A BIOCHEMICAL ASPECT OF PHYLOGENETIC STUDY OF BAMBUSACEAE IN TAIWAN^{1,2}

III. The Genera Arthrostylidium, Chimonobambusa, and Dendrocalamus

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Abstract

A biochemical approach based on phenolics and isozymes of peroxidase and esterase was conducted to study the phylogenetic relationship among species of three genera, namely Arthrostylidium naibunensis, Chimonobambusa quadrangularis, Dendrocalamus asper, D. giganteus, D. latiflorus, D. latiflorus cv. "Mei-Nung" and D. strictus. By means of paper and thin-layer chromatography and UV-visible spectrophotometry, 44 spots of phenolics including flavonoids were found in C. quadrangularis leaves, and more than 50 spots were found in the remaining species mentioned. About 19 bands of esterase isozymes and 26 bands of peroxidase isozymes were found in the genus Dendrocalamus, and less than 10 bands of both isozymes were found in the genera Arthrostylidium and Chimonobamusa by using an acrylamide gel electrophoresis. The phenolics and isozymes data were computed by a simple matching coefficient and unweighted pair-group with simple average to obtain phenograms. The findings of all characters studied concluded that the genus Dendrocalamus is phylogenetically far from the other two gerera. Within the genus Dendrocalamus there were two major clusters, in which one cluster includes D. asper and D. giganteus and another cluster comprises D. latiflorus, and D. latiflorus, cv. "Mei-Nung". D. strictus was shown to be phylogenetically far from these two clusters.

Key words: Phylogenesis; Bambusaceae in Taiwan; Dendrocalamus; Arthrostylidium; Chimonobambusa; esterase; peroxidase; phenolic compounds.

Introduction

Bamboo plants are one of the major forest in the oriental countries and widely

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planted on many hillsides of Taiwan. The taxonomic research of bamboos has extensively been taken by scientists (Hayata, 1916; Nakai 1925; Sasaki, 1933; McClare, 1957; Lin, 1961, 1978; Kiang, 1974; Liu, 1962; Kiang, 1974). In which most of taxonomic work was primarily based on a description of morphological characters. Recently, a biochemical appraoch based on plant secondary metabolites, such as phenolic compounds and flavonoids, and isozymes was introduced into the field of plant systematics, leading to a better understanding of the phylogenesis of plant kingdom (Chu et al., 1972; Hsiao, 1973, 1980, 1971; Kiang and Wu, 1979; Chou et al., 1984a, 1984b). Regarding the phenolic compounds in bamboos, we reported previously that the phenolics including flavonoids are variously distributed in 14 bamboo species (Chou and Hou, 1981) and some of the phenolics play a significant role in the regulation of understories in bamboo plantation (Chou and Yang, 1982). Because the important roles of phenolics and isozymes in plant metabolism that may lead to a differential development of phylogenesis, we thus conducted a series of studies based on these biochemical characters to elucidate the taxonomic and evolutional significance of Bambusaceae. In the past two years we have done the phylogenetic study of the genera Phyllostachys, and Bambusa (Chou et al., 1984a, 1984b). Continued to this study, we present here the third report on the preliminary identification of phenolic compounds and the isozymes distribution in genera Arthrostylidium, Chimonobambusa, and Dendrocalamus, from which we may clarify some ambiguous taxonomic position of taxa and their phylogenetic relationship among the taxa studied.

Materials and Methods

Materials

Leaves of 5 taxa of *Dendrocalamus*, namely *D. asper* (Schult) Backer ex K. Heyne. *D. giganteus* (Wall.) Munro, *D. latiforus* Munro, *D. latiforus* Munro cv. "Mei-Nung", and *D. stricius* (Roxb.) Nees, and *Arthrostylidium naibunesis* (synonym *Arundinaria naibunensis*, *Chimonobanbusa naibunensis* and *Leleba naibunensis*) and *Chimonobambusa quadrangularis*, were collected in the summer and winter of 1983 at the Chitou Forest Experiment Station of National Taiwan University and Taipei Botanical Garden of Taiwan Forestry Research Institute. The fresh leaves collected were immediately placed in an ice-box and brought back to laboratory for isozyme study, and dried leaves ground into powder for the extraction of phenolic compounds including flavonoids.

Extraction of Flavonoids from Bamboo Leaves

To 200 grams of leaves of each aforementioned taxa, 2 liters of reagent grade methanol were added and allowed to stand overnight. The methanolic extract was

obtained by filtration through Whatman No. 42 filter paper, and the residue was added with 2 liters of methanol and treated by the same way. The subsequent extracts were obtained by the same way and all combined. After the methanolic extraction, the residues were re-extracted with 80% methanol, 50% methanol till with distilled water. The entire extraction fractions were combined and concentrated to syrup-like solution by a rotatoy evaporator in vacuo. The concentrated extract was subsequently re-extracted with hexane, chloroform, and ethyl acetate followed the techniques described by Newman et al. (1979); and the re-extract soluble in each aforementioned fraction was respectively designated as Hexane fraction, Chloroform fraction, and Ethyl acetate fraction, and the insoluble fraction was called "Water fraction". The extracts of each fractionations were saved for chromatographic analyses.

Isolation of Flavonoids by Chromatography

The syrup-like extract of each fractionation mentioned was first run by paper chromatograph in order to see the pattern of fiavonoids distribution, and the appropriate fraction was chosen for further large scale isolation of compound by column chromatography. About 150 g of polyvinylpolypyrrolidone powder (Sigma Chemical Co., USA) was soaked with 1500 ml methanol or distilled water. The preparation of polyvinylpolypyrrolidone-methanol gel was followed by the techniques described by Newman *et al.* (1979). The techniques and solvent systems used for the column chromatography were described by Mabry *et al.* (1979). After several times of rechromatography, the isolated compounds became relatively pure and were finally passed through a Sephadex LH-20 column and eluted with spectroscopic grade methanol. The clean eluate was concentrated to a small volume and placed in a vial in a refrigerator to allow crystallization.

Isozymes Analyses of Bamboo Leaves

A vertical gel electrophoresis (M & S Slab Electrophoresie, Model SG-80) was employed and technique for electrophoresis of bamoo were described by Chou *et al.* (1984a), which is a modification of Brewer (1970). Two isozymes of esterase and peroxidase were investigated. The zymogram of each isozyme gel was kept in permanency by coating a cellophane after the gel was dried.

Simple Matching Coefficient and Clustering Analysis

The phenolic compounds including flavonoids and zymogram patterns of peroxidase and esterase studied were used as the characters to determine the similarity between taxa, using a formula Ssm = m/(m+u), where m is the number of matches or agreements, u is the number of mismatches, and n is equal to m+u. The data of Ssm were then set in a simple matrix table using each

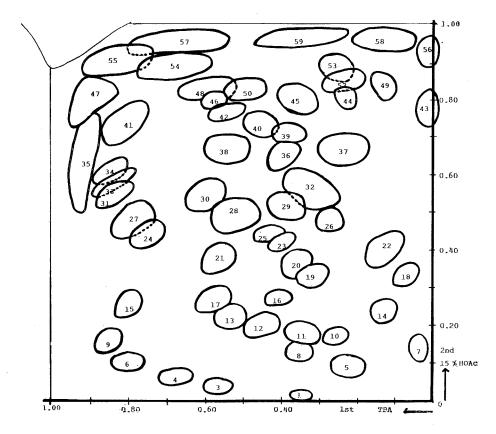


Fig. 1. Paper chromatogram of phenolic compounds in five taxa of Dendrocalamus.

Table 1. Distribution of phenolic compounds in the genus **Dendrocalamus** obtained by paper chromatography⁽¹⁾

a .	Rf c	of PC	Color re	eaction(2)			Taxa	-	
Spot no.	ТВА	15% HOAc	UV	UV/NH ₄	Da	Dg	Dl	Dlm	Ds
1	0.34	0.01	yel				+-		
2	0.87	0.02		yel			+		
3	0.56	0.04	yel gn				+		
4	0.68	0.06	pu	yel	+	+	+	+	+
5	0.23	0.09	fl yel	fl yel			+	+	+
6	0.80	0.10	pu	pu				+	
7	0.04	0.13	yel	fl yel gn	+	+	+	+	+
8	0.35	0.13		yel	+		+	+	+
9	0.85	0.15		yel			+		
10	0.26	0.17	fl yel	fl yel		+			
11	0.35	0.17	fl yel				+	+	
12	0.45	0.20		fl yel		+			-1-
13	0.54	0.22	wh yel	yel gn				+	
14	0.13	0.23		yel	+			+	

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15	0.80	0.25	pu				+	+	
16	0.41	0.27	pu		-}-			+	+
17	0.58	0.27		fl yel		+		+	
18	0.08	0.33	yel					+	
19	0.32	0.33		ye1	+		+	+	+
20	0.37	0.36		ye1					+
21	0.57	0.38	pu		+		+	+	
22	0.14	0.40	pu	yel	+	+		+	+
23	0.41	0.42		yel			+	+	
24	0.76	0.43	b1		· +				+
25	0.43	0.44	pu	br yel		+-		+	
26	0.28	0.48		yel			+	+	
27	0.80	0.48	bl		-+-	+		+	+
28	0.53	0.49	pu	yel gn			+	. +	
29	0.40	0.51	b1	b1		-+-			
30	0.64	0.54	pu	yel	+			+	+
31	0.85	0.55		fl bl	+	+	+	+	+
32	0.33	0.56	pu	br yel	+	+	+	+	+
33	0.84	0.57		yel					
34	0.85	0.60	yel	-			+		
35	0.93	0.62		yel gn	+		+		
36	0.40	0.65	yel				+		
37	0.25	0.67	yel	ye1	+	+	+	+	+
38	0.55	0.67	pu		+	+		+	+
39	0.39	0.71		yel			+		
40	0.45	0.74	pu	yel			+	+	
41	0.81	0.74	_	pu	+			+	
42	0.55	0.77	yel		+				
43	0.01	0.78	wh yel	br yel	+	-t-	+	+	+
44	0.23	0.80	bl	yel				+	
45	0.36	0.80	yel gn						+
46	0.58	0.80	bl				+		
47	0.91	0.80		yel gn	+			+	
48	0.61	0.82	pu	yel gn				+	
49	0.13	0.83	bl					+	+
50	0.50	0.83	bI	fl yel gn		+			+
51	0.25	0.85		yel gn	+				
52	0.26	0.88	bl	fl bl	+				+
53	0.30	0.88	b1	b1				+	
54	0.69	0.89	yel gn		+				
55	0.83	0.91	_	fl bl	+			+	
56	0.01	0.93	wh yel	br yel	+	+	+	+	+-
57	0.70	0.95	• -	yel gn	-+-	+	*	+	•
58	0.13	0.96		yel gn		+		+	
59	0.34	0.97	yel gn	fl yel gn		-			
Total					26	19	26	37	22

⁽¹⁾ The abbreviations of taxa: Da=Dendrocalamus asper; Dg=D. giganteus; Dl=D. latiflous; Dlm=D. latiflorus cv. "Mei-Nung". The abbreviations hereafter in the following tables are the same except otherwise mentioned.

⁽²⁾ Color reaction: bl=blue, br=brown, fl=fluorecence, gn=green, pi=pink pu=purple, wh= white, yel=yellow, or=orange.

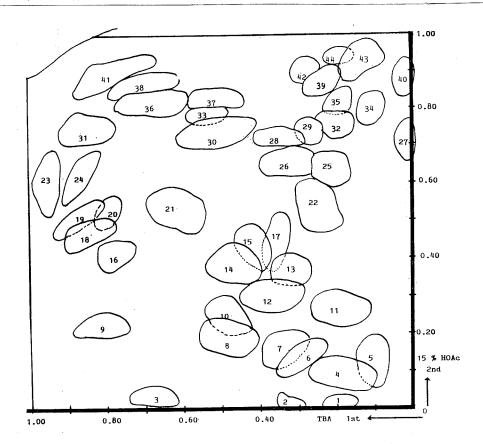


Fig. 2. Paper chromatogram of phenolic compounds in the genera Arthrostylidium and Chimonobambusa.

Table 2. Distribution of phenolic compounds in the genera **Arthrostylidium** and **Chimonobambusa** based on paper chromatography⁽¹⁾

	Rf of PC		Color	reaction	Taxa		
Spot	TBA	15%HOAc	UV	UV/NH4	A. naibenensis	C. quadrangularis	
1	0.20	0.01	yel	yel gn		+	
2	0.32	0.01	fl yel			+	
3	0.68	0.03	pu	yel	+	+	
4	0.20	0.10	fl yel	fl yel	+		
5	0.11	0.13	yel gn	fl bl		+	
6	0.30	0.13	fl yel	yel		+	
7. \	0.35	0.16	wh yel	fl yel gn	+		
8	0.50	0.20	yel	fl yel gn	+		
9	0.82	0.22	yel gn	yel		+	
10	0.49	0.23	pu			 -	

⁽¹⁾ The abbreviations of color reactions see Table 1.

species as an operational taxonomic unit (OTU). The clustering analysis between species was obtained by an unweighted pair-group method using simple arithmetric average described by Sneath and Sokal (1973).

Results

Distribution of Phenolic Compounds in Leaves of Arthrostylidium, Chimonobambusa, and Dendrocalamus

By means of two dimensional paper chromatography, 59 spots of phenolic compounds including flavonoids were found in the methanolic extracts of leaves of the genus *Dendrocalamus* (Fig. 1). The characteristics of each spot are given in Table 1, showing that there are 26 spots in *D. asper*, 19 spots in *D. giganteus*, 20 spots in *D. latiflorus* Munro, 37 spots in *D. latiflorus* Munro cv. "Mei-Nung", and 22 spots in *D. strictus*. Only 7 spots, such as spots 4, 7, 31, 32, 37, and 43 are common to all taxa of *Dendrocalamus*. In addition to that, spots 18, 22, 27, and 38 were mostly distributed in these five taxa.

On the other hand, the paper chromatogram of phenolic compounds present in

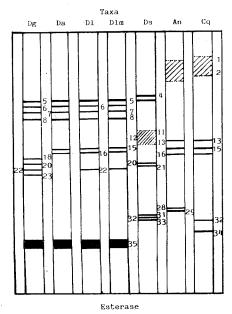


Fig. 3. The zymogram patterns of esterase present in the fresh leaves of seven taxa, namely Da=Dendrocalamus asper, Dg=D. giganteus, Dl=D. latiflorus, Dlm=D. latiflorus cv. "Mei-Nung", Ds=D. strictus, An=Arthrostylidium naibunensis, Cq=Chimonobambusa quadrangularis.

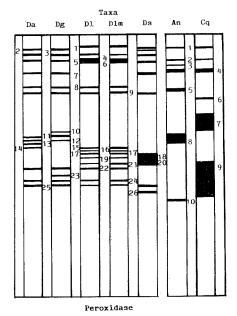


Fig. 4. The zymogram patterns of peroxidase in the fresh leaves of seven taxa, which abbreviations was described in Fig. 3.

the remaining two genera is shown in Fig. 2. There were 24 spots in Arthrostylidium naibenensis, and 23 spots in Chimonobambusa quadrangularis (Table 2). Only 3 spots were common to these two species, reflecting that these taxa should belong to a separate genus instead of in the same genus Chimonobambusa as treated by McClure and Lin (cited by BCFRA, 1980).

Distribution of Esterase And Peroxidase Isozymes in Genera Arthrostyilidum, Chimonobambusa, and Dendrocalamus

The zymogram patterns of esterase of seven taxa mentioned are shown in Fig. 3, in which 19 bands were founds in genus Dendrocalamus, 7 bands in Arthrostylidium, and 7 bands in Chimonobambusa (Table 3). The zymogram patterns of the latter two genera were quite different from these of the genus Dendrocalamus. The esterase isozyme pattern was exactly the same in taxa of D. latiflorus and D. latiflorus cv. "Mei-Nung". On the other hand, the peroxidase zymograms of the seven taxa are shown in Fig. 4, in which there are 20 bands in the genus Dendrocalamus, and less than 7 bands in the remaining genera (Table 4). Similarly, the zymogram patterns of the genus Dendrocalamus were quite different from that of gerera Arthrostylidium and Chimonobambusa, and the pattern of the latter two genera were significantly differnt (Fig. 4). Particularly, the peroxidase isozyme of the genus Chimonobambusa exhibited two distinguished bands (bands 7 and 9), which were absent from the other two genera.

Phylogenetic Relationship of Five Taxa of Genus Dendrocalamus

Based on chromatographic data of five taxa of Dendrocalamus, the simple matching coefficients are shown in Table 5, which derives to phenogram of Fig. 5. From the phenogram it was found that the taxa D. giganteus and D. strictus had high similarity, and D. latiflorus revealed a low similarity from the remaining taxa. Based on the zymogram patterns of esterase and peroxidase, the simple matching coefficients are given in Tables 6, which leads to phenograms of Fig. 6A and 6B, indicating that both taxa of D. latiflorus and D. latiflorus "Mei-Nung" has the highest similarity of Ssm = 1.00. In addition, the taxa D. latiflorus D. giganteus and D. asper also had high similarity of Ssm above 0.70. Combining both characters of esterase and peroxidase, the simple matching coefficients of the taxa of Dendrocalamus is given in Table 7, which results in Fig. 6C. From Fig. 6C, it was found that the taxa of D. asper was very close to D. giganteus, and the taxa of D. strictus was evidently far from D. asper and D. giganteus. Furthermore, when combining all characters of phenolics and isozymes of esterase and peroxidase, the simple matching coefficients are given in Table 8, derives to Fig. 7, concluding that taxa of D. latiflorus and D. latiflorus cv. "Mei-Nung" belonged to one cluster, while the taxa of D. asper and D. giganteus belonged to another cluster. However both

Table 3. The distribution of esterase isozymes in the genus **Dendrocalamus,** and **Arthrostylidium naibunensis** (An) and **Chimonobambusa quadrangularis** (Cq)

Band no.	Da	Dg	Dl	Dlm	Ds	An	Cq
1,2			-107771			+,+	,
4					- -		
5	+	+	+	+	- + -		
6	+	+	+	+			
7	+	+	+	+			
8	+	+	+	+			
11, 12					+,+		
13					+	+	+
15	+		+	+		+	+
16	+		+	+-		+	+
18		+					
20		+			+		
21					+		
22		+	·- -	+			
23		+					
28						+	
29						-+-	
31					+		
32					+		+
33					+		
34							+
35	+	+	+	+ '			

Table 4. The distribution of peroxidase isozymes in the genus **Dendrocalamus**

Band no.	Da	Dg	Dl	Dlm	Ds
1			+	+	
2	+	+			+
3	+	+	+	+	+
4 5			+	÷	
5	+	+	+	+	+
6			+	+	
7	+	+			+
8	+	+	+	+	
9	+-	+	+	+	+
10 11		+			
11		+			
12	+				
13	+	+			
14	+				•
15	+		+	-1-	
16			+	+	
17			+	+	+
18					+
19			+	+	+
20					+
21			+	+	+
22	+	+	+	+	
23		+			
24	+	+	+	+	
25	+	+	+	+	+
26					+

Table 5. The matrix of simple matching coefficients of the genus **Dendrocalamus** based on chromatographic data of phenolic compounds

OTU	OTU (operational taxonomic uni;)							
	Da	Dg	Dl	Dlm	Dз			
Da								
Dg	0.610							
Di	0.492	0.475	_					
Dlm	0.610	0.525	0.508	-				
Ds	0.695	0.712	0.525	0.508				

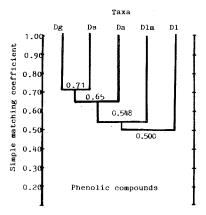


Fig. 5. The phenograms of 5 taxa of *Dendrocalamus* based on the chromatographic data of phenolic compounds. The abbreviations of taxa see Fig. 3.

Arthrostylidium naibunensis and Chimonobambusa quadrangularis were phylogenetically far from the taxa of Dendrocalamus.

Discussion

In a series of phylogenetic study of bamboo plants in Taiwan, we have previously reported that there was a substantial number of phenolic compounds and numerous bands of isozyme peroxidase present in leaves of bamboo plants. These characters were used for clustering analysis, leading to an understanding of phylogenetic relationship between taxa of Bambusaceae. We have found that there are three major clusters both in *Phyllostachys* and *Bambusa* genera (Chou *et al.*, 1984a, 1984b). Chou *et al.* (1984b) and Hsiao (1980, 1981) pointed out that the isozyme patterns revealed less confidence as compared with the phenolic compounds as

far as phylogenetic viewpoint was concerned. This is primarily due to the number of isozyme bands of peroxidase is less than that of phenolic compounds, and sometimes both major characters could not well agree with each other. At the present study, we introduce an additional enzymes of esterase to increase the number of isozyme bands, which is of great advantage for the clustering analysis and provides a more confident phenogram. As a result of all combination of characters, esterase, peroxidase, and phenolic compounds, we conclude that the

Table 6. The matrix of simple matching coefficient of the genus **Dendrocalamus** based on the zymogram patterns of esterase and peroxidase

OWI	OTU (operational taxonomic unit)						
OTU	Da	Dg	Dl	Dlm	Ds		
A: Este	ase			and and the second			
Da	_						
Dg	0.684	-					
DI	0.947	0.737					
Dlm	0.947	0.737	1.000				
Ds	0.210	0.210	0.158	0.158			
3: Perox	idase						
Da							
Dg	0.769	A					
D1	0.539	0.462					
Dlm	0.539	0.462	1.000				
Ds	0.500	0.500	0.500	0.500			

Table 7. The matrix of simple matching coefficients of the genus **Dendrocalamus** based on the zymogram patterns of esterase and peroxidase

0.771		OTU (operational taxonomic unit)							
OTU	Da	Dg	D1	Dlm	Ds				
Da									
Dg	0.733	·							
Dl	0.711	0.578							
Dlm	0.711	0.578	1.000						
Ds	0.378	0.378	0.356	0.356	-				

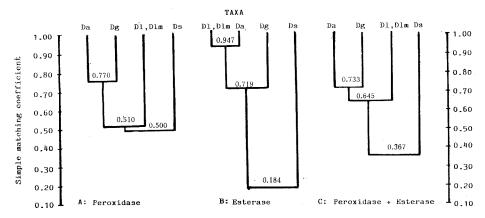


Fig. 6. The phenograms of 5 taxa of *Dendrocalamus* based on peroxidase (A), esterase (B), and the combination of peroxidase and esterase (C).

Table 8. The matrix of simple matching coefficients of the genus **Dendrocalamus** based on the characters of phenolic compounds and isozyme patterns of esterase and peroxidase

OTU	OTU (operational taxonomic unit)							
	Da	Dg	D1	Dlm	Ds			
Da								
Dg	0.663							
Dl	0.587	0.519						
Dlm	0.654	0.548	0.721					
Ds	0.558	0.567	0.452	0.442	_			

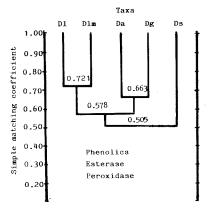


Fig. 7. The phenograms of 5 taxa of *Dendrocalamus* based on the characters of phenolic compounds and isozyme patterns of esterase and peroxidase.

taxa of *Dendrocalamus latiflorus* and *D. latiflorus* "Mei-Nung" are genetically close together, and that of *D. asper* and *D. giganteus* are also close together. But these two clusters reveal rather far phylogenetical relationship (Ssm = 0.575).

The similarity coefficients between Arthrostylidium naibunensis and Chimonobambusa quadrangularis are very low, e.g., Ssm = 0.068 based on phenolic characters, Ssm = 0.300 based on peroxidase, and Ssm = 0.556 based on esterase, indicating that their phylogenetic relationship is very far from each other. However, some taxonomists already gave several synonyms for Arthrostylidium naibunensis, such as Chimonobambusa naibunensis (Hayata) (McClure and Lin, 1974), Arundinaria naibunensis Hayata (Lin, 1978), Pseudosasa naibunensis (Hayata) Makino & Nemota (Lin, 1978), Pleioblastus naibunensis (Hayata) Kanehira & Sasaki (Lin, 1978), Bambusa naibunensis (Hayata) Nakai (Lin, 1967), Lebeba naibunensis (Hayata) Nakai (Lin, 1961; Li, 1963). These synonyms reflect the taxonmic position of this species has been so confused. However, the present evidence of phylogenetic study at least shows that the Arthrostylidium naibunensis should not be placed in the genus of Chimonobamubusa studied. Moreover, the genus name of Dendrocalamus was previously placed in the genus Bambusa, or Sinocalamus (see Lin, 1961; Li, 1963, Lin, 1978). To this point, we could clarify the ambiguous taxonomic name by studying their phylogenetic relationship. In addition, we may employ more enzyme characters, such as acid phosphatase, polyphenol oxidase, etc. to provide more biochemical characters as well as morphological characters to find out the phylogenetic relationship between taxa of Bambusaceae. Some of these enzymes have recently been found in bamboo leaves. A complete understanding of phylogenetic relationship and adequate taxonomic names for these ambiguous bamboo species could be arrived when the series of study is done. However, a difficulty of isolation and identification of flavonoids in bamboo plants is still a puzzling problem which requires more studies.

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臺灣什科植物血緣關係之生化學研究 三、麻什屬、內門什屬及寒什屬

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在臺灣大學溪頭實驗林及臺北植物園之標本園中取 蔴 竹屬(Dendrocalamus),內門竹屬(Arthrostylidium)及寒竹(Chimonobambusa)屬之新鮮竹葉,以萃取葉中之酚類化合物,並以紙色層,薄色層及紫外可視光譜儀初步鑑別酚類化合物的分佈,結果指出蔴竹屬(五種)中之酚類化合物共59個斑點。內門竹屬及寒竹屬(兩者在臺灣均單屬單種)得44個斑點。另外以聚丙烯醯胺電泳法分析過氧化酶及酯酶,並得酶譜,而發現在蔴竹屬中得過氧化酶共36個帶,內門竹屬及寒竹屬則得10個帶。酯酶分析結果得蔴竹屬中19個帶,而內門竹屬及寒竹屬僅7個帶。酶譜圖及酚類化合物之分佈依種屬而異,將酶譜圖及酚類化合物在種上分布之情形以統計相似度方法分析則得相互間之關係。此相似度之關係決定竹類植物之親緣關係。結果指出內門竹屬及寒竹屬與蔴竹屬之親緣關係相當疏遠,且相互關係小。而蔴竹屬中之五個種間有顯著相關關係;其中,蔴竹(D. latiflorus)及美濃蔴竹(D. latiflorus cv. Mei-Nung)屬一羣,馬來蔴竹(D. asper)及巨竹(D. giganteus)屬另一羣,而印度實竹(D. strictus)在親緣上則遠離上述四種。內門竹與四方竹(Chimonobambusa quadrangularis)在親緣上無甚大關係,過去人將內門竹置於寒竹屬或青籬竹屬(Arundinaria)是不安的。