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The phyto-serological study on the phylogenic relationship in *Pinus*

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Résumé

This paper reports the phyto-serological study on the phylogenic relationship and others that were investigated by the species-specificity of seed protein in *Pinus*. It consists of five Parts.

Part 1. The phyto-serological species-specificity of seed protein and its application

The serological method consisting in the medical science, its historical background and the possibility of its application were investigated through many sources and the author's earlier preliminary examinations.

The application of the phyto-serological method toward the studies on plant taxonomy was first reported by KOWARSKI in 1901. After his report, from the beginning of the 1900's, phyto-serological research was carried out by many workers rather actively in Europe, America and in Japan, but they soon fell into decay after the 1930's. Such decline is considered to be due to some defects in what is called precipitin test (classic serological technique) which puts antigen upon antiserum.

Under those circumstances, OUDIN (Frenchman) developed originally the new single agar gel diffusion method in 1946. OUCHTERLONY soon developed the double diffusion method in agar gel in 1947. These developments of immunochemical methodology advanced the progress of studies on the phylogenic relationship in plants. In addition to that, T. MATSUI tried to modify the OUCHTERLONY's method in 1959, and developed what is called "T. MATSUI's modified method (modified OUCHTERLONY's)." The development of these gel diffusion techniques made it possible to carry out more precisely the relative quantitative analysis, to investigate the phylogenic relationship of cultivated plants.

Soon, it was confirmed by many workers that his modified method is very suitable for the studies on phylogenic differentiation in cultivated plants.

To make the matter better, the immunologic-electrophoresis was added as a new remarkable technique by GRABAR and WILLIAMS. With these new techniques, it was possible to carry out more clearly the investigation on qualitative and quantitative differences in plant protein.

Under the development of gel diffusion techniques described above, the phyto-serological studies on phylogenic relationship in the several most useful

trees were first reported by MEZ and GOHLKE in 1913, but they were carried out by means of the classic serological techniques (precipitin reaction). We have as yet very little information as to the phyto-serological studies on the phylogenic relationship in the several useful trees, and then the phyto-serological studies by means of an antigen-antibody reaction in these agar-gel double diffusion were not yet discovered, so that the author tried to employ these improved techniques for his purpose.

In the preliminary reports of some of this work, the author found that those new techniques are very convenient for the phyto-serological studies on phylogenic relationship in local pine races in Japan, and then the seed protein obtained from seeds of these material trees is suitable for materials of the serological studies.

Part 2. The phyto-serological studies on the quantitative and qualitative changes of seed protein in the old and the new seeds, the process of germination and the gamma exposure

(1) The study on the seed storage

In this part, the problem of seed storage was taken up; that is, the change of seed protein in both new and old seeds was investigated. It is well known that the chemical change and the consumption in seed take place at the same time while seeds are stored. Germination energy is lost completely as soon as a certain limit is exceeded in this chemical change and consumption. In this study, what sort of component in seed protein mixture is first consumed was investigated.

Material pine used is Modomatsu (local pine race in Japan). This material consists of the following three classes, that is, (1) the oldest seed that was reserved from Autumn in 1963 to May in 1965, (2) newer seed that was reserved from Autumn in 1964 to February in 1965 and (3) the newest seed that was tested at once after the seed gathering in 1965.

These pine seeds were pulverized by a mill, and treated with petroleum—ether to remove oils contained. The defatted seed-powder was extracted with phosphate buffer (pH 7.5, 1/30 M) over-night in the refrigerator and then filtered. This phosphate buffer extract was subjected to reciprocal dialysis with ammonium sulphate over-night in the refrigerator and then dialyzed with the phosphate buffer (used for the extracts) for two days. This dialyzed extract was lyophilized. This dried powder was preserved in an ampoule and then dissolved again in distilled water before gel diffusion. This dissolved extracts were used as the antigen.

Total nitrogen of the liquid protein was estimated by the Micro-Kjeldahl

method, and then the quantity of total protein in each antigen was determined. This content was equalized among the antigens to be compared.

Antiserum was prepared against this dissolved extracts of *pinus Thunbergii* (Modomatsu, the second seed in test materials). Healthy 2 Kg rabbits were used for the preparation of the antiserum. The liquid protein used for the immunization contained 30 mg protein per ml. The method used for the immunization was carried out by adjuvant method of FREUND, that is, by intramuscular injection of "water in oil emulsion." Each animal was given 150 mg total protein, 50 mg protein in the first injection, 50 mg protein in the second injection after two weeks, 50 mg protein in the third injection after four weeks from the second injection. Ten days after the last injection the animals were bled. The blood was allowed to clot and the resultant serum centrifuged to clear up. The serum was inactivated and added 1 per cent sodium azide solution, making up 0.1 per cent concentration, and stored in a refrigerator. This stored serum is antiserum used.

The method employed is an antigen-antibody reaction in agar gel diffusion of T. MATSUI'S modified OUCHTERLONY'S method. According to his technique, the agar gel plate using four basins and arranging these basins in the 4 suitable spaced positions in an agar gel plate was prepared. Agar plates were prepared from 1.5 per cent agar containing 1 g of sodium azide, 0.02 g of methyl orange and 9 g of sodium chloride per liter of medium. Each basin of agar gel has a capacity of about 0.2 ml. The experiments were carried out by charging each of the lower two basins with 0.2 ml of the same antiserum, and one of the upper two ones was filled with 0.2 ml of the antigen solution and the another with 0.2 ml of antigen to be compared. The antigens were adjusted to their respective concentration based upon the protein content measured by the Micro-Kjeldahl method.

All the charged plates were incubated at 37° C. Antigens and their corresponding antibodies diffuse towards each other in a gel and react by forming sharply defined precipitate lines. The reaction patterns were examined after 19 days and permitted detection of minimum number of reacting components. T. MATSUI distinguished these precipitate lines to the 4 kinds of category; that is, reactions of perfect identity, imperfect identity, non-identity and partial identity. Then, he indicated that the different species contained a antigenic substance which developed thick major precipitate lines (as the major component) not in common, and some antigenic substances which gave rise to minor precipitate lines (as the minor component) in common, and, in addition, different varieties within a species contained a certain common major component and some minor ones in common but in varied quantities. He successfully discerned between the intra-specific and the inter-specific

differences and also differentiated one species from another, and similarly one variety from another.

The reaction patterns in agar gel in the present study are shown in Fig. 4. The upper left basin was filled with the Modomatsu (the second material) antigen and the upper right one was filled with antigens of each stage in seed storage. The lower two basins were filled with the anti- "Modomatsu" serum. All reagents were added to 0.2 ml. In the intervening space between the upper two antigen basins and the lower two antiserum basins, the thick major lines and various minor lines developed. And moreover, the lines were connected straightly showing a typical pattern of a reaction of identity on the one hand, and were connected in a curve on the other hand representing a reaction pattern of an imperfect identity.

With the identification of these reaction patterns, the following results were found, namely, the quantity of a component corresponds to the minor line comes to decline in the first stage of seed storage, and that of a component to the major line comes to decline in the second stage.

(2) The study on the process of germination

The study in this Part was conducted to investigate the changes of seed protein in the process of germination. Test material is the seed of Modomatsu. The seeds were sown and then collected again little by little at the 2nd, the 4th and the 8th days after the sowing. These collected materials were used for the preparation of liquid protein. The details of the preparation method were already mentioned above. But on the 8th day, the development of cotyledon was found already, so that the liquid antigen of this material was prepared by the following process. Materials were cleared by running water at once, and then frozen. The frozen materials were pulverized by a mill, and extracted with phosphate buffer (pH 7.5 1/30 M) and filtered. This phosphate buffer extract was centrifuged (25000 g). The supernatant was frogen and lyophylized. This dried powder was used for the preparation of antigen.

The phosphate buffer extracts of each treatment was used as the antigen. Anti- "Modomatsu" serum used in serological study described above was used too in this study. The method employed is T. MATSUI'S modified method.

The antigen-antibody reaction patterns in agar gel with these antigens and antisera are shown in Fig. 5. The upper left basin was filled with the Modomatsu (no treatment) antigen and the upper right one was filled with antigens from the 2nd, the 4th or the 8th days after the sowing. The two lower basins were filled with the anti- "Modomatsu" serum. All reagents were added to 0.2 ml.

The experimental results were the following. The control antigen and the antigen from the 2nd day after the sowing hold the major components in

common, and hold too some minor components in common but in different quantities. As compared with the control antigen, some minor componet of antigen from the 2nd day lessened quantitatively, while others increased. The control antigen and the antigen from the 4th day were the same as the pattern between the control antigen and the antigen from the 2nd day. The control antigen and the antigen from the 8th day did not hold some major components and the minor components in common, but it was found that there were some different minor lines in the intervening space between the antigen from the 8th day and the anti- "Modomatsu" serum.

These results may be taken to indicate that the albumin protein is resolved by enzyme as a result of water absorption when water is given to seeds, but the order is as follows; a component corresponding to a minor line is first resolved, a component of a major line is secondly resolved, and then they are absorbed as nutrient, while in embryo protein, a component corresponding to minor line is increased in opposition.

(3) The study on the gamma exposure to seeds

When the seed was exposed itself to radiation, the germination rate falls. This phenomenon was investigated by means of serological studies. The material tree investigated in this study is Modomatsu as the type of *Pinus Thunbergii*. Five kinds of gamma exposure 30 KR, 18 KR, 12 KR, 6 KR and 2 KR were carried out against the dormant seeds.

The serological method employed is T. MATSUI's modified method. The liquid antigen was prepared by the same process as described above. Antiserum was prepared against no treatment seed.

The reaction patterns in agar gel with these antigens and antiserum are shown in Fig. 7. The qualitative and quantitative changes of a component in each radial treatment were investigated by means of the identification method of reaction patterns described above. With the analytical results of these reaction patterns and the immunologic-electrophoresis patterns, the following facts were pointed out. In 2 KR and 6 KR treatments, it was not possible to find qualitative and quantitatve changes of the protein, as compared with control. In 12 KR and 18 KR treatments, the antigens hold major component in common, but some peculiar minor components came to increase quantitatively to a remarkable degree. In the next 30 KR treatment, the pattern in minor components came to be similar to non-treatment (control), while major components came find a certain quantitative change.

With these results, the author found that there is a contrary correlation between the quantitative increase of a peculiar component corresponding to the minor line and the germination rate of material seeds in each radial treatment.

Part 3. The study on the inter-specific relationship in *Pinus*

(1) The study on species hybrid

The material trees used for this work are *Pinus rigida*, *P. rigitaeda* (F_1), and *P. taeda* was obtained by crossing *P. rigida* (♀) and *P. taeda* (♂) in Korea. The first filial generation in this crossing holds in common the cold resistance of *P. rigida* and the excellent growth of *P. taeda*. The author investigated the specificity of heterosis by means of species-specificity of seed protein of this F_1 tree, and then was able to distinguish this F_1 tree from the parents with the qualitative and the quantitative differences in a component of the seed protein.

The details of the material seeds are the following;

P. rigitaeda: Osan, Kyunggido, Korea (locality)
1965 (seed year)
10 (mother tree's age)

P. taeda : South Carolina, U.S.A. (locality)
1964 (seed year)

P. rigida : Osan, Kyunggido, Korea (locality)
1965 (seed year)
10 (mother tree's age)

The method employed is T. MATSUI's modified method. These liquid antigen was prepared by the same process as described above. Antiserum was prepared against the seeds of *P. rigida*. The reaction patterns in gel by these antigens and antiserum is shown in Fig. 11.

With analysis by the reaction patterns between anti- "*P. rigida*" serum and *P. rigida*, *P. rigitaeda* and *P. taeda* antigens, and immunologic-electrophoresis patterns, the following results were obtained. In *P. rigitaeda*, the protein components correspond to some minor line is increased quantitatively as compared with *P. rigida*. It seems that the quantitative increase of this components means the specificity of heterosis. The components corresponding to the major line produce the pattern that resembles closely *P. taeda* but differs from *P. rigida*. With these results, the author found that *P. rigitaeda* resembles comparatively *P. taeda* in seed protein, and then inherits relatively the character of the father's side.

(2) The study on the inter-specific relationship in *Pinus*

The experiment in this Part was carried out to investigate inter-specific differentiation. The material species used for this study are many foreign produced pines and others, that is, *P. luchuensis*, *P. nigra*, *P. caribaea*, *P. Thunbergii*, *P. densiflora*, *P. sylvestris*, *P. pinaster*, *P. Banksiana*, *P. ponderosa*, *P. taeda*, *P. rigida*, *P. koraiensis*, *P. monticola*, *P. Strobus*, *P. pumila* and *P parviflora*. The employed method is T. MATSUI's modified method.

Antiserum was prepared against the seeds of the following species: *P. Thunbergii* (Modomatsu, local race in Japan), *P. densiflora* (Samuraihamamatsu, local race in Japan), *P. sylvestris*, *P. Banksiana*, *P. ponderosa*, *P. rigida*, *P. monticola* and *P. parviflora*.

The reaction patterns by these antigen and antisera are shown in Fig. 14-20. The phylogenic relationship in *Pinus* was investigated. Analytical results of these reaction patterns are shown in Table 1. With the analytical results of these reaction patterns and Table 2-8, it was pointed out that the phyto-serological relationship are in accordance with the morphologically classified methods by W. DALLIMORE and A. B. JACKSON, and W. M. HARLOW. Especially, they were good in accordance with the classified method that is divided into two classes; soft pine and hard pine.

With these results, the author knew that phylogenic relationship by phyto-serological reseach is related remarkably to the wood's quality. Classification with the number of needle leaf was proved serologically too, but there is such an exception as *Pinus Banksiana*. Correlation between the serological relationship and the grafting affinity was able to fined to some extent.

Part 4. The study on the inter-specific differentiation of Japanese *Pinus-Diploxylon*

(1) The phylogenic relationship among *Pinus densiflora*, *P. densi-Thunbergii* and *P. Thunbergii*

The material tree seeds investigated in the present work are *P. Thunbergii*, *P. densi-Thunbergii* and *P-densiflora*. They were gathered every tree in whole place of Shimabara peninsula in autumn 1963.

In this work, the correlation between the species-specificity of these protein and the morphological character was investigated. The differentiations by means of morphological characteristics in these mother trees given in the examples in Table 9.

The liquid antigens in these material seed were prepared in the same way with the study described above. Antiserum was prepared against the phosphate buffer extracts of seeds of *P. Thunbergii* (Modomatsu, local pine race) and *P. densiflora* (Samuraihamamatsu, local pine race).

The reaction patterns with these antigen and antiserum are shown in Fig. 24-27. No. 1-3 patterns in Fig. 24 are reactions against anti- "Modomatsu" serum, No. 1-3 patterns in Fig. 26 are reactions against anti- "Samuraihamamatsu" serum. In Fig. 24, one spur was found in pattern between Modomatsu antigen and *P. densi-Thunbergii* antigen, while two spurs were found in pattern between Modomatsu antigen and *P. densiflora* antigen. In Fig. 26, each pattern was not able to find the spur in any way.

Applying 4 kinds of categories of identification described above, the author succeeded in the distinction on phylogenic relationship in these pines, and found that the reaction patterns against *P. Thunbergii* was relative strongly to the morphological relationship, the patterns against the *P. densiflora* was not relative to these.

For that reason, the author made it a rule to employ the reaction patterns against rather *P. Thunbergii* than *P. densiflora* to investigate the phyto-serological relationship in *Pinus*.

(2) Serological studies on the qualitative and quantitative differences in a certain component of the seed protein of the same intraspecific trees growing in different habitats

In this Part, whether plants growing in vertically or horizontally different habitats have or not the qualitative and quantitative difference each other on a component of the seed protein was investigated. Plants come to have such organization, function and habitat as consistent with their environment by some style against the difference and the change of its environment. In these adaptations, there is the following; for one thing there occurs a change of genes which can be inherited, for another there is change of phenotype which is not always inherited.

The material seeds investigated in this work are pine seeds named Miyamaakamatsu that were introduced by S. MURAI and were gathered from 35 KA stand (one hundred and eighty meters above the sea level) in national forest within the jurisdiction of Mutsu-Kawauchi Forestry Office. This pine is growing in high latitude and thousand meter regions above the sea level of the main land in Japan.

The morphological examinations were 2 marks by Miyamaakamatsu, 5 marks by Samuraihamamatsu, 4 marks by Tozanmatsu and 23 marks by Modomatsu. These pines are *Pinus-Diploxylon* in Japan,

The liquid antigen was prepared by the same process as described above. Because of scanty material seeds, antiserum for the Miyamaakamatsu was not able to obtain. And so, anti- "Modomatsu," anti- "Samuraihamamatsu" sera obtained in the study described above were in use even on this work.

The reaction patterns by these antigen and antisera are shown in Fig. 28. A reaction pattern between the Modomatsu and Miyamaakamatsu holds the major line in common, and holds the minor line in common but in different quantity or quality.

With these reaction patterns for the anti- "Modomatsu" serum, it was found that the reaction patterns of this Miyamaakamatsu resemble strongly the reaction patterns of Hiyugamatsu and Tozanmatsu. On the other hand, the morphological marks of Miyamaakamatsu differ from that of Tozanmatsu or

Hiyugamatsu. It seems therefore that this serological relationship are not in agreement with this morphological analysis, but it seems impossible to tell you a conclusion at a single research.

Part 5. The study on relationship in local races of Japanese *Pinus-Diploxylon*

From this series of serological experiments, it may be concluded that there is a certain correlation between the relationship of serological species-specificity of seed protein in *Pinus* and the morphological characteristics. And then, in this Part, relationship in local races of *Pinus-Diploxylon* in Japan was investigated.

(1) The first study

The materials used for this study are many local races (local pine races in Japan, *Pinus-Diploxylon*) of *P. Thunbergii* or *P. densiflora*, that is, Modomatsu, Hiyugamatsu, Shirahatamatsu, Nameramatsu, Samuraihamamatsu, and so on.

Antigens were prepared the same way as described above. Antiserum was prepared against the phosphate buffer extracts of seeds of the following local races, Modomatsu as the typical tree of *P. Thunbergii* and Samaraihamamatsu as the one of *P. densiflora*. The method employed is the T. MATSUI's modified Ouchterlony's method. The method used for the immunization was carried out by means of the complete adjuvant method of FREUND.

The reaction patterns are shown in Fig. 30-31. The analytical results are shown in Table 13. With this results, it was pointed out that anti- "*P. Thunbergii*" serum reacts strongly with Modomatsu, Hiyugamatsu, and Tozanmatsu seed-extracts, less strongly with Samuraihamamatsu and Nameramatsu seed-extracts, and moreover these results are in accordance with the morphological relationship.

In view of these facts, is seems that phyto-serological phylogenic relationship by means of species—specificity of seed protein has the correlation strongly to the morphological characteristics, that is, the localities of resin canal and sub- "resin canal," the number of hypodermis layer, the characteristics of needle, bark and terminal bud.

(2) The second study

The materials used for this study are such many local races in Japan as described above and *Pinus Thunbergii* (Fukuoka, locality), *P. Thunbergii* (Sakata, locality), *P. densiflora* (Omagari, locality), and *P. koraiensis*.

The liquid antigens were prepared by the following process. Seeds were pulverized by a mill, and treated with ethyl-ethel to remove oils contained.

The defatted seed-powders were extracted with 0.9 per cent sodium chloride solution over-night in refrigerator and then filtered. These saline extracts were dialyzed with the saline solution for 72 hours. These dialyzed saline extracts were used as the antigen. Antigens were newly prepared as injections and tests.

Antiserum was prepared against the saline extracts of seeds of *P. Thunbergii* (Sakata, locality) and *P. densiflora* (Omagari, locality).

Each test animal was given 2 intraveneous injections every week in the ear. During four weeks the saline extract was injected, and seven days after last injection the animals were bled. The blood was treated in the common way, and then antiserum was obtained.

The reaction patterns with these antigens and antiserum are shown in Fig. 32–33.

Analytical results are shown in Table 15.

With these results, it was found that the phylogenic relationship was good in accordance with results in the first study described above, and the reaction patterns in this second study were suitable to investigate the phylogenic relationship in local races in Japan than the first study.

With these facts, it seems that the antiserum obtained by means of injections to venous blood in the ear is advantageaous for studying the phylogenic relationship in the closed related plants.

From this series of serological experiments, it may be concluded that the serological method belonging to the territory of medical science is a very useful means for the various studies in forestry.