 (77): 139-153.

Londoño, X. 1991. Estudio botánico, ecológico, silvicultural y económico-industrial de las Bambuoideae de Colombia. Cespedesia 16-17(59):51-78.

Marulanda, M.L; Márquez, P & Londoño, X. 2002. AFLP analysis of *Guadua angustifolia* (Poaceae: Bambuoideae) in Colombia with emphasis on the Coffe Region. The Journal of the American Bambú Society 16(1): 32-42.

Marulanda. M. L; López, A. M. & Claroz, J.L. 2007. Analyzing the genetic diversity of Guadua spp. in Colombia using rice and sugarcane microsatellites. Crop Breeding and Applied Biotechnoloy 7: 43-51.

Mcneely, J.A. 1995. Bambú, biodiversity and conservation in Asia en: PROCEEDINGS OF THE VTH INTERNATIONAL BAMBÚ WORKSHOP AND THE IV INTERNATIONAL BAMBÚ CONGRESS. 19 – 22 junio 1995. Ubud, Bali, Indonesia. International Network for Bambú and Rattan, Environmental Bambú Foundation, International Plant Genetic Resources Institute, International Development Research Centre. Vol. 2: 23-44

Nayak, S & Rout, R.2005. Isolation and characterization of microsatellites in Bambusa arundinacea and cross species amplification in other bamboos. Plant Breeding 124: 599-602.
Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583-590.
Ouborg, N.J; Piquot, Y & Van Groenendael, J. M. 1999. Population Genetics, Molecular Markers and the Study of Dispersal in Plants. The Journal of Ecology, 87(4): 551-568.
Parker, P.G; Snow, A.A; Schug, M.D; Booton, G.C &. Fuerst, P.A. 1998. What molecules can tell us about populations: choosing and using a molecular marker. Ecology, 79 (2): 361-382.
Potosí, C. T; Vallejo, F. A & Palacio-Mejía, J. D. 2006. Estimación mediante RAPD´s de la diversidad genética en guadua en el departamento del Cauca, Colombia. Acta Agronómica 55(2): 43-47.
Paetkau, D & Strobeck, C.1994. Microsatellite analysis of genetic variation in black bear populations. *Molecular Ecology* **3**:489–495.
Roa, A. C; Chavarriaga-Aguirre, P; Duque, M. C; Maya, M. M; Bonierbale, M. W; Iglesias, C & Tohme, J. 2000. Cross-Species Amplification of Cassava (Manihot esculenta) (Euphorbiaceae) Microsatellites: Allelic Polymorphism and Degree of Relationship. American Journal of Botany,87(11):1647-1655.
Stapleton, C.M.V & Ramanatha RAO, V. 1995. Progress and Prospects in Genetic Diversity. Studies on Bambú and its conservation en: PROCEEDINGS OF THE VTH INTERNATIONAL BAMBÚ WORKSHOP AND THE IV INTERNATIONAL BAMBÚ CONGRESS. 19 – 22 junio 1995. Ubud, Bali, Indonesia. International Network for Bambú and Rattan, Environmental Bambú Foundation, International Plant Genetic Resources Institute, International Development Research Centre. Vol. 2: 23-44
Villegas, M; Villegas, B; Villegas, L & Londoño, X. 2003. Guadua: arquitectura y diseño. Colombia, Villegas editores. 208p.
Young, S.M. & Judd, W. S. 1992. Systematics of the Guadua angustifolia Complex (Poaceae: Bambuoideae). Annals of the Missouri Botanical Garden, 79 (4): 737-769.
Zhao, X & Kochert, G. 1992. Characterization and genetic mapping of a short, highly repeated, interspersed DNA sequence from rice (Oryza sativa L.). Molecular & general genetics 231(3):353-9.

Occurrence of filamentous fungi on Brazilian giant bamboo

[1] Rodolfo Gomes da Silva; [1] Antonio Ludovico Beraldo; [2] Milena Binatti Ferreira; [2] Rafaella Costa Bonugli-Santos; [2] Lara Durães Sette

1 – School of Agricultural Engineering – FEAGRI/UNICAMP

2 – Division of Microbial Resources- CPQBA/UNICAMP

Abstract

Bamboo has many economical and environmental advantages compared with other materials commonly employed in construction. However, bamboo is handicapped by the low natural durability of the most of species. According to optimal environmental conditions, several insects or fungi decay bamboo.

The aim of this research was to identify taxonomically some filamentous fungi that decay bamboo in contact with the soil. Fungi were collected from samples of bamboo strips expose to outdoor conditions. Isolated fungi were taxonomically characterized based on morphological and genetic (Amplified Ribosomal DNA Restriction Analysis-ARDRA) approaches.

Ten isolates of filamentous fungi were obtained. Data derived from ARDRA analyses showed the presence of seven different taxonomic groups (ribotypes). Based on microscopic and macroscopic analysis, fungi were identified as belonging to cellulolytic genera: *Arthrinium*, *Fusarium*, *Acremonium*-like and *Trichoderma*, and an unidentified isolate. As there was no fungal mycelial growth of green in samples of bamboo, *Trichoderma* sp. may have been originated from the proper soil. In addition, the fungus that was evaluated separately showed morphological characteristics similar to those of basidiomycete (Basydiomycota).

Introduction

In global terms, 40% of energy consumption and carbon emission in the world are caused by construction (Ferraz 2008). This situation is exacerbated by the use of native timber for building. According to Kageyama (1987), the deforestation of tropical rainforest may cause the extinction of entire species. The market preference by certain tropical woods because of its high quality, provoke its intensive use and became a serious problem, especially at Sao Paulo State, Brazil.

The solution to this problem involves the use of materials less harmful to the environment than that conventional ones. The possibility of applying bamboo, therefore, appears as an alternative to the tropical wood.

However, bamboo applications are hampered by the low natural durability of the most of specie. Decay caused by physical, chemical and biological agents associate bamboo as a low quality material, creating the false idea that bamboo should be employed only in the scarcity of most appropriate materials.

Fungi were considered plants for a long time. Unlike plants, fungi are heterotroph and do not have clorophyll or other photosynthetic pigment. Their cell walls are composed by chitin, cellulose does not, unless some aquatic fungi.

Fungi can decompose dead matter (saprotrophic) or obtain its nutrients from living organisms (parasitic), preferring simple carbohydrates, but may also use more complex sources, as starch and cellulose (Burton & Engelkirk 2005). Basidiomycetes fungi are the most responsible by decay materials composed by lignin and cellulose. This group is represented by mushroons, puffballs and bracket fungi, most of them known for its economic importance, provoking plant diseases, or acting as decomposers of organic matter and for its culinary potential. However, representatives of others fungi groups, such as ascomycetes are able to colonize and degrade lignocelullosic material (Sette et al. 2008).

According to Highley (1999), "fungus damage to wood may be concerned to three general causes: lack of suitable protective measures when wood storing, improper seasoning, storing, or handling of the raw material produced from the log and failure to take ordinary simple precautions in using the final product".

From the 1980's, many studies on the degradation of wood by fungi were performed. Auer et al. (1988) associated the monoculture of eucalyptus and the high incidence of fungi. Wood has great potential as building material since it is well applied to buildings and since they were well designed, constructed properly and adequately maintained. However, any of these aspects is often overlooked at the construction, allowing the attack of the decay agents, such insects and fungi (Nunes et al. 2000).

The objective of this research was to identify taxonomically some fungi that decay bamboo in contact to the soil. In a next step, intends to inoculate these fungi on bamboo, seeking to evaluate the effectiveness of some treatments applied to bamboo strips.

Materials and Methods

Figure 1 shows the flowchart of the steps undertaken during the development of this research.

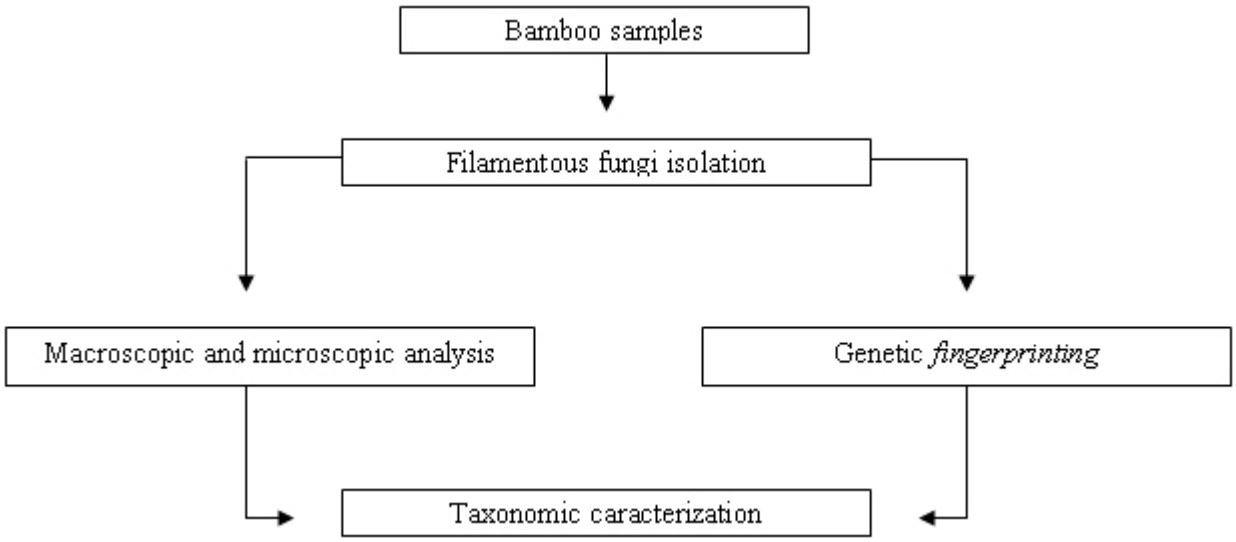


Figure 1 - Flowchart for the implementation of isolation and identification of fungi associated with bamboo.

Strips of 15 cm x 3 cm x 3 cm were obtained from a 5 years old culm of giant bamboo (*Dendrocalamus giganteus* Munro) Strips were exposed to an oxisol type, simulating the decay by wet soil fungi, allowing the colonization of several species (Figure 2).

Figure 2 – Aspect of bamboo strip after 15 days of exposition.

After 15 days of exposure, strips were numbered and delivered to Division of Microbial Resources (CPQBA/UNICAMP) for filamentous fungi isolating and identifying.

A visual inspection of the bamboo strips indicated the development of several fungi (Figure 3), wich were readily separated by the morphological characteristics of the colonies. Bamboo strips were washed with sterile distilled water to remove the soil and to isolate only the fungi associated with bamboo. Filamentous fungi were plated by swab technique on culture media MA2 (Malt Extract Agar 2%) and SDA (Sabouraud Dextrose Agar) added 300 mg/L riphampicin, antibiotic to prevent bacteria proliferation. The plates were incubated at laboratory temperature (28 ± 1 °C) for 15 days. Isolation of colonies was conducted daily and pure cultures were obtained after serial transfers on MA2 medium (Figure 4).

Figure 3 – Fungi spores (dark spots) and mycelia (white areas) infesting bamboo.

Figure 4 – Culture of bamboo-derived fungus grown in Petri dish.

The colonies were observed by stereoscope to the fungi identification. Microscope slides were prepared by scrubbing technique, stained with lactophenol cotton blue and visualized in optical microscope. These observations, using the morphological criteria determined by the literature, allowed the preliminary identification of some genera. The identification of species requires molecular techniques (sequencing and phylogenetic analyses) and additional macro and microscopic analyses.

Isolates were subjected to ARDRA analyses (Amplified Ribosomal DNA Restriction Analysis) to identify possible different taxonomic groups. Filamentous fungi were cultured on MA2 medium and after culture growth, genomic DNA extraction was performed according to Raeder & Broda (1985). The 28S rRNA D1/D2 region of the filamentous fungi were amplified from genomic DNA by Polymerase Chain Reaction (PCR) using the following set of primers, NL-1m (5' GCA TAT CAA TAA GCG GAG GAA AAG 3') and NL-4m (5' GGT CCG TGT TTC AAG ACG 3'). PCRs were performed according to Sette et al. (2006). PCR products were digested using the restriction enzymes *Msp*I, *Hha*I, *Hae*III and AluI (GE Healthcare). Restriction reactions were carried out in 2h at 37 °C and the electrophoresis were performed on a 2.5% agarose gel, with a 100-bp DNA ladder, for 2.5h at 230 V.

In addition to the filamentous fungi that have developed in bamboo, it was obtained a fungus fruit body, probably a basidiomycete, from one sample of decayed bamboo.

Results and Discussion

From two bamboo samples, ten isolates of filamentous fungi were identified, based on microscopic and macroscopic analysis, as belonging to the genera: *Arthrinium* (F1, F2, F4, F8, F9 e F10), *Fusarium* (F3), *Acremonium*-like (F5) and *Trichoderma* (F6), and an unidentified isolate (F7) (Table 1). In addition, the fungus that was evaluated separately from a decayed bamboo (F11) showed morphological characteristics similar to those of basidiomycete (Basydiomycota), a well known lignocelullolytic degraded group of fungi.

Table 1 - Data from the morphological characterization and genetic fingerprinting.

Isolates	*Hae*III	*Msp*I	*Hha*I	*Alu*I	Ribotypes	Morphologic id.
F1	A	A	A	A	1	*Arthrinium* sp.
F2	A	A	A	A	1	*Arthrinium* sp.
F4	A	B	A	A	1A	*Arthrinium* sp.
F8	A	A	A	A	1	*Arthrinium* sp.
F9	A	B	A	A	1A	*Arthrinium* sp.
F10	A	A	A	A	1	*Arthrinium* sp.
F3	B	C	B	B	2	*Fusarium* sp.
F5	A	B	C	C	3	*Acremonium*-like
F6	C	D	D	C	4	*Trichoderma* sp
F7	D	E	E	D	5	NI*
F11	A	F	F	C	6	NI*

NI * Non identified.

According to Morakotkam et al. (2007), *Arthrinium* (Xylariales) are a dominant genus in bamboos. Representatives of this genus and its telemorph (*Apiospora*) have been reported as fungi associated with bamboo from New Zealand and Japan (Morakotkam et al. 2007). The genus *Fusarium* (Hypocreales) were also reported as fungal associated with bamboo plants (Hino & Katumoto 1961; Morakotkam et al. 2007). *Arthrinium* and *Fusarium* are soil-inhabiting fungi that could be found in decomposing plant material. Both are cellulolytic, but this activity for *Arthrinium* is rarely reported. *Fusarium* and its anamorph *Giberella* have been isolated from many plants and cause some plant diseases (Rubini et al. 2005).

There are no data in the consulted literature concerning *Acremonium* (anamorphic fungi) and *Trichoderma* (Hypocreales) derived from bamboo samples. As there was no fungal mycelial growth of green in samples of bamboo in the present study, *Trichoderma* sp. (F6) may have been obtained from the proper soil where the bamboo was removed. It is worth to mention that representatives of *Trichoderma* and *Acremonium* are very common in soil and are also able to produce cellulolytic enzymes, which are responsible for cellulose degradation (Nakari-Setälä & Petillä 1995; Stemberg 2004; Ikeda et al. 2007).

Some of isolated fungi showed morphological features very similar and to verify the genetic diversity (polymorphism) of them ARDRA analyses were carried out (Figure 5 and Figure 6). The band pattern (ribotype) generated by enzymatic digestion allowed the differentiation of taxonomic groups previously obtained by conventional taxonomy. Seven different ribotypes were obtained: ribotype 1, 1A, 2, 3, 4, 5 and 6 (Table 1).

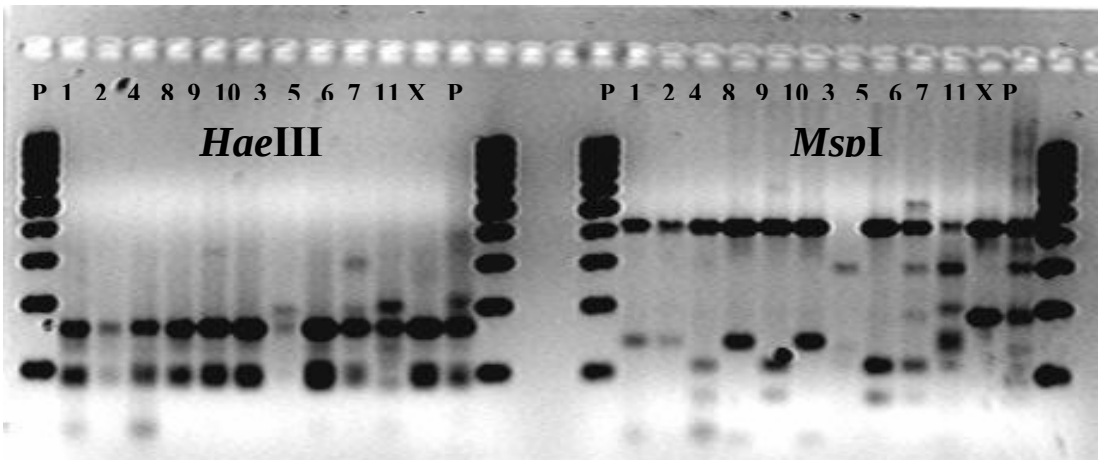


Figure 5 – Restriction profile from the eleven isolateds after digestion with the *Hae*III and *Msp*I enzymes. The numbering at the top of the figure represents the fungi order of application on the agarose gel. P = Standard molecular weight (1kb). X = Sample to be disregarded because it is not part of this project.

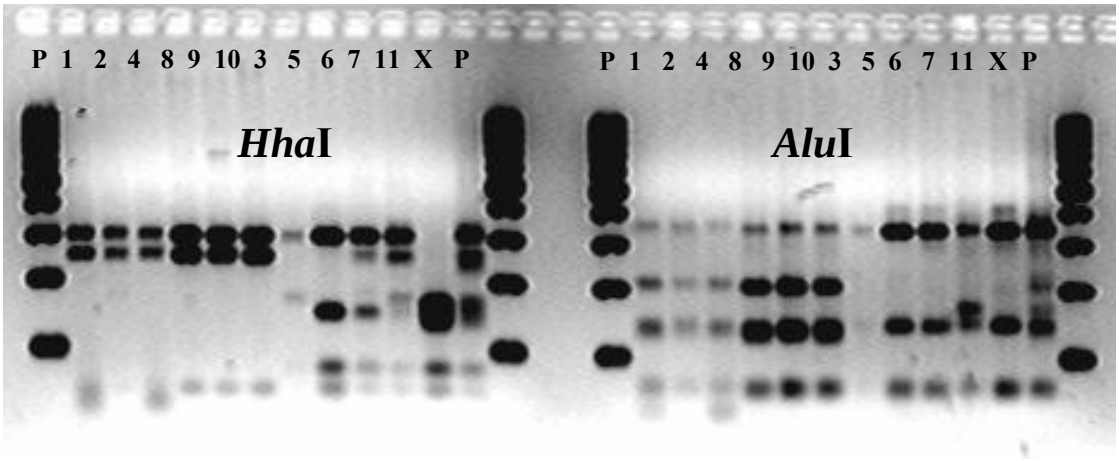


Figure 6 – Restriction profile from the eleven isolateds after digestion with the *Hha*I and *Alu*I enzymes. The numbering at the top of the figure represents the fungi order of application on the agarose gel. P = Standard molecular weight (1kb). X = Sample to be disregarded because it is not part of this project.

Ribotypes 1 and 1A showed little difference in the restriction profile when *Msp*I enzyme was used. As both isolates showed morphological (macroscopical and microscopical) characteristics similar to the genus *Arthrinium*, the polymorphism may not be representative or may indicate strains of different species.

On the other isolates, the results of ARDRA corroborated the morphological analysis, since the fungi showed different restriction profiles and were classified morphologically as belonging to different genera. The isolated fungi unidentified by conventional taxonomy (F7 and F11) showed morphological characteristics and restriction profile different from the others, suggesting that it should belong to different filamentous fungi genera.

Aiming at a more accurate identification of different ribotypes obtained in this work, as well as the identification of ribotypes F7 and F11 (not identified by conventional taxonomy), further studies of sequencing and comparative analysis should be performed.

Conclusions

Although a definitive taxonomic assignment of the fungi isolated and characterized in this study was not always possible, these data present an emerging view of filamentous fungi from Brazilian bamboo samples, since, to our knowledge, there were no previous reports on fungi isolated from bamboo in Brazil. Based on the literature, the genera *Arthrinium* and *Fusarium* have been reported as fungi associated with bamboo in other countries. However, it is important to highlight that it is the first report concerning *Acremonium* from bamboo samples.

The occurrence of cellulolytic fungi in bamboo was expected, since these fungi are able to use the bamboo cellulose as carbon source. The filamentous fungi isolated in the present study will be deposited in the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI) for further research on effectiveness of some treatments applied to bamboo strips against these cellulolytic filamentous fungi.

References

Auer, C.G.; Krugner, T.L.; Barrichelo, L.E.G. Fungos termófilos em pilhas de cavacos de Eucalyptus spp. com auto-aquecimento. Instituto de Pesquisas e Estudos Florestais, Piracicaba, n. 38, p. 28-32, abr. 1988. Disponível em: <http://www.ipef.br/publicacoes/scientia/nr38/cap04.pdf>. Acesso em 14 jul.2008.

Burton, G.R.W.; Engelkirk, P.G. Microbiologia para as ciências da saúde. Rio de Janeiro: Guanabara Koogan, 2005.

Ferraz, M. Uma casa para o semi-árido. Ciência Hoje, Rio de Janeiro, v. 42, n. 249, p. 50-51, jun. 2008.

Highley, T.L. Biodeterioration of wood. In: United States Department of Agriculture. Wood handbook: wood as an engineering material. Madison, WI: United States Department of Agriculture Forest Service, Forest Products Laboratory, 1999. p. 13.1 – 13.16.

Hino, I.; Katumoto, K. Icones Fungorum Bambusicolorum Japonicorum. The Fuji bamboo garden, Gotenba, Japan, 1961.

Ikeda, Y.; Hayashi, H.; Okuda, N.; Park, E.Y. Efficient Cellulase Production by the Filamentous Fungus Acremonium.cellulolyticus. Biotechnol. Prog v. 23, p. 333-338, .2007

Kageyama, P.Y. Conservação "in situ" de recursos genéticos de plantas. Instituto de Pesquisas e Estudos Florestais, Piracicaba, n. 35, p. 7 – 37, abr. 1987.

Morakotkarn, D.; Kawasaki, H.; Seki, T. Molecular diversityof bamboo-associated fungi isolated from Japan. FEMS Microbiol Lett, v. 266, p. 10–19, 2007.

Nakari-Setälä, T.; Penttilä, M. Production of Trichoderma reesei cellulases on glucose-containing media. Applied and Environmental Microbiology. v. 61, p. 3650–3655, 1995.

Nunes, L.; Nobre, T.; Saporiti, M. Degradação e reabilitação de estruturas de madeira. Importância da acção de térmitas subterrâneas. In: Encontro Nacional sobre Conservação e Reabilitação de Estruturas, 2000. Laboratório Nacional de Engenharia Civil, Lisboa, p. 167-175.

Raeder, J.; Broda, P. Rapid preparation of DNA from filamentous fungi. Letters in Applied Microbiology, v.1, p. 7-20, 1985.

Rubini, M.R.; Silva-Ribeiro, R.T.; Pomella, A.W.V.; Maki, C.S.; Araujo, W.L.; Santos, D.R.; Azevedo, J.L. Diversity of endophytic fungal community of cacao (Theobroma cacao L.) and biological control of Crinipellis perniciosa, causal agent of Witches' broom disease. Int J Biol Sci v.1, p. 24–33, 2005.

Sette, L.D.; Passarini, M.R.Z.; Delarmelina, C.; Salati, F.; Duarte, M.C.T. Molecular characterization and antimicrobial activity of endophytic fungi from coffee plants. World J. Microbiol. Biotechnol., 22, 1185-1195, 2006.

Sette, L.D.; de Oliveira, V.M.; Rodrigues, M.F.A. Microbial lignocellulolytic enzymes: Industrial applications and future perspectives. Microbiology Australia, v.99, p. 18-20, 2008.

Sternberg, D. A method for increasing cellulase production by Trichoderma viride. Biotechnology and Bioengineering, v. 18, p. 1751-1760, 2004.

Consideration of the flowering periodicity of *Melocanna baccifera* through past records and recent flowering with a 48-year interval

Shozo Shibata
Field Science Education and Research Center, Kyoto University, Japan

Abstract

The gregarious flowering of *Melocanna baccifera* has been recorded in its native area mainly since 2003 and this period of dynamic flowering is now coming to an end. For over 100 years, the flowering periodicity of the species has been estimated by many researchers as being in the vicinity of 30 – 45 years. However, local information from the author's detailed interviews with farmers in the Mizoram area of northeast India indicates that a more accurate estimation is 48-year. Based on this estimation, the author and his colleagues conducted important ecological research on the flowering and fruiting process in this area, while flowering information for 2008 was obtained from Taiwan and that for 2009 from Japan. These two data sets present accurate records of the last fruiting year, and flowering occurred as expected in the 48th year after seeding. This outcome suggests that understanding the true flowering periodicity of bamboo requires the monitoring of seedling with accurate fruiting data in addition to vegetative information.

Introduction

For more than 100 years, bamboo researchers have been interested in estimating the flowering periodicity of bamboo (cf. Seifriz 1923; Raizada & Chatterjii 1956; McClure 1966; Janzen 1976). Although bamboo flowering is gregarious and synchronized in many cases, its prediction is exceptionally hard work because of the very long flowering periodicity involved. This also means that it is difficult for a single researcher to carry out observation of bamboo flowering and confirm its periodicity.

Bamboo flowering is mainly classified into the two types of gregarious and sporadic flowering. Many cases of sporadic flowering are seen, but, its exact definition is not clear. Some such flowering occurs in small-scale vegetation and is even seen in parts of the culm. Although the relationship between the flowering periodicity and simultaneousness remains unclear, it is currently considered that gregarious flowering with periodicity usually occurs simultaneously. In the past many triggers for flowering have been discussed, including drought, burning, trimming, disease (Hori 1911), transplant and injury (Seifriz 1923). However, at present, the flowering for these reasons is regarded as a different physiological occurrence from the gregarious and simultaneous phenomenon brought about by the biological clock.

Gregarious flowering of bamboo results in the death of the parent population, and this occurrence has long been recognized as continuing for several years as whole in tropical areas. In Japan, the flowering of *Phyllostachys bambusoides* was reported in around 1970, and continued for about ten years (Kasahara 1971). Similarly, the recent flowering of *Sasa veitchii* var. *hirsuta* recorded near Kyoto city continued over a period of four years (Abe & Shibata 2007).

Melocanna baccifera is a species that has extensive past flowering records. The form of its characteristic fruit and the culm neck are worth noticing, and the first taxonomical records of this species also refer to these points naturally (Roxburgh 1814). Its flowering periodicity has been estimated many times, but, as the distinction between sporadic flowering and gregarious or extensive flowering was insufficient, these estimations can be considered capricious. The reason for this problem is a lack of surveys on flowering vegetation from seedling stage. *M. baccifera* flowered gregariously in 2005 – 2008 in its native region, with the flowering area reaching up to several tens of thousands of square kilometers. Ecological research in this native area by the author and his colleagues revealed that sporadic flowering occurs one year before and after gregarious flowering, and that the area covered by gregarious flowering moves from the northeast to the southwest over a period of several years. This has also been noted in Bangladesh by Alam (2008).

In relation to the recent flowering, some flowering records derived from fruits taken from native areas during the last flowering are being collected. These examples include cases reported from Japan and Taiwan, where seedlings are maintained in a pure condition without being mixed with other seedlings. These plants clearly flowered in the 48th year after seeding. Referring to these examples, the author use this paper to consider the relevant points in order to gain an accurate understanding of *M. baccifera*, and in turn, its flowering periodicity, and re-inspects the past flowering records of this species.

Flowering records of *M. baccifera* seedlings with a 48-year interval

In Japan the flowering of *M. baccifera* was observed in May, 2009. The plantation in question is derived from a seeding brought in 1961 by Koichiro Ueda, who was performing a bamboo resource survey, from what is now Bangladesh (former East Pakistan) (Ueda 1968) to the former Shirahama Experimental Station of the Field Science Education and Research Center at Kyoto University. In those days Ueda was the only Japanese person to have visited East Pakistan, so it is clear that the flowering periodicity of the species is 48 years, at least, for the genealogy he brought into Japan.

In Taiwan, flowering was recorded in 2008. Lu (2009) reported that the flowering plantations in question were derived from two seedlings introduced from a group of 58 from USA through USDA, which collected fruits in Puerto Rico from a fruiting plantation in 1960 after the flowering of 1959. This flowering record also supports the estimation that the species flowers every 48 years.

Flowering records of *M. baccifera* in the past reports

There are many bamboo flowering records in the world, but it is thought that the reports recorded by observers themselves are rare, especially for tropical bamboo species. In the case of *M. baccifera*, the first record by

Roxburgh (1814) and even that of Staff (1904) noting the detail of the fruit shape and inflorescence were not observed directly in the native flowering area. Most past records are simply general descriptions formulated by referring to lots of information from native areas.

Although the accuracy of these data resources is uncertain, in Mizoram, India, records provided by the state government indicate gregarious flowering in 1815, 1863, 1911 and 1958 – 1959. In this information supplied on the website of International Network of Bamboo and Rattan (http://www.bambootech.org/), the oldest flowering record is from 1815. This information is not enough to rely on because the records it is based on are unknown, and the recorded year is similar to that of the first notification of this species. However, there is still the possibility of pinpointing this flowering occurrence from the record of Brandis referred to by Blatter (1929), which reported flowering at Chittagong, Bangladesh, in 1811 without consideration of whether it was gregarious flowering or not (Table 1). Below, the author introduces past flowering records according to information from the Mizoram state government based on the supposition of a 48-year flowering interval.

The next flowering records after those of 1815 are based on Munro (1868), who recorded flowering in 1864 – 1865 in Arracan. Riviere & Riviere (1878) also recorded flowering for similar years in present-day Bangladesh and reported a scarcity of bamboo timber resulting from the death of bamboo forest following the flowering. Staff (1904) noted that the flowering area covered about 6,000 square miles. All flowering records from the 19th century after Munro referred to his records (Gamble 1896; Brandis 1899). For subsequent flowering, we see an occurrence in 1901 – 1902 in the Chittagong area of what is now Bangladesh from reporting on fruit and inflorescence by Staff (1904). Although this flowering does not fit the 48-year interval, it is estimated that the area it covered was relatively large.

The next gregarious flowering, thought to have occurred in 1911, can be understood from the reporting of Troup (1921) in Chittagong and the Arakan mountains as referred to by Blatter (1929, 1930a, 1930b) (Table 1), followed by the reports of Parry (1931) in Assam, India and Hossain (1962) in the former East Pakistan as referred to by Janzen (1976) (Table 2). Camus (1913) did not mention this flowering despite publishing in the same year. Concerning Mizoram, India there are two mentions of the flowering in 1911 by Rokhuma (1988) and in 1911 – 1912 by Thanchuanga (2004). On the other hand Janzen (1976) did not refer to the three intimate records of Blatter (1929, 1930a, 1930b). As a result it seems that there was gregarious flowering around 1911 from Bangladesh to Myanmar through northeast India.

There are many records of the last gregarious flowering from the latter half of the 1950s to the beginning of the 1960s. I has already been mentioned that Ueda also came across this flowering in Bangladesh in 1961 as the first Japanese person there (Ueda 1968). Janzen (1976) referred to two records: the flowering in 1960 at Mizoram, and that in 1958 – 1959 at Chittagong (Table 2). In Mizoram, Rokhuma (1988) recorded flowering in 1958 – 1959, and Thanchuanga (2004) in 1959 – 1960. In addition to these, there is another flowering report for 1957 – 1960 by Rain Forest Research Institute of Jorhat in Assam (2003). Alam (2008) noted that gregarious flowering in the Chittagong area of Bangladesh occurred in 1960 – 1961 after a period of sporadic flowering from 1952 to 1958 or 1959 and that this occurrence covered an estimated area of 1,000 square miles.

Although many flowering reports exist, they are inadequate for detailed consideration of flowering periodicity because they lack important information such as whether the flowering of all vegetation was seen or not.

Specifically, many flowering records for *M. baccifera* do not offer enough information to allow estimation of the true flowering periodicity. In addition to this, we need to understand the flowering phenology by which the species flowers at the end of the year and fruiting is seen in the following year. This means that the recorded year in past records is that of fruiting rather than that of fruiting. In the temperate zone, however, the timing of flowering tends to be delayed, and the flowering year becomes the same as the fruiting year.

There are also many flowering records for *M. baccifera* outside the 48-year interval for flowering. This kind of flowering was recorded in 1801, 1849, 1889, 1892 and 1900 – 1902 by Staff (1904). As shown in Table 1 Blatter (1929) also noted flowering in 1889, 1892, 1900 – 1902 and 1904 – 1905. In addition to these records Nath (1968) reported flowering in 1967 at Manipur, India, and Alam (2008) reported an instance in 1901 – 1905 in Chittagong, Bangladesh. However, it is not possible to ascertain whether the flowering in these records is gregarious or otherwise.

Flowering reports of *M. baccifera* outside its native area

M. baccifera has been recognized as a useful resource of bamboo timber and food, which has prompted planting around the world outside its native area. Instances of planting are especially high in regions that neighbor the native area, such as India and Nepal. One flowering report from Blatter (1929) comes from the records of the botanic garden in Kolkata. This kind of reporting is found for many places. McClure (1966) reported the flowering and fruiting of plantation in Jamaica and Puerto Rico in 1957 and 1958 (a flowering record that matches the 48-year interval) as well as flowering in Honolulu in 1948 and 1949. Furthermore, flowering in 1990 in northern Queensland, Australia (Poudyal 2006) and in 2003 at a Sri Lanka plantation introduced in 1910 (possibly introduced with fruit) (Ramanayale & Weerawardene 2003) are recorded.

Past discussion on the flowering periodicity of *M. baccifera*

Since the end of the 19th century, many estimations concerning the flowering periodicity of *M. baccifera* have been suggested. However, the quantity of records available has been insufficient except for those after the last flowering around 1960. The first reference to flowering periodicity is thought to have been made by Kurz in 1876 (Gamble 1896). This estimation was based on flowering records for Arakan (Staff 1904) and was 30 years. Brandis (1899) also referred to the observation of Gamble. Staff referred to Kurz's estimation but also noted that the information was insufficient for full discussion. Blatter (1929) introduced two estimations of 30 years from Kurz (1876) and 45 years from Troup (1921). Blatter (1930a) also pointed out that regular flowering periodicity is difficult to find when the interval of all flowering records over a broad area are dealt with on the same level, and induced the need to distinguish the "extensive flowering" and "gregarious flowering". He also noted that the periodicity estimated only from "gregarious flowering" is around 50 years. On the other hand McClure (1966) made reference only to past estimations and the introduction of a gardener's estimation in Jamaica of an approximately 60-year periodicity. Godesberg (1969) proposed an estimation of 45 years.

Janzen (1976) referred to the flowering records of the species from two areas in India and one area in present-day Bangladesh (Table 2). His discussion offered no definite estimate of the flowering interval in these three areas and as a result it was impossible to estimate flowering periodicity as a species. He indicated some

estimated periodicity as 7 – 10 years, 26 – 30 years, 27 years, 31 – 33 years, 42 – 49 years, 47 – 49 years and 46 – 51 years by the simple calculation. In recent years there are some estimations such as 40 – 45 years by Seethalakshmi et. al (1996), and 48 – 50 years as well as 40 – 47 years by Banik (1998) based on records from Mizoram, India and from northeastern Bangladesh, respectively. In addition, Rao et. al (1998) noted three periodicities of 30 – 35 years, 45 – 48 years and 60 – 65 years. On the other hand Alam (2008) estimated the flowering periodicity in Chittagong Hill Tracts, Bangladesh as 50±5 years.

As outlined above, the flowering periodicity of *M. baccifera* was previously estimated as between 30 and 45 years. In more recent years, other periodicity estimations of around 50 and 60 years have been presented. However, no exact estimation matching the 48-year periodicity referred to here is found.

Discussion – Requirements for understanding the true flowering periodicity of bamboo

The flowering periodicity of bamboo differs by species, and seems to have a shorter tendency for those mentioned tropical area. On the other hand the periodicity in temperate bamboo species longer and in Japan is commonly said to be either 60 or 120 years. In this country, there have been ongoing trials to identify the true bamboo flowering periodicity of bamboo since the 19th century, and two flowering records have been obtained for *Phyllostachys pubescens* with a 67-year interval after seeding (Watanabe et. al 1982; Shibata 2002).

The flowering periodicity of *M. baccifera* has recently prompted detailed discussion, e.g., Alam (2008). However, as past flowering records for the species do not seem to be based on first-person observation at flowering sites, it is difficult to conclude that the reporters understood the true ecological process of flowering in detail. According to the author and his colleagues' ecological research at Sairang in India's Mizoram area, sporadic flowerings on a small scale has been observed one year before and after gregarious flowering. If we look at this three-year flowering phenomenon on the same level, while the overall flowering area moves from the northeast to the southwest, the overall flowering period can be understood as 8 – 10 years.

The flowering of bamboo is observed on various scales. In the case of *M. baccifera*, this scale is very large. In its vegetation area, non-flowering bamboo groves (called Mauhawk in Mizoram) and shifted-flowering bamboo groves may be mixed in with gregarious flowering bamboo vegetation. These groves should not be confused with the three-year flowering period that including gregarious flowering in the second year as mentioned above. It is clear that this kind of confusion will hinder the process of understanding bamboo's actual flowering periodicity. It is important to pinpoint the real year of gregarious flowering year by omitting the small-scale flowering that takes place before and after it and extracting the true flowering periodicity and flowering year.

The area of gregarious flowering for *M. baccifera* is unique. The phenomenon transits over a period of four yearscovering an area of more than 10,000 square kilometers annually in the whole of the native area. This is a dramatic vegetation change that can be seen from space. It is clear and relevant that the factor behind this phenomenon is slash-and-burning agriculture implemented by farmers.

Conclusion

The results obtained from research in Mizoram, India show that the flowering of this species clearly occurs every 48 years on a large scale. However, the transition of the flowering area over a period of several years – referred to as "the flowering wave" (Alam 2008) – skews information that would enable identification of the true flowering periodicity. To obtain the true vakue, it is necessary to carry out detailed ecological research at the flowering sites.

The estimation of a 48-year flowering periodicity for *M. baccifera* was supported by ecological research in Mizoram, India and by flowering records from Japan and Taiwan on 2008 and 2009. To confirm the true flowering periodicity of bamboo, the growth of bamboo plantations needs to be accurately monitored from the seedling stage to the flowering stage.

This research was supported by Grant-in-aid for scientific research (A) from Japan's MEXT (No. 17255007; representative: Shibata).

References

Abe Y. and Shibata S. 2007. Observation of sporadic flowering in dwarf bamboo, *Sasa veitchii* Rehder var. *hirsuta* - Flower and seed production habits. Bamboo J, 24, 12-16 (in Japanese with English abstract)

Alam M. K. 2008. Recent flowering of Muli bamboo (*Melocanna baccifera*) in Chittagong Hill Tracts and Rat Infestation: Eco-environmental aspects. USAID Task no. 388-c-00-03-00050-00

Banik R. L. 1998. Reproductive biology and flowering populations with diversities in muli bamboo, *Melocanna baccifera* (Roxb.) Kurz. Bangladesh Journal of Forest Science, 27(1), 1-15

Blatter E. 1929. The flowering of Bamboos. Part I. J. Bombay Nat. Hist. Soc., 33, 899-921

Blatter E. 1930a. The flowering of Bamboos. Part II. J. Bombay Nat. Hist. Soc., 34, 135-141

Blatter E. 1930b. The flowering of Bamboos. Part III. J. Bombay Nat. Hist. Soc., 34,447-467

Brandis D. 1899. Biological notes on Indian bamboos. Indian Forester, 25(1), 1-25

Camus E. G. 1913. Les Bambusees: Monographie, Biologie, Culture, Principaux Usages.

Chatterjii D. 1960. Bamboo fruits. J Bombay Nat Hist. Soc, 57. 451-453

Gamble J. S. 1896. Bambuseae of British India (Annals of RBG, Calcutta 7)

Godesberg A. L. B. 1969. Bamboo. CIBA Review 1969/3. Basel, Switzerland, pp2-39

Hori S. 1911. Factor in bamboo flowering. Reports of Agricultural Station, Japan, 38 (in Japanese)

Hossain K. M. I. 1962. Bamboos of East Pakistan with particular reference to muli bamboo and its flowering. Pakistan J. For., 12, 194-201

Janzen D. H. 1976. Why bamboos wait so long to flower. Ann Rev Ecol Syst, 7. 347-392

Kasahara K. 1971. Bamboo flowering. *Iden*, 25(8), 83-90 (in Japanese)

Kurz S. 1876. Bamboo and its use. Indian Forester, 1, 219-235

Lu J. 2009. Flowering, Fruiting and recovery by seedling of *Melocanna baccifera*. Bamboo J. 26 (in Japanese)

McClure F. A. 1966. The Bamboos. Harvard Univ. Press

Munro C. 1868. A monograph of the Bambusaceae (Transaction of the Linnean Society No. 26)

Nath G. M. 1968. Flowering of Muli bamboos (*Melocanna bambusoides*). Indian Forester, 94, 346

Parry N. E. 1931. On the flowering of bamboos. J. Bombay Nat Hist Soc, 1099-1101

Poudyal P. P. 2006. Bamboos of Sikkim (India), Bhutan and Nepal

Rain Forest Research Institute, Jorhat, Assam 2003. Selected facts about bamboo flowering in the northeast India.

Raizada M. B. and Chatterjii R. N. 1956. World distribution of bamboos with special reference to the Indian species and their more important uses. Indian Forest Leaflet, 151 (Botany)

Ramanayake S. M. S. D., Weerawardene T. E. 2003. Flowering in a bamboo, *Melocanna baccifera* (Bambusoideae: Poaceae). Botanical Journal of the Linnean Society, 143, 287-291

Rao A. N., Rao V. R.and Williams J. T. 1998. Priority species of bamboo and rattan. IPGRI & INBAR

Riviere E. A. and Riviere M. C. 1878. Le Bambous: vegetation, culture, multiplication en Europe et en Algerie, et generalement dans tout le basin mediterreneen.

Rokhuma C. 1988. *Tam do pawl in evenge a tih?* Aizawl

Roxburgh W. 1814. Hortus Bengalensis, a catalogue of plants growing in the honourable East India Company's botanic garden at Calcutta, Serampore

Seethalakshmi K. K., Muktesh Kumar M. S., Sarojam N., and Sankara P. K. 1996. Status of information on bamboos occurring in India. Proceedings of National Seminar on Bamboo, 41-56

Seifriz W. 1923. Observation of the causes of gregarious flowering in plants. Am. J. of Botany, 10, 93-112

Shibata S. 2002. Flowering of *Phyllostachys pubescens* Mazel ex Houzeau Lehaie in the sixty-seventh year, and germination of caryopsis produced at Kamigamo, Kyoto University Forests, Japan. *In* Bamboo for sustainable Development, 345-365

Staff O. 1904. On the fruit of *Melocanna bambusoides* Trin., an endospermless, viviparous, genus of Bambuseae. Transaction of the Linnean Society of London (2nd Ser) Botany, 6, 401-425

Thanchuanga C. 2004. Bamboo flowering in Mizoram. Magazine of the American Bamboo Society, 25(1-3)

Troup R. S. 1921. The silviculture of Indian trees. 3. Oxford University Press London

Ueda K. 1968. Bamboo Material. *In* Utilization of Bamboo Resource in South-east Asia to Promotion of Bamboo Pulp Industry. Japan Agency of Resources Vol. 19 (in Japanese)

Watanabe M., Ueda K., Manabe I. and Akai T. 1982. Flowering, seedling, germination, and flowering periodicity of *Phyllostachys pubescens*. J Jpn. For. Soc., 64(3), 107-111

http://www.bambootech.org/ National Mission on Bamboo Applications, India 2009/02/20

Table 1. Flowering records of *Melocanna baccifera* in Blatter(1929)

Flowering year	Reporter	Flowering area	Flowering type and remarks
1811	Brandis	Chittagong	?
1863 – 1866	Gamble	Chittagong, Arakan.	Gregarious flowering
		Bot.G.Calcutta	?
1889	Gamble	Garo and Khasia Hills	Gregarious flowering
1892	Troup	Assam	Gregarious flowering
1900 & 1902	Troup	Garo and Khasia Hills	Gregarious flowering
1901 & 1902	Troup	Chittagong, Arakan	Flowering in limited area
1904 & 1905	Troup	Chittagong, Arakan	Flowering in limited area
1908 – 1912	Troup	Chittagong	Extension of flowering area
1909 – 1910	H. de L.	Calcutta Bot. G. (cultivated)	□
1910 – 1913	Troup	Arakan	Extension of flowering area in
			1912-1913 to the eastearn area of Yoma
1910 – 1911	Troup	Silhet (Assam)	Gregarious floweirng
1911 – 1912	Troup	Garo Hills, Cachar, Sylhet,	Gregarious flowering
		Lushai Hills (Assam)	
1912 – 1913	Troup	Bamonpokri plantation(Kurseong)	?
1915 – 1916	Troup	Arakan	Gregarious flowering

Table 2. Flowering records of *Melocanna baccifera* in Janzen(1976)

(Figures in parentheses represent the year after the last recorded flowering)

Flowering area	Flowering year and related reports on flowering periodicity
Mizo Hills, Assam	1863 – 66, 1892 – 93(26 – 30), 1900 – 02(7 – 10), 1933(31 – 33), 1960(27) from Chatterjii(1960)
Lushai Hills, Assam	1864, 1911 – 12(47 – 48) from Parry.(1931)
Chittagong, East Pakistan	1863 – 66, 1908 – 12(42 – 49), 1958 – 59(46 – 51) from Hossain(1962)

Gregarious flowering of *Melocanna baccifera* around north east India
Extraction of the flowering event by using satellite image data

Murata Hiroshi*, Hasegawa Hisashi, Kanzaki Mamoru, Shibata Shozo
Kyoto University, Japan

Abstract

For the identification and mapping of the gregarious flowering area of *Melocanna baccifera*, the most common bamboo species ranged from Myanmar to Bangladesh, an object-based land cover classification was conducted for a QuickBird image covering 1.5km^2. The segmentation of the image well corresponded with the actual land cover, and the bamboo flowering area was successfully extracted. The method is expected to be applicable to the low resolution satellite images covering the larger spatial scales, and to enable the visualization of the geographical sequential flowering pattern of the bamboo from east to west in its distribution range.

Keywords: bamboo flowering, *Melocanna baccifera*, remote sensing, QuickBird, object-based classification, Mizoram,

Introduction

Melocannna.baccifera is distributed in North east India, Myanmar, and Bangladesh (Alam 1995). From past study, it flowered in 1765, 1815, 1863, 1911, and 1959 in Mizoram State, showing 48 years interval. As predicted from the interval, *M. baccifera* flowered in 2006 to 2007 around Aizawl, the capital of Mizoram state. Flowering started from November 2006, and bamboo clumps died and defoliation started from January 2007. In February seeds were grown up on the bamboo and seeds fallen in May 2007. Then seed emerged in rainy season started from June 2007.

In Mizoram, the shifting cultivation is most common cropping system until now. The quick regeneration from the rhizome after the slush-and-burn cropping preferred *M. baccifera* and the bamboo has increased with expanding shifting-cultivation. Mizoram state has an area of 21,081km^2 and half of it is covered by bamboo forest of which *M. baccifera* stands account for 90% area (Report on Bamboo Resources Inventory). The death of *M. baccifera* after gregarious flowering, therefore, had catastrophic impact on the agriculture, local vegetation and human society. In past flowering events, rats consumed bamboo seeds and increased explosively, then, they shifted their food source to agricultural products, mainly rice. Therefore serious famine has been repeated in 48 years cycle. In 2008, the serious decrease of rice production was also reported.

The bamboo ranged from Myanmar to Bangladesh and actually flowering started from eastern part of their distribution range and flowering area moved to west. The last flowering event started probably from 2005 in the eastern part such as Myanmar and flowering in Bangladesh confirmed in 2009. Even though it is clear that this flowering event occurred in vast area, probably several tens of thousands km^2, exact flowering range in each year has not yet been clarified and flowering wave from east to west also has not yet been visualized. Accurate identification of geographical range of flowering event during successive gregarious flowering of *M. baccifera* is quite important to understanding the ecology of *M. baccifera* and making counter measure for the catastrophic damage to the society and local people.

We challenge the problem using remote-sensing technique. Our ultimate purpose is to clarify the flowering sequence of the bamboo in geographical scale. In this paper, we report the methodology to identify the flowering area in satellite images.

Research site

This study focused on Mamit in Mizoram, India (Fig.1) where we have continuously monitored the flowering and regeneration process of the bamboo. Main form of agriculture in research site is shifting-cultivation called as 'Jhum' in local and most of fallow stands consists of *M. baccifera*. Around Mamit, flowering started in 2008.

Satellite image analysis

In order to extract flowering area of *M.baccifera*, land cover classification map was made from satellite image taken by QuickBird satellite (Digital Globe). The image covered $1.5km^2$ and was taken on January 25, 2009 (Fig.2) when inflorescence was made already and most bamboo leaves turned brown and easily distinguished by the other vegetation or non-flowered bamboo stand. Resolution of QuickBird is 2.5m (multispectral) or 0.6m (panchromatic) and it enables the precise mapping of flowering area.

For image analysis, ENVI 4.5 and IDL 6.0 (ITT Visual Information Solutions) were used. Object-based classification method was carried out for mapping ground cover. In the object-based classification, DN (digital number) of pixels and shade pattern were analyzed. In addition to these data, size of object and pattern of texture are considered in classification. An extension tool of ENVI 4.5, Feature extraction was used for the classification.

Result and discussion

Fig. 3 is classification map obtained by the object-based classification of the area shown by Fig. 2. In the study site, fallow lands of various ages make a patch mosaic pattern. Such a small scale mosaic pattern was successfully visualized by the segmentation map. The segmentation map was then subjected to supervised classification based on ground truth data. In Fig. 4, the extracted flowering area of *M. baccifera* obtained by the classification was shown.

Thus the use of high resolution satellite image can successfully identify the flowering area of the bamboo successfully. The object-based classification method was quite powerful for our purpose. However to identify the flowering area of each year in a successive flowering event in large spatial scale ranging from Myanmar to Bangladesh, low resolution images taken with high frequency and covered large area must be used. The current results using QuickBird image will be utilized for the development and validation of the analyses using low resolution images.

Acknowledgement

This research was supported by Grant-in-aid for scientific research (A) of Ministry of Education, Culture, Sports, Science and Technology, Japan (No. 17255007, representative: Shibata).

Reference

Alam.M.K. 1995. Melocanna baccifera (Roxb.) Kurz. In Plant Resources of South-East Asia, No. 7, Bamboos, edited by Dransfield.S and Widjaja.E.A, PROSEA Network Office, Bogor, Indnesia, pp.126-129.

Environment & Forest Department. 2002. Report on Bamboo Resources Inventory of Aizawl, Champhai, Darlawn, Kawrthanh, Kolasib, Mamit, N. Vanlaiphai and Thenzawl Forest Divisions. Aizawl, India.

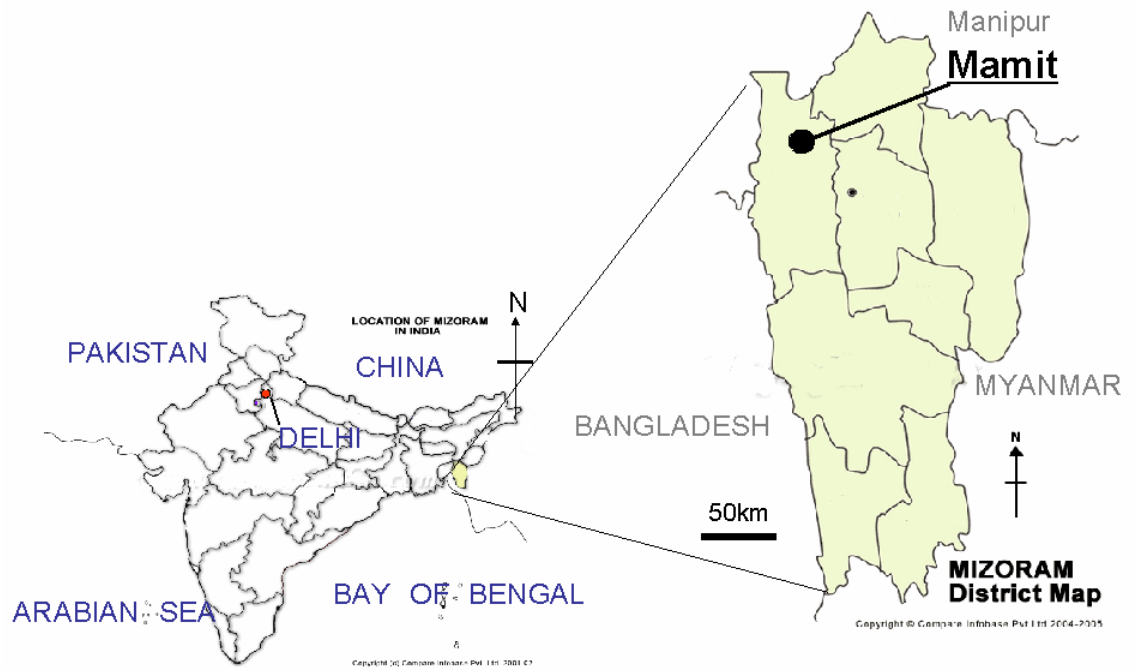


Fig. 1. Maps showing the research site in Mizoram, India.

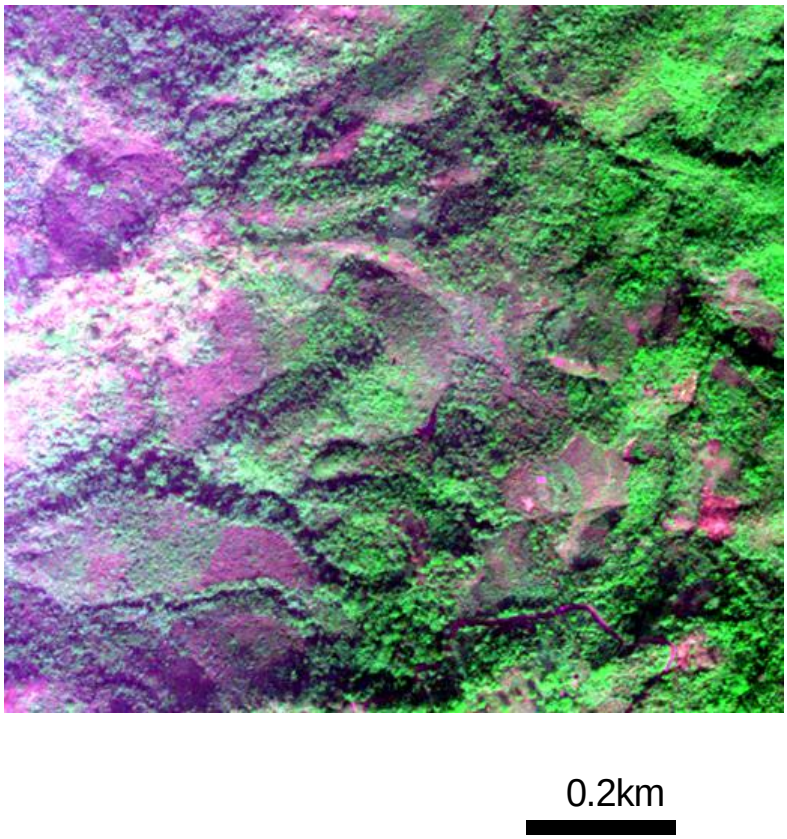


Fig. 2. QuickBird image of Mamit study site.

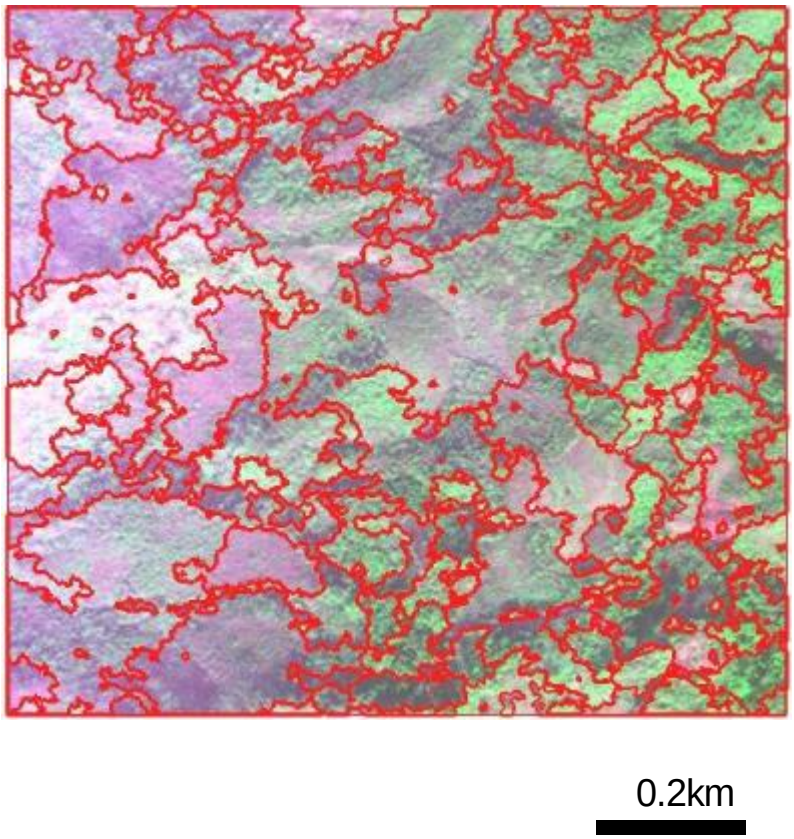


Fig. 3. Image segmentation in study area. Lines are borders of segmented objects.

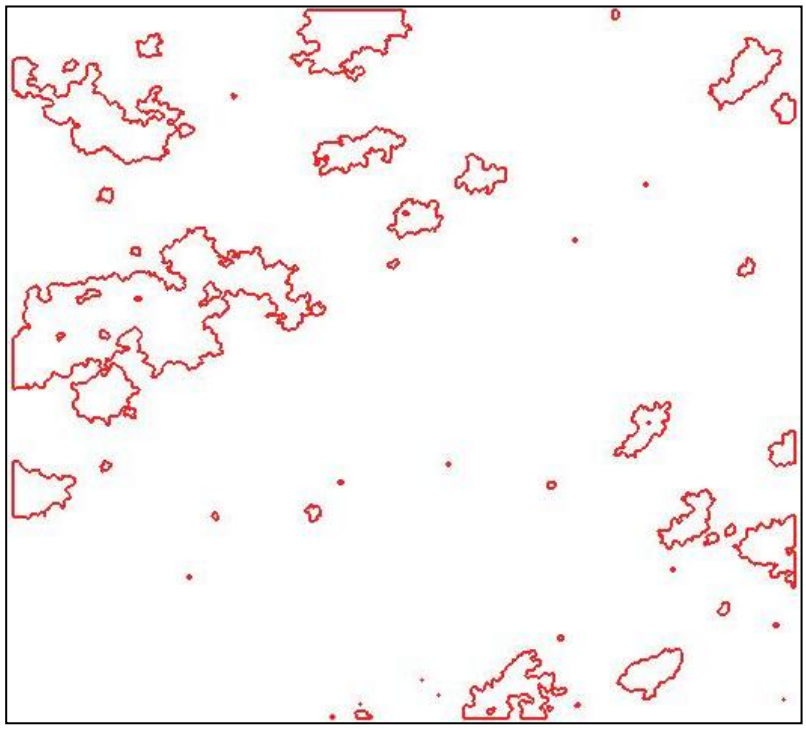


Fig.4 Extracrted bamboo flowering area