

A BIOCHEMICAL ASPECT OF PHYLOGENETIC STUDY OF BAMBUSACEAE IN TAIWAN

II. The Genus *Bambusa*^{1,2}

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Abstract

A biochemical approach based on phenolic compounds and peroxidase isozymes was conducted to study the phylogenetic relationship among eleven taxa of *Bambusa* planted in Chitou, Taiwan. By means of paper and thin-layer chromatography, 42 spots including phenolic acids and flavonoids were variously distributed in 11 taxa of *Bambusa*. In addition, by using an acrylamide gel electrophoresis, 26 bands of peroxidase isozyme were also found in the genus. The data of phenolics and peroxidase isozymes distributions in the genus were computed by a simple matching coefficient and unweighted pair-group method; thus the phenograms were obtained. Within taxa of each species, such as *B. multiplex* and *B. vulgaris*, the phenetic relationship is evidently close together. Combining both characters of phenolics and isozyme patterns, 11 taxa of *Bambusa* studied can be grouped into three clusters: one cluster includes *B. multiplex*, its cultivars and *B. oldhamii*; the second cluster comprises *B. beecheyana*, *B. dolichoclada*, *B. ventricosa*, *B. vulgaris* and *B. vulgaris* var. *striata*, and the remaining taxa are the third cluster, which is genetically far from the above two clusters mentioned. Further study of identification of flavonoids in *Bambusa* genus is in progress.

Key words: Bambusaceae; *Bambusa* taxa; phylogenetic study; phenolics; flavonoids; peroxidase zymogram; phenogram; operational taxonomic unit.

Introduction

Bamboo plants are one of the major forests in the oriental countries and widely planted on many hillsides of Taiwan. The taxonomic research of bamboos has

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extensively been taken by scientists of the world (Hayata, 1916; Nakai, 1925; Sasaki, 1933; McClure, 1957; Lin, 1961; Kiang, 1974). In which most of taxonomic work was primarily based on a description of morphological characters; however, the phylogenetic aspect of bamboo study has little been attempted. Recently, a biochemical approach based on plant secondary metabolites, such as phenolic compounds and flavonoids, and isozymes were introduced into the field of plant systematics, leading to a better understanding of the phylogeny of plant kingdom (Chu *et al.*, 1972; Hsiao, 1973, 1980, 1981; Kiang and Wu, 1979). Regarding the phenolic compounds in bamboos, we have previously reported 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 phylogeny, we thus conducted a series of studies based on these biochemical characters to elucidate the taxonomic and evolutionary significance of Bambusaceae. The first report of the study revealed a significant evidence of phylogeny among seven species of *Phyllostachys* (Chou *et al.*, 1984). Continued to this study, we present here the second report of the work on the eleven taxa of *Bambusa* in Taiwan.

Materials and Methods

Materials

Leaves of 12 taxa of *Bambusa*, namely *B. beecheyana*, *B. dolichoclada*, *B. edulis*, *B. oldhamii*, *B. multiplex*, *B. multiplex* cv. "Alphonse Karr", *B. multiplex* cv. "Fernleaf", *B. pachinensis*, *B. stenostachya*, *B. ventricosa*, *B. vulgaris*, and *B. vulgaris* var. *striata*, were collected in the summer and winter of 1982 in the Chitou (溪頭) Forest Experiment Station of National Taiwan University. The fresh leaves collected were immediately placed in an ice-box and brought back to laboratory for isozyme study, and dried leaves were ground into powder for the extraction of phenolic compounds including flavonoids. Due to insufficient quantity of sample of *B. edulis*, its chromatographic study was unfortunately not carried out.

Extraction of Flavonoids from Bamboo Leaves

To 200 gram of leaves of each aforementioned taxa, except *B. edulis*, 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 all combined and concentrated to syrup-like solution by rotatory evaporator in vacuo. The concentrated extract was re-extracted with hexane and the hexane soluble fraction was designated as *Hexane fraction*. The hexane insoluble fraction was re-extracted with chloroform and the chloroform soluble fraction was designated as *Chloroform fraction*; then the chloroform insoluble fraction was re-extracted with ethyl acetate, which fraction was designated as *Ethyl acetate fraction*, and the insoluble fraction was called *Water fraction*. These fractionations of extracts were saved for chromatographic analyses.

Isolation of Flavonoids by Chromatography

The syrup-like extract of each fractionation mentioned was first run by paper chromatography in order to see the pattern of flavonoids distribution, and the appropriate fraction was chosen for further large scale isolation of compound by column chromatography. About 150 g polyvinylpyrrolidone powder (Sigma Chemical Co., USA) was soaked with 1,500 ml methanol or double distilled water. The preparation of polyvinylpyrrolidone—methanol gel was followed by the techniques described by Neuman *et al.* (1979). The techniques and solvent systems used for the column chromatography were described by Chou *et al.* (1984). After several times of re-chromatography, 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.

Peroxidase Analysis of Bamboo Leaves

A vertical gel electrophoresis (M & S Slab Electrophoresis, model SG-80) was employed and techniques for electrophoresis of bamboo leaves were described by Chou *et al.* (1984).

Simple Matching Coefficient and Clustering Analysis

The phenolic compounds including flavonoids and zymogram patterns of peroxidase studied were used as the characters to determine the similarity between taxa, using a formula $S_{sm} = m/n = m/(m + u)$, 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 S_{sm} were then set in a simple matrix table using each species as an operational taxonomic unit (OTU). The clustering analysis between species was obtained by an unweighted pair-group method using simple arithmetic average described by Sneath and Sokal (1973).

Results

Distribution of Phenolic Compounds in Leaves of Bambusa Taxa

By means of two dimensional paper chromatography, 42 spots of phenolic compounds including flavonoids were found in the methanolic extracts of leaves of the genus *Bambusa* (Fig. 1). The characteristics of each spot are given in Table 1, showing that there are 13 spots in *Bambusa beecheyana*, 13 spots in *B. dolichoclada*, 25 spots in *B. multiplex*, 24 spots in *B. multiplex* cv. "Alphonse Karr", 29 spots in *B. multiplex* cv. "Fernleaf", 27 spots in *B. oldhamii*, 19 spots in *B. pachinensis*, 26 spots in *B. stenostachya*, 15 spots in *B. ventricosa*, 22 spots in *B. vulgaris*, and 17 spots in *B. vulgaris* var. *striata*. Among these compounds, spots 2, 3, 5, 7, 10, 11, 17, 18, 19, 25, 26, 27, 28, and 38 are common to most of taxa studied. Most likely, *B. multiplex* and its varieties have similar chromatographic patterns. However, *B. vulgaris* var. *striata* reveals rather different chromatographic results and the former taxa has five more spots than the latter (Table 1). Due to a great number of flavonoids present in the genus *Bambusa*, it is difficult at the present time to isolate all compounds from each plant. However, studies have been focused on *B. multiplex* and *B. oldhamii* in order to isolate a significant amount of flavonoids for structural identification, which is in progress.

Distribution of Peroxidase Isozymes in the Genus Bambusa

The zymogram patterns of peroxidase of 11 taxa of *Bambusa* collected from the

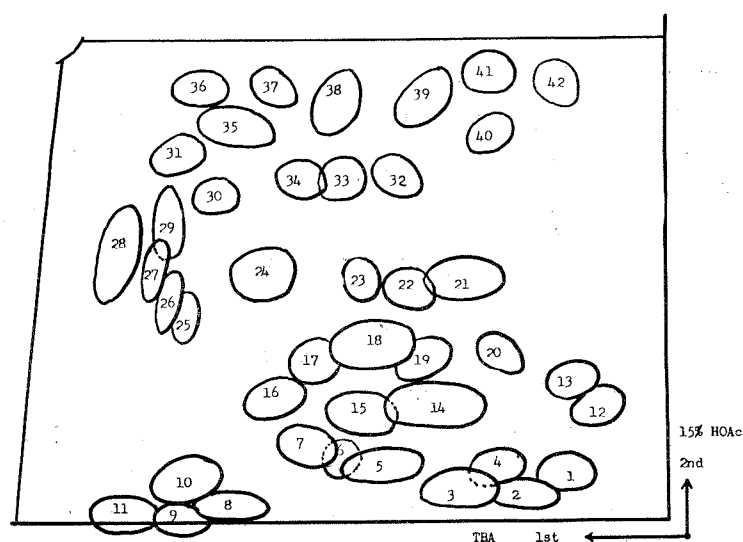


Fig. 1. Paper chromatogram of phenolic compounds in 11 taxa of *Bambusa*.

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

Spot No.	R _f of PC		Color reaction ²		Taxa										
	TBA	15% HOAc _c	UV	UV/NH ₃	Bb	Bd	Bm	Bma	Bmf	Bo	Bp	Bs	Bve	Bvv	Bvs
1	0.13	0.15	l yel	bl gn	+						+	+			
2	0.27	0.06	l yel	yel	+	+	+	+	+	+		+	+	+	+
3	0.37	0.07	or	yel	+	+	+	+	+	+	+	+	+	+	+
4	0.31	0.13	or	yel or					+	+	+	+		+	
5	0.47	0.12	or	or		+	+	+	+	+		+	+	+	+
6	0.56	0.17	yel gn	gel gn				+	+	+		+		+	
7	0.56	0.25	or	yel	+	+	+		+	+	+	+	+		
8	0.72	0.02	pur	yel	+	+				+	+	+	+	+	+
9	0.74	0.02	or	bl		+									
10	0.78	0.09	bl	bl			+	+	+	+	+	+		+	+
11	0.84	0.02	pur	or								+			
12	0.10	0.23	pk	pk								+			
13	0.12	0.28	or	pk						+		+			
14	0.32	0.25	or	or			+	+	+	+		+			
15	0.44	0.25	pur	or			+	+		+	+			+	
16	0.64	0.25	pur	l yel	+		+	+				+	+		
17	0.56	0.36	pur	yel			+	+	+	+	+			+	
18	0.48	0.35	pur	yel	+		+		+	+	+	+			+
19	0.24	0.28	or	yel					+			+	+	+	+
20	0.26	0.33	or	or		+					+	+	+	+	+
21	0.39	0.50	pk	or			+	+	+	+	+	+			
22	0.39	0.42	or	yel	+				+				+		
23	0.50	0.45	or	yel	+		+	+	+			+		+	
24	0.60	0.48	pur	or	+		+	+	+	+		+	+		
25	0.78	0.39	bl	bl		+	+	+	+	+		+	+	+	+
26	0.91	0.41	or	sk bl	+	+	+	+	+	+	+	+	+	+	+
27	0.83	0.46	or	bl	+	+	+	+	+	+	+	+	+	+	+
28	0.87	0.51	or	bl			+	+	+	+	+		+	+	+
29	0.74	0.56	bl	bl			+	+		+				+	
30	0.61	0.60	pur	yel		+					+		+		
31	0.76	0.67	pur	yel	+	+								+	
32	0.45	0.58	pur	or					+			+		+	
33	0.53	0.64	pur	or					+	+	+	+			
34	0.55	0.69	pur	or			+	+	+	+		+			
35	0.67	0.76	pur	yel			+	+	+	+				+	+
36	0.80	0.82	bl	bl		+		+	+						+
37	0.64	0.85	bl						+						
38	0.53	0.84	or	sk bl			+	+	+	+	+			+	+
39	0.40	0.85	bl	sk bl			+	+	+	+	+				+
40	0.31	0.75	bl	bl			+	+			+				+
41	0.27	0.88	bl	bl			+	+	+	+					
42	0.19	0.86					+		+	+					
Total number of spot					13	13	25	24	29	27	19	26	15	22	17

(1) The abbreviations of taxa: Bb=*B. beecheyana*; Bd=*B. dolichoclada*; Be=*B. edulis*; Bm=*B. multiplex*; Bma=*B. multiplex* cv. "Alphonse Karr"; Bmf=*B. multiplex* cv. "Fernleaf"; Bo=*B. oldhamii*; Bp=*B. pachinensis*; Bs=*B. stenostachya*; Bve=*B. ventricosa*; Bvv=*B. vulgaris*; Bvs=*B. vulgaris* var. *striata*;

(2) Color reaction: l=light, yel=yellow, bl=blue, gn=green, or=orange, pk=pink, pur=purple, sk=sky.

Chitou area are given in Fig. 2, in which 26 bands were found. There are 17 bands in *B. beecheyana*, 15 bands in *B. dolichoclada*, 9 bands in *B. edulis*, 12 bands in *B. multiplex* cv. "Alponse Karr", 12 bands in *B. multiplex* cv. "Fernleaf", 10 bands in *B. oldhamii*, 15 bands in *B. pachinensis*, 18 bands in *B. stenostachya*, 16 bands in *B. ventricosa*, 16 bands in *B. vulgaris* and its variety *striata* (Table 2). Among these 26 bands, only bands 10 and 18 are common to all taxa studied. It was also obvious that varieties *B. multiplex* (Bma, Bmf) as well as those of *B. vulgaris* (Bvv, Bvs) in Fig. 2 revealed the same zymogram pattern within the species. *B. oldhamii*

Table 2. The distribution of peroxidase isozymes in 11 bamboo plant leaves of the genus *Bambusa**

Bands	Bb	Bd	Be	Bma	Bmf	Bo	Bp	Bs	Bve	Bvv	Bvs
1	+		+	+	+	+	+				
2	+	+	+				+	+	+	+	+
3	+	+	+				+	+	+		
4	+	+					+		+	+	+
5							+				
6										+	+
7	+			+	+	+		+	+	+	+
8	+	+	+	+	+		+	+	+	+	+
9	+	+						+		+	+
10	+	+						+		+	+
11		+						+		+	+
12		+									
13									+	+	+
14	+			+	+	+	+	+	+	+	+
15	+			+	+		+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	+	+
17	+	+		+	+	+		+	+	+	+
18	+	+	+	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+	+	+	+
20								+			
21								+			
22				+	+	+	+		+	+	+
23									+	+	+
24	+	+	+	+	+	+	+	+	+		
25	+	+					+	+		+	+
26	+	+	+	+	+	+	+	+		+	+
Total (+)	17	15	9	12	12	10	15	18	15	19	19

* The abbreviations of taxa, see Table 1.

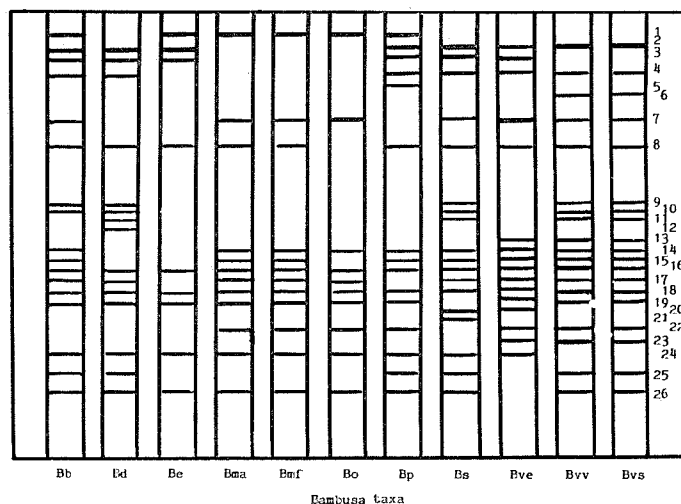


Fig. 2. The zymogram patterns of peroxidase of the genus *Bambusa* grown in Chitou Forest Experiment Station. The abbreviations of taxa see Table 1.

revealed a high similarity to *B. multiplex*. We also collected leaves of *B. oldhamii* from various locations in Taipei Botanical Garden, Pingtung and Kaohsiung areas, and found that the zymogram patterns of the leaves exhibited the same as shown in Fig. 2. (Chou, 1984 unpublished data), suggesting that the zymogram pattern of peroxidase will not vary with geographic location in Taiwan.

Phylogenetic Relationship of the Genus Bambusa

Based on chromatographic data and zymogram patterns of eleven taxa, the simple matching coefficients were obtained (Tables 3 and 4), which derived to phenograms of Figs. 3, 4, and 5. By using the phenolic characters, it was found that *B. multiplex* and its cultivar "Aphonse Karr" revealed a high similarity ($S_{sm}=0.880$), in addition, two other taxa, namely *B. multiplex* cv. "Fernleaf" and *B. oldhamii* were also genetically close to the former two taxa (Fig. 3a and 3b). It is the same to those of *B. vulgaris* and its variety. There are three major clusters in the genus *Bambusa*; one cluster includes *B. multiplex*, its cultivars and *B. stenostachya*; the second cluster comprises *B. dolichoclada*, *B. ventricosa*, *B. beecheyana*, and *B. vulgaris* and its cultivar. The remaining taxa belong to the third cluster, which is rather genetically far from the former two clusters mentioned (Fig. 3a). When the data of Bm was not treated in the clustering analysis, the phenogram of Fig. 3b was also exhibited the same as that of Fig. 3a.

On the other hand, based on the matrix data of isozyme patterns, two phenograms of Figs. 4a and 4b were obtained, in which within the taxa of same species the S_{sm} was 1.00 for both species of *B. multiplex* and *B. vulgaris*. In addition,

Table 3. *The matrix of simple matching coefficient of the genus Bambusa based on the chromatographic data of phenolic compounds*

OTU	OTU (operational taxonomic unit)*										
	Bb	Bd	Bm	Bma	Bmf	Bo	Bp	Bs	Bve	Bvv	Bvs
Bb	—										
Bd	0.714	—									
Bm	0.523	0.428	—								
Bma	0.452	0.407	0.880	—							
Bmf	0.428	0.381	0.714	0.690	—						
Bo	0.428	0.428	0.809	0.738	0.761	—					
Bp	0.571	0.571	0.571	0.500	0.476	0.691	—				
Bs	0.595	0.476	0.500	0.476	0.595	0.595	0.500	—			
Bve	0.761	0.809	0.523	0.500	0.476	0.476	0.571	0.595	—		
Bvv	0.548	0.595	0.547	0.619	0.547	0.595	0.595	0.571	0.595	—	
Bvs	0.571	0.714	0.619	0.643	0.571	0.571	0.665	0.500	0.714	0.690	—

* The abbreviations of OTU, see Table 1.

Table 4. *The matrix of simple matching coefficient of the genus Bambusa based on the zymogram patterns of peroxidase*

OTU	OTU (Operational taxonomic unit)*										
	Bb	Bd	Be	Bma	Bmf	Bo	Bp	Bs	Bve	Bvv	Bvs
Bb	—										
Bd	0.769	—									
Be	0.692	0.692	—								
Bma	0.731	0.500	0.731	—							
Bmf	0.731	0.500	0.731	1.00	—						
Bo	0.654	0.500	0.731	0.923	0.923	—					
Bp	0.769	0.615	0.769	0.731	0.731	0.654	—				
Bs	0.808	0.731	0.500	0.538	0.538	0.462	0.577	—			
Bve	0.692	0.538	0.615	0.731	0.731	0.654	0.692	0.577	—		
Bvv	0.692	0.615	0.385	0.577	0.577	0.500	0.538	0.654	0.692	—	
Bvs	0.692	0.615	0.385	0.577	0.577	0.500	0.538	0.654	0.692	1.00	—

* The abbreviations of OTU, see Table 1.

B. oldhamii was found to be genetically close to *B. multiplex*, while *B. beecheyana*, *B. stenostachya*, and *B. dolichoclada* are genetically far from *B. multiplex*. It is generally agreeable that when the data of Be was not included in the clustering analysis, the phenogram (Fig. 4b) is also shown to be the same as those of Fig. 4a.

Furthermore, when all characters of phenolics and zymogram patterns were run for the clustering analysis, a simple matching coefficient is given in Table 5, leading to a phenogram of Fig. 5. It is evident that the phenetic relationship between taxa of *Bambusa* shown in Fig. 5 is more agreeable with that of Fig. 3 than that of Fig. 4, indicating that the present findings of phenolic characters could be more reliable for the phylogenetic study than the isozyme characters. Nevertheless, both two characters of phenolic compounds and zymogram patterns are of particular significance regarding the phylogenetic study of Bambusaceae.

Discussion

In the course of phylogenetic study of bamboo plants in Taiwan, we have reported that there are 39 compounds of phenolics and 19 bands of zymogram

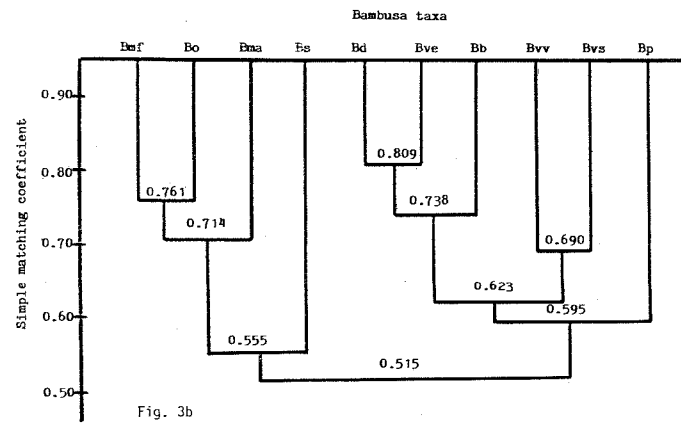
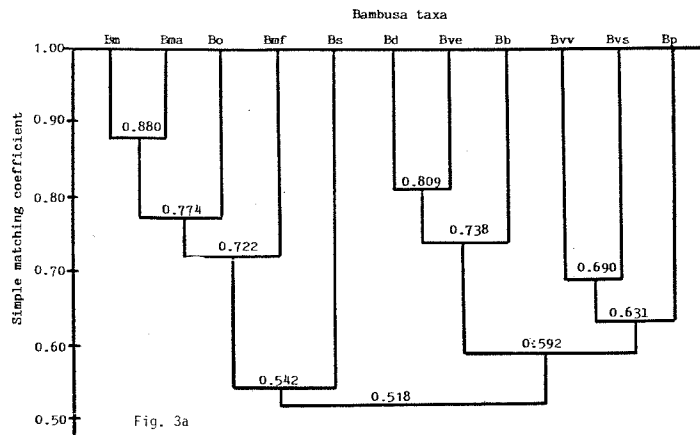


Fig. 3. The phenograms of 11 taxa (Fig. 3a) and 10 taxa (Fig. 3b) of *Bambusa* based on the chromatographic data of phenolic compounds. The abbreviations of taxa see Table 1.

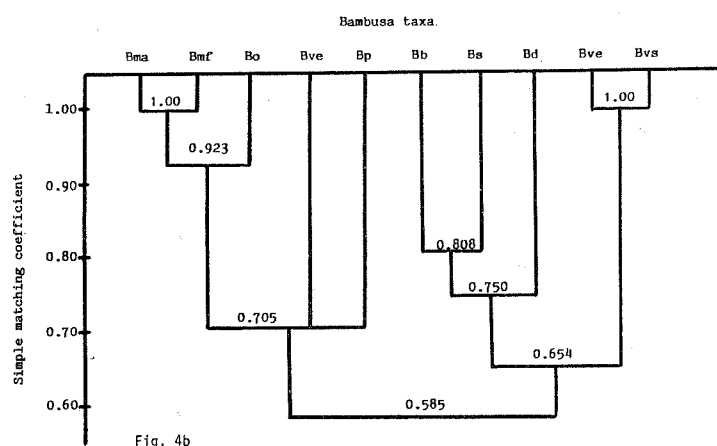
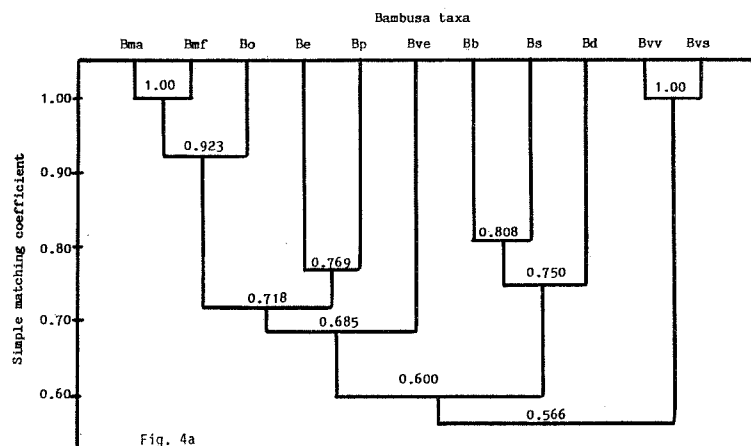


Fig. 4. The phenograms of 11 taxa (Fig. 4a) and 10 taxa (Fig. 4b) of *Bambusa* based on zymogram patterns of peroxidase. The abbreviations of taxa see Table 1.

patterns in the genus *Phyllostachys* (Chou *et al.*, 1984). In this study we found 42 compounds and 26 bands in the genus *Bambusa*. There are three major clusters both in *Phyllostachys* and *Bambusa* genera. Combining both characters of phenolics and isozyme patterns for clustering analysis, it is evident that the isozyme patterns reveal less confidence as compared with the phenolic compounds as far as phylogenetic viewpoint is concerned. This work is closely parallel to that of Hsiao (1980, 1981), who studied the genus *Chamaecyparis* in Taiwan. Hsiao (1981) pointed out that the genetic difference reflected by peroxidase characters could not be expected to be the same as those reflected by phenolic characters because the number of peroxidase characters is very small and the production of these isozymes is generally under the control of only a few genes. In the first report of this series of studies when the number of characters of phenolic compounds was artificially

reduced by employing chromatographic patterns, a better correlation of phenograms was obtained between phenolics and zymograms (Chou *et al.*, 1984). The present study generally shows a good correlation of phenograms derived from both the two characters although little difference of similarity was found between phenograms.

Nevertheless, one may argue that the zymogram pattern of a species might vary with habitats. We have found that the peroxidase zymograms of *B. oldhamii* grown in various locations are insignificantly different as compared with that grown

Table 5. *The matrix of simple matching coefficient of the genus **Bambusa** based on the phenolic compounds and isozyme patterns of peroxidase*

OTU	OTU (Operational taxonomic unit)*									
	Bb	Bd	Bma	Bmf	Bo	Bp	Bs	Bve	Bvv	Bvs
Bb	—									
Bd	0.735	—								
Bma	0.559	0.441	—							
Bmf	0.544	0.574	0.809	—						
Bo	0.515	0.456	0.588	0.824	—					
Bp	0.647	0.588	0.602	0.574	0.632	—				
Bs	0.676	0.574	0.500	0.574	0.544	0.529	—			
Bve	0.735	0.706	0.588	0.574	0.544	0.618	0.588	—		
Bvv	0.603	0.602	0.602	0.559	0.559	0.574	0.602	0.632	—	
Bvs	0.618	0.676	0.618	0.574	0.544	0.618	0.559	0.706	0.809	—

* The abbreviations of OTU, see Table 1.

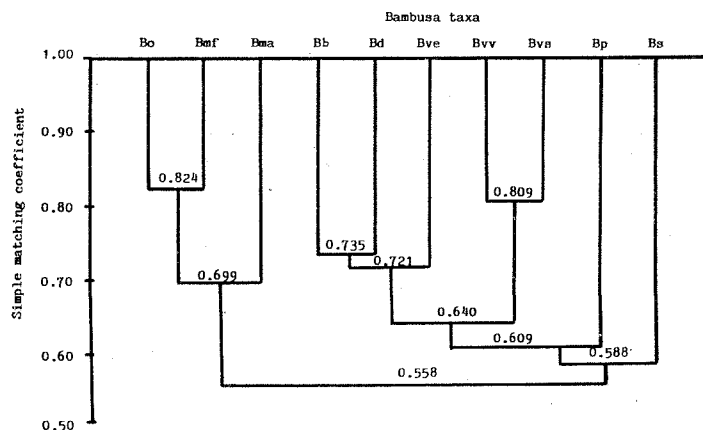


Fig. 5. The phenogram of 10 taxa of *Bambusa* based on both the characters of phenolic compounds and zymogram patterns of peroxidase. The abbreviations of taxa see Table 1.

at the Chitou experimental site (Chou, 1984 unpublished data). In addition, within the same population or different population, the peroxidase zymogram may vary quantitatively but not qualitatively. However, a better understanding of phylogenesis among bamboo plants in Taiwan will arrive when the complete investigation of the series of studies is done. At the moment, we still face with difficulty of isolation and identification of all flavonoids in bamboo plants due to insufficient quantity of pure isolates.

Acknowledgement

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臺灣竹科植物血緣關係之生化學研究

二、蓬萊竹屬

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在臺灣大學溪頭竹類標本園取十二種蓬萊竹屬之植物，並分析其葉中之酚類化合物及過氧化酵素之分布，藉以了解其血緣關係。以甲醇萃得之化合物經紙色層，薄色層及紫外光——可視光譜儀鑑得42個酚類化合物，另以聚丙烯醯胺電泳法分析得26個帶分布於11種蓬萊竹屬。以上述兩種特性之性狀以數量分類之方法得不同種間之相似度而導出種間之血緣關係。結果得知，十一種中可分三個分類羣。其中蓬萊竹，蘇仿竹，鳳凰竹及綠竹之血緣關係最近屬第一個分類羣，南洋竹，長枝竹，佛竹，泰山竹及金絲竹屬第二個分類羣，另外八芝蘭竹，刺竹，及烏腳綠竹屬另一個分類羣。此研究報告乃初步之血緣關係探討，詳細的關係須待整個研究完成後才能定論。