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소나무屬 23樹種에 있어서 Peroxidase 同位酵素의 変異*1

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Comparative Electrophoretic Studies of Isoperoxidase for 23 Species in the Genus *Pinus**1

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摘 要

소나무屬 23樹種의 針葉 Peroxidase同位酵素를 澱粉겔 전기영동법으로 분석하였다. 각種마다 特有의 밴드型을 가지고 있었으며 이들 밴드型은 7個群으로 나눌 수 있었다. 이가운데 제Ⅱ群은 어느 樹種에서나 나타나서 소나무屬의 固有型으로 推定하였다. 이들 7個群은 古典的 形態分類群과는 確実한 関係를 発見할 수 없었다.

SUMMARY

The patterns of isoperoxidase in needle-leaves of 23 species of the genus *Pinus* were analyzed by means of starch gel electrophoresis. Each species had a unique band pattern. In all, 56 isoperoxidase bands were identified, of which 9 to 35 isoperoxidase bands were possessed by single species. No single band was common to all *Pinus* species but when band patterns were grouped into 7 types, type II was considered to be the specific to genus *Pinus*. The results of this experiment indicated that various *Pinus* species had their more or less specific band patterns of peroxidase.

INTRODUCTION

There are not a few papers reporting that isozymes are capable of pioneering a powerful approach to genetic studies of wild organisms. This is because isozymes are likely to be little affected by outer environmental conditions, on the one hand and probably are free from any selective pressure, on the other (Park, 1977; 1979).

Isozyme markers have been used with varying degrees of efficiency to distinguish within and between species. Torres (1978) and Esen (1977) reported the leaf-isozymes as genetic markers between *Citrus* species. Pea (Daves, 1976), *Petunia* (Natarella, 1975), and *Chenopodium* (Crawford, 1979) were studied to identify between species by means of isozyme variation. Bonnet (1978) have successed in making use of isozyme of glutamate-oxaloacetate-transaminase for identifying four subspecies within *Pinus nigra*.

It is widely accepted that genus *Pinus* is composed of numerous species, say more than 100, which are distributed mainly in the northern hemisphere (Mirov, 1967; William and Little, 1966). Within this expanded range of distribution they dominate the natural vegetation in many regions. The genus includes some of the most valuable timber trees in the world, and is readily distinguishable from other genera.

The zymography techniques, using starch or

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acrylamide gels, has been effectively applied to detect the relationship in many species of plants. The purpose of this study was to survey the zymographic variation of peroxidase in 23 pine species.

Fortunately, I was able to obtain experimental materials from the forest stands of 21 introduced and two pine species native to Japan which were planted in the Forest Experimental Station of Kyoto University. I would like to express sincere thanks to Dr. Sakai for guiding me to this study and to Dr. Iyama for computing the data. I have also to extend my heartiest thanks to gentlemen of the office of the Forest Experimental Station of Kyoto University, especially to Dr. Akai and Dr. Furuya for their generosity and kindness in allowing me to make use of materials from their forest.

MATERIALS AND METHODS

Needle-leaves for the present electrophoretic study were collected from trees of 21 introduced pine species and 2 species native to Japan. The age of the trees was approximately 10 years old in most species. The total number of samples was 526 twigs with needles which were picked from individual sample trees. Those needle-leaves were brought back to the laboratory and stored in a deep freezer kept at −20℃ until they were used for electrophoretic study.

Horizontal starch gel electrophoresis was carried out following the method described in Miyazaki and Sakai(1969). 56 grams of hydrolyzed starch were heated in 500ml of 0.03M borate buffer(pH8.5) containing 0.105 grams sodium hydroxide and 0.9grams boric acid. When this became a viscous mixture, the gel was degassed and poured into 12 gel molds, allowing it to become hard at room temperature. A crude extract from needle-leaves of approximately 0.3grams was taken up in a small piece of filter paper which was inserted into a slit of the starch gel at 8cm of distance from the catodic edge. The gel mold was than subjected to electrophoresis with electrode tanks containing the borate buffer (3.94 grams sodium hydroxide and 18.5 grams boric acid per liter of distilled water, with the adjusted pH 8.5). The electrophoresis was run in an incubator at a temperature of 7℃, under applied voltage gradient of 20 v/cm about 100 minutes when BPB (Bromphenol Blue) had migrated about 11cm from the origin. Following electrophoresis, the gel mold was removed from the electrode tanks and the gel was sliced horizontally with a gel cutter. Using benzidine acetate as the hydrogen donor, the bottom pieces were stained for peroxidase, 0.2% benzidine acetate and 0.0625 M tris-acetate acid buffer (0.01M tris and 0.0525M acetate acid) with pH of 4.0. The reaction was stopped in water by pouring off the stain when the bands get stained light-blue, around 15 minutes after staining.

RESULTS AND DISCUSSION

The range of distribution of the isozyme bands of peroxidase counted in needle-leaves of *Pinus* 23 species was from 2 to 56. Of those bands high variation were always shown between individual trees within same species. In table 1 the distribution of the number of bands per individual of 23 pine species is shown. The mean number of bands, 14.08 in *P. sylvestris* was the highest, while the mean number of bands 5.61 in *P. ayachuite* was the lowest of 23 *Pinus* species. *P. patula* showed the highest standard deviation 2.97, while *P. koraiensis* showed the lowest standard deviations 0.75 of 23 *Pinus* species.

In table 2 the banding patterns in 23 pine species are summarized. It is shown that each species is composed of bands less than 56 in number. *P. strobus* and *P. sylvestris* showed more than 35 bands, while *P. ayachuite* revealed only 9 bands.

The highest frequency of occurrence of certain bands in each species is shown in table 2. In 14 species some bands appeared with 100 percent occurrence, while it was not in the remaining 9 species. During observation on the patterns of isozyme bands, it was noticed that there were several groups which were characterized by possessing a certain combination of isozyme bands. 7 band types were grouped by the number of 8, 9, 10 and 11 band

in type Ⅰ, of 14, 15, 16 and 17 band in type Ⅱ, of 19 band in type Ⅲ, of 23 and 24 band in type Ⅳ, of 28, 29, 30 and 31 band in type Ⅴ, of 46 and 47 band in type Ⅵ and of 53 band in type Ⅶ. If one individual of same species have only one band of the four, that is, 8, 9, 10 and 11, the individual was classified into type Ⅰ. The actual number of the occurrence of species was 5 in type Ⅰ, 15 in type Ⅱ, 1 in type Ⅲ, Ⅳ and Ⅶ, 7 in type Ⅴ and 2 in type Ⅵ. Type Ⅱ represented by 15 species might be considered to be specific band type of the genus *Pinus*.

Rate of fixed band was calculated by the number of band occurring at 100 percent devided by the number of total bands. *P. nigra*, *P. nigra* v. *austriaca*, *P. pungens* and *P. laricio* showed the high rate of fixed band. According to the classical taxonomy *P. nigra*, *P. nigra* v. *austriaca* and *P. laricio* have been regarded as different varieties of the same species.

Not a few works using zymography were carried out in order to study the phylogenetical relationship within and between species. Esen *et al.* (1977) studied the variation of amylase band patterns from

Tab. 1. The distribution of the number of bands per individual in 23 pine species

Species	Number of Individual	Number of band 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20	Mean number of band	Standard deviation	Provenance
P. sylvestris	24	1 2 2 10 4 1 1 3	14.08	1.85	France Paris
strobus	38	1 - 3 2 6 5 4 8 6 1 2	13.74	2.34	Canada Toronto
koraiensis	38	16 20 - 2	12.68	0.75	Japan Native
excellsa	15	1 3 7 4	11.93	0.89	France Nogent
peuce	19	6 1 6 3 - 3	11.84	1.75	Netherlands Amsterdam
pungens	25	5 6 11 2 1	11.52	1.05	U. S. A. Cambridge
nigra	9	2 2 - 2 3	11.22	1.72	Canada Toronto
pinea	13	1 - 3 2 2 1 2 1 - 1	11.08	2.47	France Paris
rigida	12	1 - - 2 1 1 3 4	11.08	2.20	Canada Toronto
banksicna	18	4 1 1 1 4 2 2 2 - 1	10.72	2.77	Canada Montreal
nigra v. *austriaca*	13	1 4 2 2 2 2	10.46	1.90	Netherlands Wageningen
pinaster	34	1 4 3 5 5 8 5 3	10.00	1.94	Spain Madrid
laricio	12	2 2 4 1 1 1 - 1	9.42	2.07	France Nogent
palustris	15	3 5 4 1 1 - - 1	9.07	1.83	U. S. A. Lisle
densiflora	39	1 1 5 11 6 5 8 1 - 1	9.03	1.83	Japan Native
massoniana	15	1 2 3 5 1 2 1	8.87	1.65	Hongkong
taeda	47	3 7 9 8 9 7 2 - 2	8.15	1.92	U. S. A. Washington D. C.
echinata	21	1 - 4 6 3 - 4 1 - 2	8.14	2.38	U. S. A. Placerville
radiata	33	2 6 1 6 3 4 4 3 3 1	8.12	2.60	Spain Madrid
patula	12	1 - 3 2 - 1 2 1 2	7.33	2.97	Mexico Mexico City
virginiana	20	3 7 7 2 - - 1	6.65	1.35	U. S. A. Berkely
elliottii	36	2 1 4 8 5 4 7 3 1 1	6.25	2.16	Australia Sydney
ayachuite	18	4 2 9 3	5.61	1.04	U. S. A. Placerville

Tab. 2. The summarized banding patterns in 23 pine species

Species	Number of total bands	Highest frequency of occurrence	Band type							Rate of fixed band
			I	II	III	IV	V	VI	VII	
P. strobus	35	1.00	+							.029
sylvestris	35	1.00		+						.057
pinaster	27	1.00		+						.037
densiflora	23	1.00		+						.043
pinea	22	1.00							+	.045
koraiensis	21	1.00		+				+		.095
peuce	21	1.00	+							.095
massoniana	20	1.00		+						.050
nigra	19	1.00		+		+	+			.316
nigra v. *austriaca*	19	1.00		+			+			.210
patula	19	1.00		+						.048
pungens	18	1.00	+	+				+		.278
laricio	16	1.00		+			+			.188
excellsa	16	1.00	+	+						.038
radiata	27	.85		+						.000
virginiana	27	.80					+			.000
echinata	26	.81		+			+			.000
banksiana	26	.78			+					.000
taeda	26	.76					+			.000
elliottii	26	.69		+						.000
rigida	25	.91		+						.000
palustris	21	.75					+			.000
ayachuite	9	.94	+							.000
			5	15	1	1	7	2	1	

Remarks: I type include 8, 9, 10 and 11 band, II type 14, 15, 16 and 17, III type 19, IV type 23 and 24, V type 28, 29, 30 and 31, VI type 46 and 47, VII type 53 band.

5 species of *Citrus* cultivars for detecting the relationship between cultivars. *C. medica* and *C. paradidisi* showed different patterns except three cultivars. The variation of protein and peroxidase banding patterns was studied by disc electrophoresis of leaf extracts from flowering plants of *Petunia*. Natarella *et al.* (1975) insisted that the electrophoresis of proteins and isozymes appear to be of potential taxonomic value in *Petunia*. Crawford *et al.* (1979) reported that the phylogenetic relationships of *Chenopodium* species are fully corroborated by enzyme profiles.

Concerning enzymes, there are a number of studies for enquiring the relationship between species. For instance Sheen (1970) reported that the variation of peroxidase bands in 60 species of the genus *Nicotiana* was not agreeable with the relationship between ploidy level and peroxidase banding patterns but was closely related in phylogeny. Smith *et al.* (1970) announced that the variation of peroxidase

and esterase in the genus *Nicotiana* was agreeable with the established taxonomy of the genus. Bonnet *et al.* (1979) asserted that the variation of GOT isozymes make use of identifying four subspecies within *Pinus nigra*. Davies (1976) characterised the storage proteins present in pea seeds in order to try and determine the proportions of proteins present which are enriched in sulphur amino-acids. Some of eight varieties examined were shown to differ in their proportions of the various storage proteins.

The results of the present study is not satifactory to decide whether the variation of isoperoxidase in genus *Pinus* is agreeable with established taxonomy and with phylogenetic problems. Merely I am to state that the variation of isoperoxidase was very great between as well as within species. When band patterns were, however, grouped into 7 types, type II was considered to be specific to the genus *Pinus*. From the results of this experiment it is indicated that various *Pinus* species had their more or less specific band patterns of peroxidase.

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