JARQ 32, 7-14 (1998)

Mitochondrial Genome Differentiation in the Genus *Phyllostachys*

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Abstract

Mitochondrial genome differentiation in the genus *Phyllostachys* was investigated by restriction fragment length polymorphism (RFLP) analyses of the restriction fragment patterns or Southern hybridization patterns of endonuclease-treated mitochondrial (mt) DNA. First, intraspecific variation of mtDNA in 3 species, *P. pubescens, P. nigra* and *P. bambusoides*, was studied using a large number of samples collected from different locations in Japan. Little intraspecific differentiation was detected in *P. nigra and P. bambusoides*, whereas *P. pubescens* showed some intraspecific variation. Second, the restriction fragment patterns and Southern hybridization patterns of mtDNAs of 13 *Phyllostachys* species were analyzed. Their comparison indicated that *P. nigra* and *P. dulcis*, and *P. angusta* and *P. propinqua* have the same mitochondrial genome, respectively. The restriction patterns allowed the identification of 9 out of 13 species. Based on the percentage of common restriction fragments, all the species except for *P. aureosulcata* were clustered into the following 3 groups by the UPGMA method; (1) *P. angusta, P. propinqua, P. pubescens* and *P. praecox*, (2) *P. nigra, P. dulcis P. humilis* and *P. aurea*, and (3) *P. bambusoides, P. bissetii, P. viridis and P. makinoi*. Clustering of 13 species based on the results of Southern pattern analysis led to the identification of the following 4 groups; (1) *P. angusta, P. propinqua, P. aureosulcata, P. pubescens, P. praecox* and *P. bambusoides*, (2) *P. nigra* and *P. dulcis*, (3) *P. bissetii*, *P. viridis* and *P. makinoi*, and (4) P. humilis and *P. aurea*. The results of the 2 methods differed in the following aspects; (1) affiliation of *P. bambusoides* differed, (2) cluster 2 in the restriction pattern analysis was divided into 2 clusters by Southern pattern analysis, and (3) intercluster relationships were considerably different between the 2 dendrograms. To develop reliable phylogenetic relationships in the genus *Phyllostachys*, it is necessary to increase the number of restriction enzymes used in the restriction pattern analysis as well as the number of probes used in the Southern pattern analysis.

Discipline: Genetic resources/Forestry and forest products
Additional key words: bamboo, Bambuseae, mitochondrial DNA, restriction pattern analysis, phylogeny

Introduction

Woody bamboos form large forests in most Asian countries. The genus *Phyllostachys*, in the tribe Bambuseae, subfamily Bambusoideae, contains about 45 species, which are distributed from the Himalayas to Japan. Many taxonomic studies on bamboos have been carried out, which were mainly based on morphological characters[7,2]. However, identification of species is difficult because their flowering interval covers a period of 20-120 years.

Phylogenetic relationship among various genera in the tribe Bambuseae has already been analyzed from the morphological point of view[4,10,11]. The tribe Bambuseae consists of woody bamboos, and is considered to be monophyletic. To clarify the phylogenetic relationship between species within a genus or between genera, analyses of secondary metabolites and isozymes were applied to 25

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Taiwanese species containing 7 *Phyllostachys* species[1-3]. Restriction fragment length polymorphism (RFLP) analysis of nuclear DNA and chloroplast DNA was also applied to 23 species, 17 of which were *Phyllostachys* species[5]. Southern hybridization patterns of nuclear DNA gave species-specific patterns of RFLP, which were useful for the identification of the species, whereas, those of chloroplast DNA showed little polymorphism. It is suggested that RFLP analysis of Southern hybridization patterns is not suitable for organellar DNA because of the smaller variation in their genome compared with nuclear DNA.

We performed an RFLP analysis of mtDNA of 13 *Phyllostachys* species produced in Japan and China, to clarify their interspecific relationships, and to reveal intraspecific differentiation in each of the 3 *Phyllostachys* species grown in Japan. Compared with the Southern hybridization pattern, restriction pattern analysis enables to detect more variation easily. Our objectives were (1) to clarify the intraspecific differentiation of 3 *Phyllostachys* species produced in Japan, (2) to identify 13 *Phyllostachys* species and (3) to analyze their phylogenetic relationship.

Materials and methods

Young bamboo shoots of 13 *Phyllostachys* species (Table 1) were used as the source of mtDNA. One mtDNA sample was prepared from 1 bamboo shoot, except for 5 samples, each of which consisted of mixed samples of mtDNA extracted from some bamboo shoots. Nine varieties and forms of the 3 species, *P. pubescens, P. nigra* and *P. bambusoides*, were also used (Table 1). Bamboo shoots of these 3 species were collected from 29 sites in Japan (Table 2, Fig. 1).

Intact mitochondria were isolated from young bamboo shoots which were sliced into pieces. The procedure for the isolation of intact mitochondria and extraction of their DNAs were the same as

Table 1. *Phyllostachys* species, varieties and forms used as the source of mtDNA

Species, variety and form	Abb.	Japanese name	Source[a)]	Collection site[b)]
P. pubescens Mazel	Pb	Mosochiku	I	24
var. *heterocycla*	–	Kikkochiku	III	7
var. *nabeshimana*	–	Kinmeimosochiku	V	8
P. nigra Munro var. *henonis*	N	Hachiku	II	3
var. *nigra*	–	Gomadake	III	7
var. *nigra*	–	Kurochiku	V, VI	8, 29
var. *tosaensis*	–	Tosatorafudake	III	7
f. *boryana*	–	Unmonchiku	III	7
f. *megurochiku*	–	Megurochiku	III	7
P. bambusoides Sieb. *et.* Zucc.	Ba	Madake	II	3
var. *castillonis*	–	Kinmeichiku	V	8
f. *Kashirodake*	–	Kashirodake	II	3
P. dulcis McClure	D	–	III	7
P. humilis Munro	H	Himehachiku	III	7
P. bissetii McClure	Bi	–	III	7
P. viridis McClure	V	–	IV	17
P. makinoi Hayata	M	Taiwanmadake	III	7
P. aurea Carr.	Au	Hoteichiku	V	8
P. praecox Chu *et* Chao	Pc	–	VII	31
P. angusta McClure	An	–	VII	31
P. propinqua McClure	Pr	–	VII	31
P. aureosulcata f. *spectabilis* Chu *et* Chao	As	–	VII	31

a): I; S. Takahashi, Sapporo Breweries Ltd., Nitta, Gunma Pref.
II; S. Nonaka, Fukuoka Forestry Research Center, Kuroki, Fukuoka Pref.
III; M. Watanabe, Kamigamo Experimental Forest Station, Kyoto Univ., Kyoto Pref.
IV; H. Kashiwagi, Fuji Bamboo Garden, Nagaizumi, Shizuoka Pref.
V; Kyoto City Rakusei Bamboo Park, Kyoto Pref.
VI; M. Niizeki, Hirosaki Univ., Aomori Pref.
VII; F. Zhou, Nanjing Forestry Univ., Nanjing, Jiangsu Province, China.
b): Refer to the site number indicated in Table 2.

those reported by Terachi and Tsunewaki[13]. MtDNAs were digested with 5 restriction enzymes (*Bam*HI, *Hind*III, *Pst*I, *Pvu*II and *Xho*I) as described by the manufacturer's instructions (Takara Shuzo and Nippon Gene). Electrophoresis of the digests was carried out at 1.3 V/cm for 20–24 h using 0.8% agarose slab gel in TAE (40 mM Tris, 20 mM sodium acetate and 2 mM EDTA, pH 8.0). The digests were stained with ethidium bromide (0.5 μg/ml), and photographed under UV light illumination. *Hind*III-digested λ-phage DNA was used as a molecular size marker.

After taking photographs, the mtDNA restriction fragments were transferred to Biodyne A nylon membrane (Pall Ultrafine) as described by Maniatis et al.[6]. Three mitochondrial genes were used as probes; pea ATPase α subunit gene (*atp*A) and cytochrome oxidase subunit II gene (*cox*2), and wheat (18+5)S ribosomal RNA gene (*rrn*18+5). The nylon membrane was hybridized with the [α-^{32}P] dCTP-labeled probe at 65°C for 18–21 h according to the method of Maniatis et al.[6]. The radioactive DNA fragments on the filter were detected by autoradiography, by exposing an X-ray film in a cassette equipped with an intensifying screen at −80°C.

Table 2. Collection sites of bamboo shoots used as the source of mtDNA, and number of mtDNA samples

Site No.	Collection site		Number of mtDNA samples		
	City, town or village	Prefecture	Pb[a]	N[a]	Ba[a]
1	Akune	Kagoshima	3	–	–
2	Gamo	〃	1	1	3
3	Kuroki	Fukuoka	3	3	1
4	Kuwano	Tokushima	4	–	–
5	Anan	〃	2	3	1
6	Kameoka	Kyoto	1	1	2
7	Kamigamo	〃	1	1	1
8	Oeda	〃	1[b]	–	–
9	Ono	Fukui	4	1	–
10	Kakusha	Ishikawa	2	1	2
11	Toyama	Toyama	6	–	–
12	Hirata	Gifu	2	–	–
13	Kiyomi	〃	–	1(1)[c]	–
14	Namai	〃	–	3(1)[c]	–
15	Nyuukawa	〃	–	3(1)[c]	–
16	Nakagawa	Nagano	–	3	–
17	Nagaizumi	Shizuoka	–	–	–
18	Ichihara	Chiba	2	–	–
19	Kawaguchi	Saitama	4	–	–
20	Yatabe	Ibaraki	2	–	2
21	Omiya	〃	6	–	–
22	Miwa	〃	1	–	–
23	Utsunomiya	Tochigi	1	–	–
24	Nitta	Gunma	6	–	–
25	Marumori	Miyagi	2	–	–
26	Natori	〃	2	–	–
27	Hiraizumi	Iwate	2	–	–
28	Tsuruoka	Yamagata	2	–	–
29	Hirosaki	Aomori	2(1)[c]	–	–
30	Misawa	〃	1(1)[c]	–	–
31	Nanjing	Jiangsu (China)	–	–	–
		Total	63	21	12

a): Refer to Table 1 for abbreviations.
b): Taken from the bamboo forest originating from a single seed collected in Jiangsu Province, China.
c): Number in parenthesis indicates the number of samples extracted from more than 2 shoots in the samples.

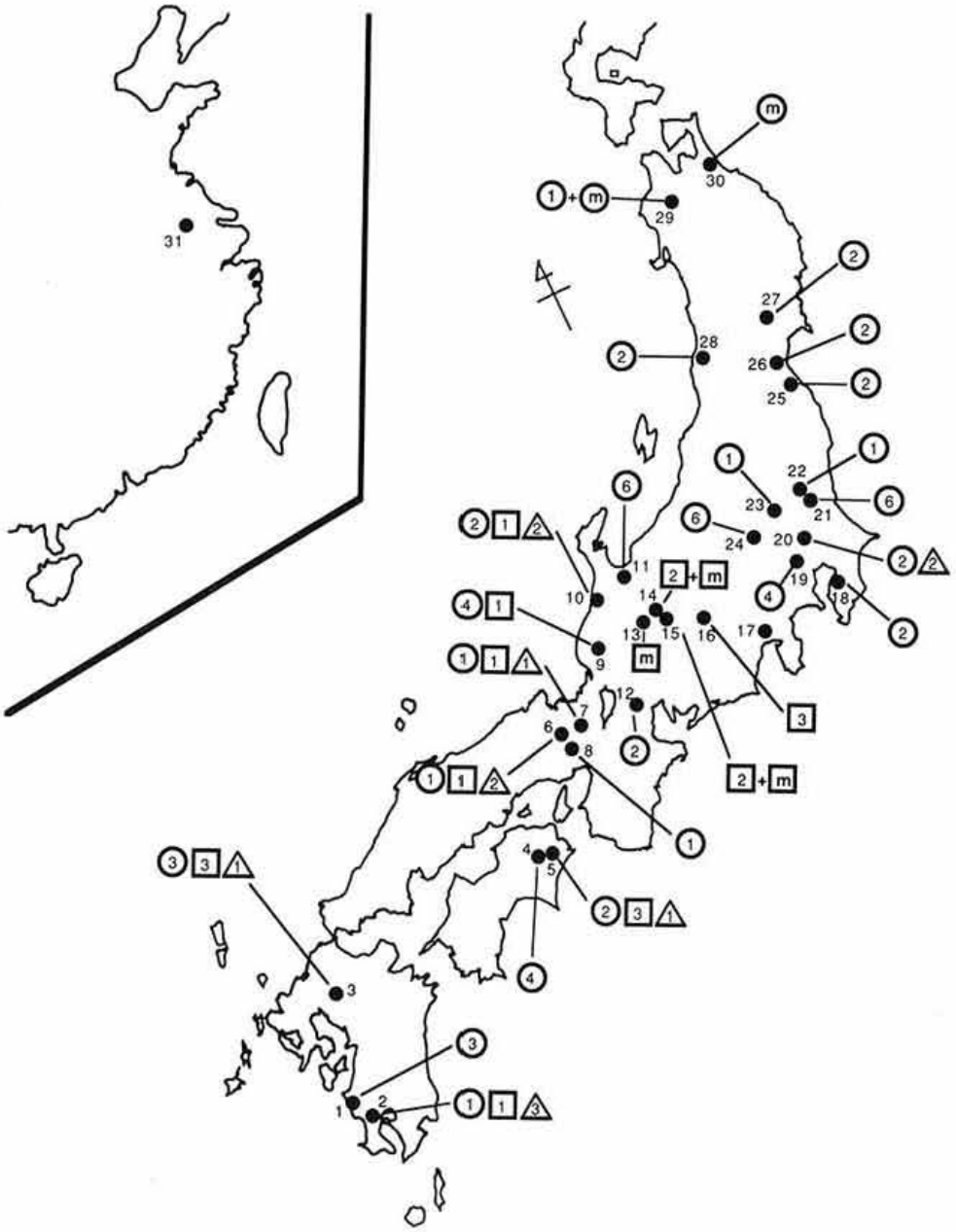

Fig. 1. Collection sites of the bamboo shoots
No. of collection sites: Refer to Table 2.
○: *P. pubescens*, □: *P. nigra* var. *henonis*, △: *P. bambusoides*.
No. in a mark: No. of samples studied.
In the case of *P. pubescens* and *P. bambusoides*, samples of varieties and forms were excluded.
m: Mixed samples, instead of the individual samples in other cases.

Results

1) Intraspecific differentiation of mtDNA from 3 Phyllostachys *species produced in Japan*

MtDNAs from 3 *Phyllostachys* species, *P. pubescens*, *P. nigra* and *P. bambusoides*, were analyzed for their restriction fragment patterns using 5 restriction enzymes (data not shown). In these species, some restriction fragments showed 2 types of intraspecific differences. One was a difference in the quantity of a DNA (or copy number) fragment

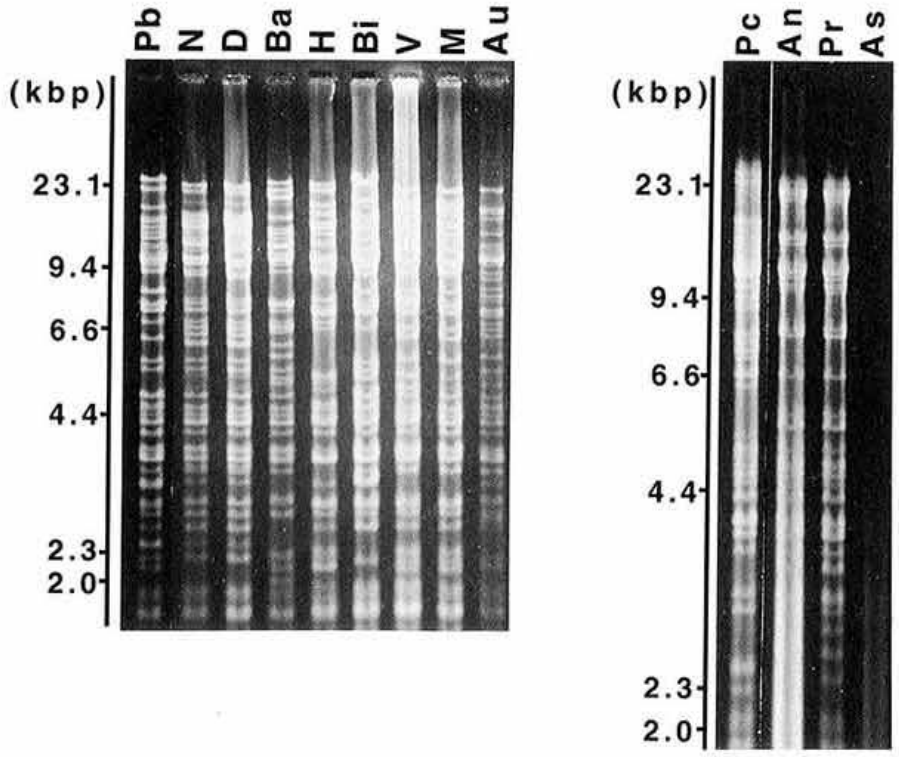

Fig. 2. Restriction fragment patterns of mtDNAs from 13 *Phyllostachys* species digested with *Pst*I

Abbreviated species name is given in each lane (refer to Table 1). One sample was chosen to represent *P. pubescens*, *P. nigra* and *P. bambusoides*, respectively, because they produced the smallest number of restriction fragments among the samples of the same species. *P. pubescens*: Site No. 24, *P. nigra*: Site No. 3, and *P. bambusoides*: Site No. 3 (refer to Table 2).

of the same size between 2 samples. Here, this difference was neglected, because no effective means of evaluation were available. The other type was the presence/absence of difference. A fragment of this type was referred to as "variant fragment".

In *P. pubescens*, 63 samples from 25 collection sites and 2 samples of 2 varieties were analyzed. The mtDNA digests with 5 enzymes produced 196 fragments in total, of which 17 were variants. The samples from the same collection sites tended to give similar patterns. In *P. nigra*, 21 samples from 11 collection sites and 5 samples of 5 varieties/forms were analyzed. Only 1 was found to be a variant among the 194 fragments observed in total. In *P. bambusoides*, 12 samples from 7 collection sites and 2 samples of 2 varieties/forms produced 4 variant fragments in a total of 192 fragments.

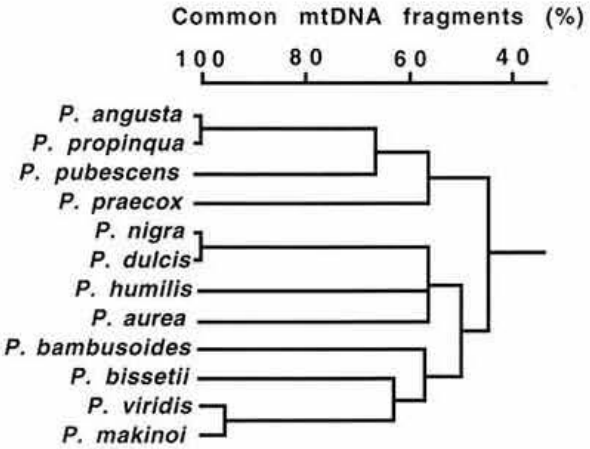

Fig. 3. Dendrogram showing interspecific relationships between mitochondrial genomes of 12 *Phyllostachys* species based on the percentage of common mtDNA fragments

2) Identification of 13 Phyllostachys *species*

The restriction fragment patterns of mtDNAs digested with 5 enzymes were compared between 13 *Phyllostachys* species. All the patterns were complex, showing many fragments, whose copy numbers were non-stoichiometric. Fig. 2 shows the restriction

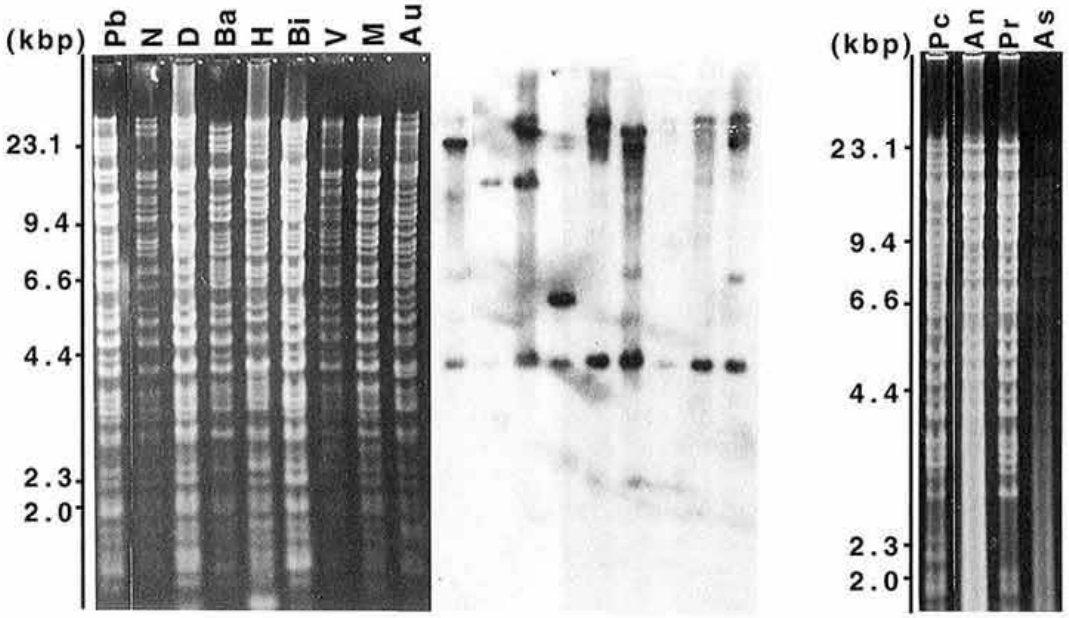

Fig. 4. Hybridization of a probe containing the pea *cox2* gene with the Southern blot of *Pvu*II-digested mtDNA from 13 *Phyllostachys* species

fragment patterns obtained from the *Pst*I-digested mtDNAs of the 13 species. *P. nigra* and *P. dulcis*, and *P. angusta* and *P. propinqua* gave the same restriction patterns for all 5 enzymes, respectively. Except for these cases, each species showed distinctly different restriction patterns.

3) Phylogenetic relationship among mtDNAs from 13 Phyllostachys *species*

Fragments having the same molecular size were considered to be common fragments. Here, the percentage of common fragments between 2 species was calculated from the numbers of total and common fragments in every pair of species.

Fragments between 2.0 and 9.4 kb in size were counted, because their adequate separation in agarose gel enabled to confirm the data. However, mtDNA from *P. aureosulcata* was excluded, because it did not allow the detection of fragments smaller than 4.4 kb. Based on the percentage of common fragments, cluster analysis using the UPGMA method[9] was carried out to construct a dendrogram showing mitochondrial genome similarity among the 12 species (Fig. 3). In this dendrogram, 3 clusters were identified; (1) *P. angusta*, *P. propinqua*, *P. pubescens* and *P. praecox*, (2) *P. nigra*, *P. dulcis*, *P. humilis* and *P. aurea*, and (3) *P. bambusoides*, *P. bissetii*, *P. viridis* and *P. makinoi*.

Three mitochondrial genes were hybridized to the restriction fragments of mtDNA, to identify their

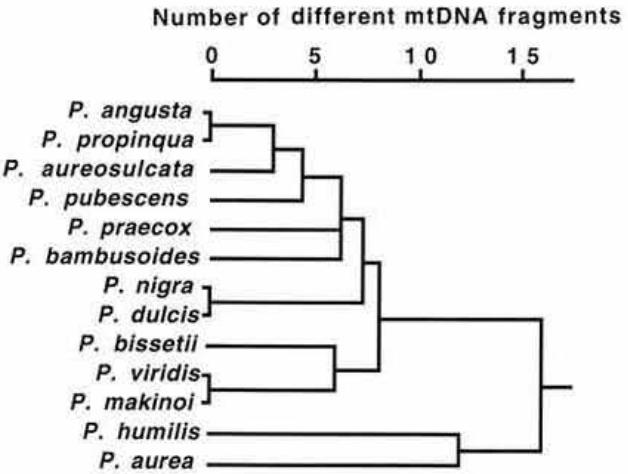

Fig. 5. A dendrogram showing interspecific relationships between mitochondrial genomes of 13 *Phyllostachys* species based on the Southern hybridization patterns, using wheat *rrn18+5*, and pea *atp*A and *cox2* as the probes

homology. Fig. 4 shows the Southern hybridization patterns of the pea *cox2* gene hybridized with *Pvu*II-digested mtDNAs from 13 *Phyllostachys* species. Based on the number of fragments which differed in size between the Southern hybridization patterns of every species pair, cluster analysis using the UPGMA method[9] was carried out, and a dendrogram was drawn that showed interspecific relationships between mtDNAs from 13 species (Fig. 5). In this dendrogram, 4 clusters were identified; (1) *P.*

angusta, *P. propinqua*, *P. aureosulcata*, *P. pubescens*, *P. praecox* and *P. bambusoides*, (2) *P. nigra* and *P. dulcis*, (3) *P. bissetii*, *P. viridis* and *P. makinoi*, and (4) *P. humilis* and *P. aurea*.

Discussion

A single sample was used to represent each species, although this procedure may not be suitable for clarifying relationships among the species, in which intraspecific variation is unknown. However, this procedure was found to be suitable at least for 3 species, *P. pubescens*, *P. nigra* and *P. bambusoides*, all of which exhibited a low level of or little intraspecific variation. Each species showed unique restriction fragment patterns except for *P. nigra* and *P. dulcis*, and *P. angusta* and *P. propinqua*. The present results demonstrated that restriction pattern analysis is a powerful tool to identify *Phyllostachys* species.

P. nigra and *P. dulcis*, and *P. angusta* and *P. propinqua* seemed to have the same mitochondrial genome, respectively. Although these 2 sets of species differed in their morphological characteristics, they are considered to have originated from the same maternal species.

Two dendrograms showing interspecific relationships of the mitochondrial genomes were obtained by restriction pattern analysis (Fig. 3) and Southern pattern analysis (Fig. 5). Three clusters are depicted in Fig. 3, whereas 4 in Fig. 5 (refer to Results). In these dendrograms, some species groups occurred in common; the group of *P. angusta*, *P. propinqua*, *P. pubescens* and *P. praecox*, and the group of *P. bissetii*, *P. viridis* and *P. makinoi*. Two different groups shown in Fig. 5, namely, *P. humilis* and *P. aurea*, and *P. nigra* and *P. dulcis*, were united into 1 group shown in Fig. 3. Thus the 4 groups depicted in Fig. 5 are considered to be essentially the same as the 3 groups in Fig. 3, although the intergroup relationships were somewhat different from each other.

Southern hybridization experiment was carried out to identify fragment homology from both the size and nucleotide sequence. The probes used in this investigation generally hybridized to the fragments with the same size, indicating their sequence homology. This fact confirms the results of the restriction pattern analysis.

To identify species such as *Phyllostachys* species, application of 2 methods has been reported; analysis of secondary metabolites and isozymes[3], and RFLP analysis of Southern hybridization patterns of nuclear and chloroplast DNAs[5]. We could not compare the estimated phylogeny of *Phyllostachys* species between these 2 reports and ours because only 4 or 5 species were analyzed in common. Since the quantity of secondary metabolites and isozymes tend to change with environmental conditions[3], DNA is considered to be a better objective for analysis. In the application of RFLP analysis based on Southern hybridization patterns, chloroplast DNA gave much less information than nuclear DNA[5]. Since the genome diversity of chloroplast is much lower than that of mitochondria[13], we should concentrate on highly variable regions in the chloroplast genome, if variability is to be studied. The use of such regions as probes for Southern hybridization, or their sequencing could provide more information. Although the mitochondrial genome shows a much larger diversity than the chloroplast genome, nucleotide substitution rates are lower in mtDNA than in chloroplast DNA[8]. Since the variation in the mitochondrial genome structure is considered to be more remarkable than that in nucleotide sequence, the restriction pattern analysis of mtDNA is a powerful tool for the detection of differences among mitochondrial genomes. To develop reliable phylogenetic relationships in the genus *Phyllostachys*, it is necessary to increase the number of restriction enzymes used in the restriction pattern analysis as well as the number of probes used in the Southern pattern analysis.

We wish to express our deepest appreciation to Dr. M. Watanabe, Kamigamo Experimental Forest Station, Kyoto University, for his generous gift of bamboo shoots and advice. We also thank Dr. S. Takahashi, Sapporo Breweries Ltd.; Dr. S. Nonaka, Fukuoka Forestry Research Center; Dr. H. Kashiwagi, Fuji Bamboo Garden; Kyoto City Rakusei Bamboo Park; Prof. F. Zhou, Nanjing Forestry University, China; and many other people, all of whom kindly supplied us the bamboo shoots used in the present investigation.

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(Received for publication, April 22, 1997)