A PAPER CHROMATOGRAPHIC STUDY OF PHENOLIC COMPOUNDS IN *ORYZA*[1]

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Introduction

With the advent of paper chromatography, a relatively simple technique was introduced for more and more investigations aiming to help solve taxonomic and evolutionary problems in various organisms. Although many compounds can be isolated by this method, Erdtman (1956) stressed the importance of secondary constituents, particularly polyphenols for the use in biosystematic studies in plants. Taxonomically, the most valuable substances seem usually to be those which are the involved in the primary metabolic processes and which do not have any special task to fulfill; in short, natural products which have been regarded as important and which are, in their biological environment, relatively stable by-products, are often denoted by the term "secondary constituents". Alston and Irwin (1961) also showed in their study of Cassia that the patterns of variation of secondary substances substantiated a greater potential in taxonomic work than amino acids. In the investigation on taxonomic affinities with certain plant forms (Riley and Bryant 1961), the patterns among phenolic compounds were generally consistent, hence they were reliable species indicators.

On the nature of hybrid populations, the passible use of chromatographic patterns of phenolic compounds as indicators of species affinities was emphasized (Alston and his coworkers 1962, 1964, 1959, Harney and Grant 1964 a, b). On the internal features of some polyploid taxa (Smith and Levin 1963, Stebbins *et al.* 1963, Torres and Levin 1964) the conclusions from the chemotaxonomic technique by polyphenols agreed in each instance with those from other approaches and substantiated the previously determined genomic relationship. Such chromatographic data were used as a reliable criterion for the

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classification in a certain group or among groups of species, subspecies or of varieties (Fukushima and Iwasa 1966).

Polyphenols contained in rice were studied (Wakimoto and Yoshi 1958). In this paper, the result of using polyphenols as a taxonomic indicator will be reported.

Materials

Since 1961, in this laboratory of cytogenetics have started the investigation of cytogenetical studies of rice and its related species. Many species of rice widely distributed in the whole world were collected, hybridized and studied. The material which we used in this experiment stemmed from the results of those cytogenetical investigations aiming to differentiate the differences among the varieties and species of different genomic constitution by the use of paper chromatography of their phenolic compounds. Twelve strains of rice were chosen (Table 1), aiming to compare the differences between varieties of the same species and among 10 species belonging to different genomic constitutions as well as among diploids and tetraploids.

Table 1. *The genomic constitution, chromosome number and distributions of rice species*

Species	Genome	Chromosome No. (2n)	Distribution
O. sativa (Taichung 65) *Japonica*	AA	24	Taiwan
O. sativa (Pamifen) *indica*	AA	24	Taiwan
O. sativa (Nanteh) *indica*	AA	24	China mainland
O. glaberrima C7692*	A^gA^g	24	West Africa
O. breviligulata Af-17	A^gA^g	24	West Africa
O. officinalis 2004	CC	24	Southeast Asia
O. australiensis W008-1	EE	24	Australia
O. brachyantha Af-15	FF	24	West and Central Afirica
O. latifolia W1173	CCDD	48	Central and South America
O. grandiglumis Manaos 7	CCDD	48	South America
O. eichingeri W043	BBCC	48	Ceylon and East Africa
O. minuta W0016	BBCC	48	Philippines

* accession number.

Methods

In our experiment the seeds of different varieties or species were germinated in petri-dishes at the same time. After that the seedlings were transplanted to glazed pots in the green-house. In order to avoid variation coming from

environmental difference the experiments were performed under relatively similar environmental conditions.

In general, polyphenol contents of the leaves of rice plants increased gradually with the growth of plants and reached the maximum at the tillering stage. Usually, leaves contained higher contents of polyphenols than other parts of the culm (Wakimoto and Yoshi, 1958). When the experiment was performed, fresh and mature leaf blades from the plant of about 90 days old were cut into pieces after the leaf sheath was removed. Ten grams of these were weighted out and shreded. One hundred mililiters of methyl alcohol were added and was reflexed at 75°C for 30 min. (Harborne, 1964). Then it was filtered after sufficient cooling. The filtrate was concentrated at 45°C under reduced pressure, washed with petroleum ether until the chlorophyll was completely disappeared. Petroleum ether was immisicible with methanol. Under this circumstance it was a very satisfactory solvent for chlorophyll. Therefore, relatively pure methanol soluble phenolic compounds were obtained which were devoid of chlorophyll. The extract was quantatively spotted on Whatman No. 1 filter paper with micropippette. It was then developed into one or two dimensional chromatograms by ascending method. By this, more satisfactory and clear-cut spots were obtained than when the descending method was tried at first.

Many kinds of developing solvents were tried (Blook *et al.* 1958). Among the seven different developing solvents, namely: distilled water; 15% acetic acid; distilled water saturated with phenol; iso-butanol; *n*-butanol; *n*-butanol plus acetic acid and water (*4:1:1 v/v*). It was found that when the combination of *n*-butanol plus acetic acid and water (*4:1:1 v/v*) for the first dimension (23 hours) and 15% acetic acid for the second dimension (7 hours) would give the best results, showing many widely spread spots visible to the naked eye. Those relatively hazy and not clear-cut were omitted in the final analysis. After several trials, 15% of acetic acid was chosen as the most appropriate in rice.

Each dried chromatogram was examined under ultraviolet light with the presence of ammonia vapor. By and large, specific and universal test for phenols is the production of an intense green, brown or blue color with ferric chloride (Bate-Smith and Westall 1950). This test was performed in 70% ethyl ethanol soltuion with freshly diluted 2% ferric chloride. In our experiment we also detected the phenolic compounds under diazotized sulfanilic acid, a common reagent for the detection of phenolic compounds (Smith 1958). However, the results were obtained exclusively by observation under ultraviolet light with the presence of ammonia vapor.

The photography of one dimensional chromatogram under ultraviolet light was taken by adding a Nikon XO yellow filter so as to cut down the reflected

blue light. Black and white (Kodak safety film, exposure 3.5 min. a distance of 4 ft. from the chromatogram) and color (Kodak color-X film, exposure 3.5 min. at a distance of 4 ft. from the chromatogram) were used.

Reactions of treated chromatograms suggested that the majority of the observed compounds were phenolic or polyphenolic substances. It was not considered essential at the present to attempt to identify the various compounds because the patterns were produced under uniform extraction and development. The identification of these different spots on the chromatograms will be studied later.

Results

A photograph of one-dimensional paper chromatogram of eight different diploid species in *Oryza* is shown in Fig. 1. It can be seen that between varieties or species which belonged to the same genome were rather similar in pattern. Conversely, species belonging to different genomes would vary widely in pattern.

The one-dimensional paper chromatograms of five species is shown in Fig. 2. All of them have the CC genome in common. The pattern of the tetraploid species *O. latifolia* and *O. grandiglumis* is rather similar to that of diploid species *O. officinalis*. On closer examination however, the pattern of the later two species are more similar to each other than to that of *O. officinalis*. When the patterns of *O. eichingeri* and *O. minuta*, both with genome BBCC, are examined, the patterns of these two are very similar but are way different from those the other species.

From analysis of the two-dimensional chromatograms, 53 different spots could be detected in the 10 species of *Oryza* and each species had varying number of spots of its own, ranging from 14 to 26. (Table 3) The spots detected through all the chromatograms could be distinguished by their fluorescent colorings (Table 2). Each spot was given a numerical number. There were six spots (No. 1, 2, 3, 4, 5 and 15) found to be present in all the 12 kinds of rice. The black spots on the diagrams represent the common spots in all the 12 kinds of rice as showed in Fig. 3. Spot No. 11 was recognized in all species except *O. officinalis* and spot No. 18 was not found only in *O. australiensis*.

There seems to have very little difference in the number and type of spots among the three varieties of the cultivated rice *O. sativa*. There is only the presence of spot No. 22 in variety Pamifen, whereas in variety Nanteh this spot is difficient. But all the other spots are identical in this subspecies *indica* as far as these two varieties are concerned.

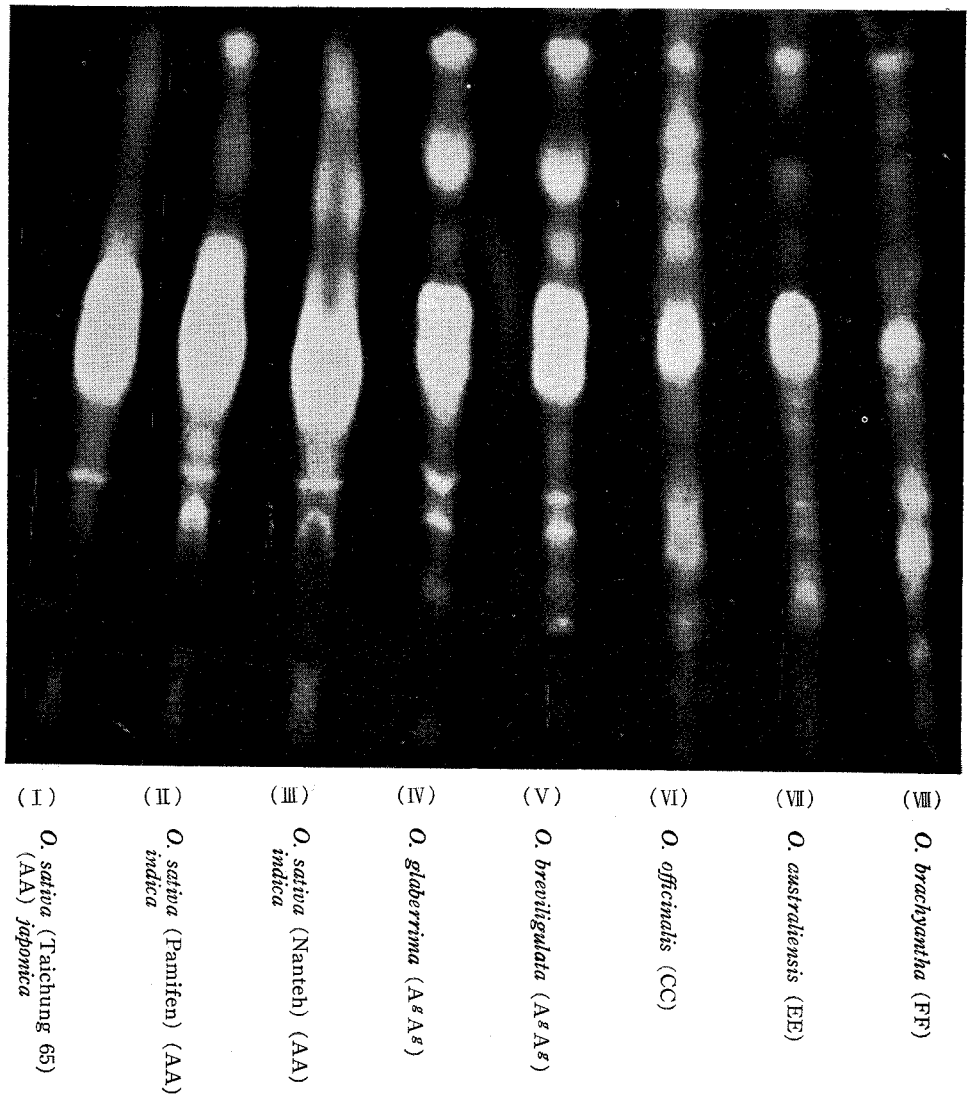


Fig. 1. One dimensional chromatogram of phenolic compounds extracted from diploid rice leaf blades and detected under ultraviolet light with the presence of ammonia vapor.

Many of the spots are similar in *O. glaberrima* and its closed related *O. breviligulata* but it is more varied than those spots of *O. sativa* even though these three species have identical genome cytologically.

The spots detected in distantly related species with different genomes and with diploid and tetraploid would vary greatly from the spots detected with *O. sativa*. There are many spots in common. However, there are many spots of different genome differ more widely than those of three varieties in *O. sativa*.

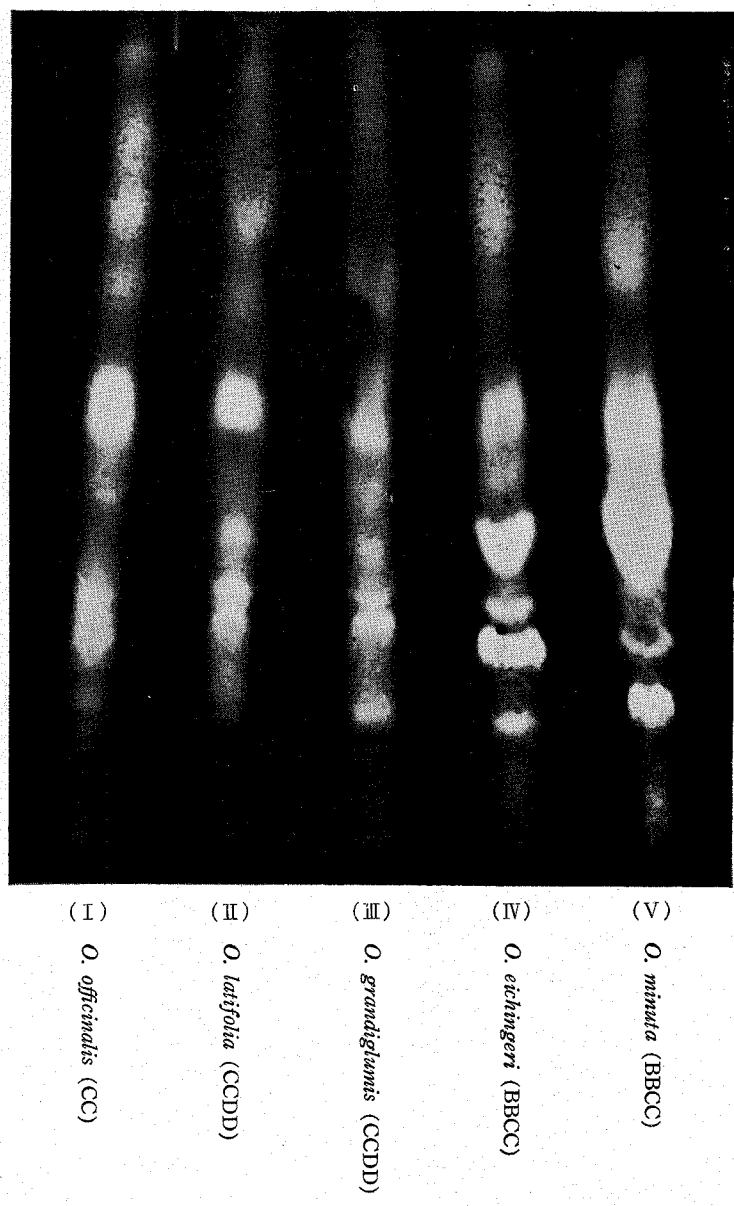


Fig. 2. One dimensional chromatogram of phenolic compounds extracted from diploid and tetraploid contain CC genome rice leaf blades and detected under ultraviolet light with the presence of ammonia vapor

Summary and Conclusion

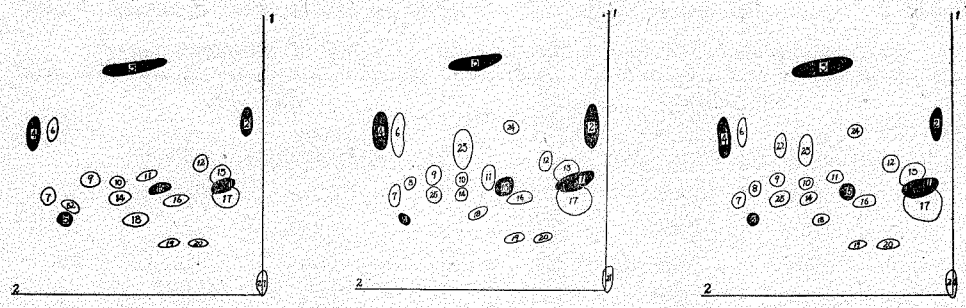
There were ten species of rice used in this experiment. One dimensional as well as two dimensional chromatographic methods were used. The Rf values of each spot in the two-dimensional chromatograph was calculated and each spot was given an asigned number. There were in a total of 53 different

Table 2. *Spots of phenolic compounds contained in leaves of 12 different species detected nnder ultraviolet light with the presence of ammonia vapor*

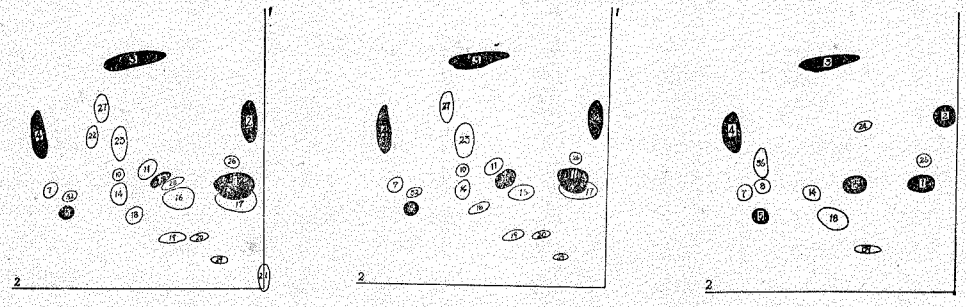
Spot No.	RF Value		Color*	Spot No.	FR Value		Color*
	First dimentional	Second dimentional			First dimentional	Second dimentional	
1	0.49	0.30	Y	28	0.41	0.41	W
2	0.78	0.09	Y	29	0.14	0.16	B
3	0.86	0.67	B	30	0.52	0.87	BG
4	0.73	0.93	BG	31	0.72	0.59	bR
5	0.32	0.83	B	32	0.72	0.62	DB
6	0.72	0.85	W	33	0.30	0.73	bR
7	0.42	0.88	B	34	0.67	0.39	bR
8	0.47	0.81	DB	35	0.20	0.82	R
9	0.50	0.74	DB	36	0.60	0.71	WY
10	0.52	0.64	bR	37	0.60	0.08	bR
11	0.51	0.50	Y	38	0.51	0.21	BG
12	0.56	0.27	B	39	0.23	0.50	Y
13	0.52	0.22	YG	40	0.28	0.96	WY
14	0.42	0.64	DB	41	0.42	0.33	YG
15	0.44	0.42	Y	42	0.17	0.32	Y
16	0.40	0.38	GY	43	0.57	0.47	DB
17	0.42	0.20	WY	44	0.41	0.49	DB
18	0.33	0.56	YG	45	0.55	0.63	DB
19	0.24	0.41	bR	46	0.66	0.85	YG
20	0.28	0.29	bR	47	0.44	0.58	bR
21	0.09	0.04	W	48	0.66	0.31	B
22	0.67	0.72	B	49	0.42	0.73	bR
23	0.68	0.60	WY	50	0.53	0.52	BG
24	0.65	0.44	bR	51	0.25	0.73	Y
25	0.40	0.75	DB	52	0.45	0.79	BG
26	0.53	0.20	W	53	0.86	0.55	B
27	0.72	0.71	DB				

* Y=yellow WY=white yellow B=blue BG=blue green W=white DB=dark brown bR=brick red

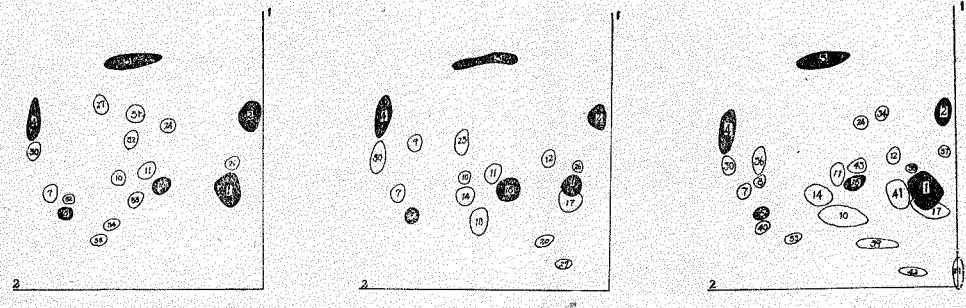
spots can be found. Each species would have as low as 14 and as high as 26 spots. Spots No. 1, 2, 3, 4, 5, and 15 were founs commonly to all these species studied. Spot No. 11 and 18 were common to all the species except one. There were three varieties studied in the cultivated rice *O. sativa*. The number and type of spots were very similar among all them. However, the spots found would vary widely from one another when the species studied were of different genomes and of different ploidy.



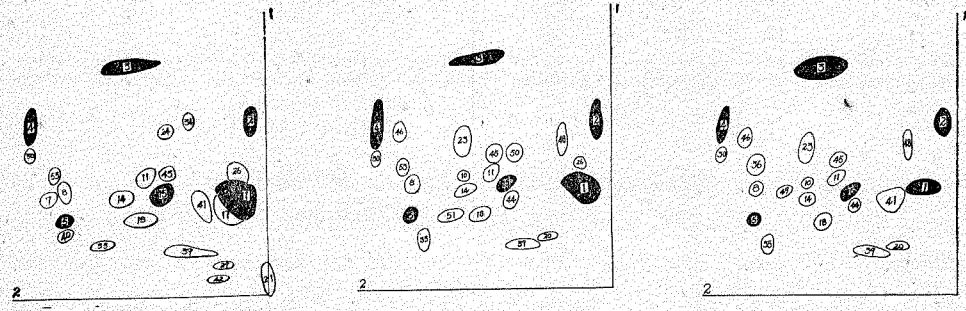
O. sativa (Taichung 65) *O. sativa* (Nanteh) *O. sativa* (Pamifen)



O. glaberrima C7692 *O. breviligulato* Af-17 *O. officinalis* 2004



O. australiensis W008-1 *O. brachyantha* Af-15 *O. minuta* W0016



O. eichingeri W043 *O. latifolia* W1137 *O. grandiglumis* Manaos

Fig. 3. Two dimensional chromatogram of phenolic compounds extracted from rice leaf blades and detected under ultraviolet light with the presence of ammonia vapor.

Table 3. *The spots distribution of 10 different species in Oryza*

Species	Spot Number																																																				Total
	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	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	
O. sativa Taichung 65	×	×	×	×	×	×	×		×	×	×	×	×	×	×	×	×	×	×	×	×																															×		21
O. sativa Pamifen	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×																													25
O. sativa Nanteh	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×		×	×	×																													24
O. glaberrima C7692	×	×	×	×	×					×	×			×	×	×	×	×	×	×	×	×	×			×	×	×	×																							×		22
O. breviligulata Af-17	×	×	×	×	×		×			×	×			×	×	×	×	×	×	×			×			×	×		×																							×		20
O. australiensis W008-1	×	×	×	×	×		×			×	×				×									×		×	×			×	×	×	×	×													×					×		19
O. officinalis 2004	×	×	×	×	×		×	×						×	×			×						×		×										×			×															14
O. brachyantha Af-15	×	×	×	×	×		×			×	×	×		×	×		×	×		×			×			×			×	×																								19
O. latifolia W1173	×	×	×	×	×			×	×	×	×			×	×			×		×			×			×				×					×				×					×	×			×		×	×		×	23
O. grandiglumis Manaos 7	×	×	×	×	×			×		×	×			×	×			×		×			×							×					×	×			×		×			×	×	×		×	×					23
O. eichyngeri W043	×	×	×	×	×		×	×			×			×	×		×	×			×			×		×			×	×			×	×					×	×	×	×	×										×	25
O. minuta W0016	×	×	×	×	×		×	×			×	×		×	×		×	×			×			×		×				×			×	×		×	×	×	×	×	×	×	×											26

用濾紙分離法 (Paper Chromatography) 對稻屬 (*Oryza*) 種間之研究

吳 麟 朱名玉 李先聞

此實驗乃是利用生物化學（Biochemiscal）的方法，做稻屬（*Oryza*）種間關係的比較。自本所細胞遺傳研究室選取十個不同的稻種（Species）其中包括兩個亞種（sub species），六種不同的染色體組（Genome）。

植株自發芽後，在同樣的環境下培養90天將葉片自葉舌的上部剪下，用甲醇（Methyl alcohol）抽出其中的芳香族化合物（Phenolic compound）濃縮後，用濾紙分離法（Paper chromatography）展開，見圖1及圖2，分離的結果顯示出二品種間所含化合物的種類最相似，如南特（Nanth）和白米粉（Pamifan）二品種間只有一個相異的化合物。其次是相同染色體組，較不同染色體組更爲相近，在單相分離的照片上（圖2）可看出含有C染色體組的共同性質。又在雙向分離（Two dimension）圖3上及第三表上發現有六種化合物是十個種間所共有者，由此可表現出稻屬種間之共同性質。

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