

CLASSIFICATION OF THE KOJI MOLD

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A taxonomical study of the *Aspergillus* was made centering on 403 strains of koji mold, with some other molds as reference. Morphological, physiological, and cultural characters were compared, and 20 mycological characters of each strain were submitted to component analysis by a computer, and the following characters were selected as the most significant key for taxonomical differentiation: Seriation of sterigmata, roughness of conidial walls, color of old cultures, diameter of conidia, and pink coloration of conidia in the anisaldehyde medium.

In addition, the strains RIB 430 and Thom No. 113 were found to be the original isolate of AHLBURG and COHN; the strain Thom No. 108 was proved to be *A. oryzae*, and *A. sojae* SAKAGUCHI et YAMADA was definitely discriminated from *A. parasiticus* SPEARE.

As a result, all the strains were proved to belong to two different clusters by the computer analysis, one being the *A. oryzae* group and the other, *A. flavus* group. The koji molds were placed in *A. oryzae* group including *A. sojae* SAKAGUCHI et YAMADA, *A. tamarii* KITA, *A. oryzae* (Ahlburg) COHN, *A. oryzae* var. *viride*, var. nov., and *A. oryzae* var. *brunneus*, var. nov., and the strains other than koji mold were placed in *A. flavus* group including *A. flavus* LINK, *A. parasiticus* SPEARE, and *A. toxicarius*, sp. nov.

I. HISTORICAL REVIEW

Among the innumerable kinds of fungus found in nature, some *Aspergilli* used widely for the manufacture of Japanese food, such as *Saké*, *Miso*, *Shoyu*, *Mirin*, *Amazaké**, and so on, have long been called koji mold.

* *Saké*=An alcoholic beverage made by the saccharification of rice by mold and the fermentation by yeast.

Miso=A paste made by grinding a mixture of rice koji, cooked soybeans, and salt, and fermenting in brine. Used in preparing soups and other foods.

Shoyu=The so-called 'soy sauce.' Made by subjecting koji of soybeans and roasted wheat to long fermentation and then to long digestion in brine.

Mirin=A sweet, alcoholic seasoning for food. Made by standing steamed rice, rice koji, and *Shochu* (kind of distilled liquor). Contains 13-22% alcohol and 25-38% sugar.

Amazaké=Sweet beverage made by standing a mixture of boiled rice and rice koji at 60° overnight.

AHLBURG (1), in 1876, isolated for the first time a yellow-white mold from koji for *Saké* making which was first called *Eurotium oryzae* AHLBURG though it was renamed *Aspergillus oryzae* (Ahlburg) COHN by COHN (2) in 1884, owing to its non-production of perithecia. From then, many strains have come to be isolated by many workers from kojis of Japan and they were called *A. oryzae* as a whole. Although *A. oryzae* was described in detail by WEHMER (3) in 1895, it was the beginning of 20th Century that the koji mold was found to contain multiple kinds of microbe when TAKAHASHI (4) in 1909 isolated many different molds from koji used for the manufacture of *Saké*, *Miso*, and *Shoyu*, and found that there are varieties of *A. oryzae*.

THOM and CHURCH (5, 6), in 1921, studied TAKAHASHI's strains named alphabetically and placed them in *A. flavus-oryzae* series because they showed some intermediate forms between strain F resembling *A. oryzae* of WEHMER (Thom No. 113) and strain C resembling *A. flavus* of BREFELD and WEHMER (Thom No. 108).

Indistinguishability of *A. oryzae* from *A. flavus* has sometimes been discussed by BLOCHWITZ (7) and SAITO (8) from the point of natural variation of *A. flavus* by long-term successive cultivation in rice. SAKAGUCHI (9) found in 1933 some strains showing echinulate conidia in addition to smooth conidiophores which do not belong to *A. flavus-oryzae* series of THOM and CHURCH, and identified them with *A. sojae* SAKAGUCHI et YAMADA in 1944 (10). OHTANI (11), in 1937, classified the koji molds from morphology and physiology; THOM and RAPER (12), in 1945, discriminated *A. oryzae* of mostly uniseriate sterigmata from *A. flavus* of mostly biseriate sterigmata; OHARA (13), in 1952, used as a key the productivity of kojic acid from different carbohydrates; NEHIRA (14), in 1957, regarded *A. oryzae* and *A. sojae* as a variety of *A. flavus* and a form of *A. parasiticus*, respectively, from their enzyme activity; and RAPER and FENNELL (15), in 1965, placed *A. flavus*, *A. oryzae*, *A. parasiticus*, and *A. tamarii* in the *A. flavus* group adding 5 new species. The present author (16, 17), in 1959, identified the koji mold for *Saké* making with *A. oryzae* var. *globosus* SAKAGUCHI et YAMADA, and, in 1966, identified an aflatoxin-producing strain, *A. flavus* ATCC 15517, with *A. parasiticus* var. *globosus*.

The present author considers, in surveying taxonomic studies made to date on koji mold, that it is of major importance to study the two original isolates of AHLBURG and LINK, and to clarify which of mycological characteristics is the most significant for taxonomy of koji molds.

II. MATERIALS AND METHODS

Molds studied. Over six hundred strains of *Aspergilli* were studied, including 406 strains isolated from koji, 68 strains received from abroad, and 185 strains of artificial mutants of the koji molds and other molds.

Culture medium and temperature. The molds were usually incubated in Czapek's agar at 24° for morphology and 34° for physiology.

Slant cultures. For the determination of color of conidial heads in old and pigment productivity, the molds were cultured in the slant of Czapek agar at 30° for 1 month, and then observed by the naked eye.

Colonies. For the determination of colony diameter, wrinkling on the reverse side, and some physiological activities, giant colonies were incubated at 34° for 7 days. When some sectors were found in the colony, the strain was purified because the sector was produced by either variation of a strain or contamination with a different strain (Plate 1).

Microstructures. The microstructure of molds on the slide glass varies considerably even in the same culture and the microstructure of a strain was determined by observations on as large a microorganism as possible, excluding that deformed. Seriation of sterigmata was observed by addition of lactophenol solution (6) and roughness of conidial walls was observed on the strain incubated for 3 weeks.

Physiological characters of colonies. Several pieces were collected from a colony incubated at 34° by piercing with a cork borer of 12 mm in diameter, including the medium (Plate 2). Amylolytic activity of a strain was determined by the usual method of adding water extract of a piece of the colony in starch solution, and activity of reducing Methylene Blue was determined by keeping a piece in 3 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.0001% Methylene Blue at 30°, followed by observation of the decolorization time of the solution. The remaining colony was sprayed 5 ml of 1% aqueous solution of hydroquinone, kept at 25° for 1–2 days, and the activity to colorize hydroquinone was determined by observation of the pale to dark red color around the colony (Plate 3).

Pink coloration of conidia by anisaldehyde. The molds were incubated in the Czapek agar slant containing 0.05% anisaldehyde (*p*-methoxybenzaldehyde) at 30°, and pink coloration of conidia was observed daily for 1 month.

Kojic acid production. In each of 2 test tubes, 10 ml of the rice-koji extract (Ballg. 6–8) was placed, and the mold was incubated stationary at 30° for 10 and 20 days. After removing the mold, 1 ml each of 1% FeCl₃ solution was added to the medium and the appearance of red color was taken as the production of kojic acid (Plate 4).

Aflatoxin productivity. The molds were incubated with shaking in a medium containing a trace of heavy metals (18) and/or with polished rice in a flask (19, 20). Aflatoxins were extracted with CHCl₃ and purified by repeated precipitation by hexane and dissolution into CHCl₃, and then subjected to thin-layer chromatography, or to determination of UV absorption spectra.

Browning of rice koji and production of deferriferrichromes. Ten grams of steamed rice was placed in a 100-ml flask, inoculated with the mold, and incubated at 34° for 40 to 42 hr. The content was soaked in 50 ml of water and filtered. Color of rice grains on the filter paper sometimes turned brown

gradually within a few days at room temperature (Plate 5, 6). Colorless deferriferrichrysine in the filtrate was determined by colorimetry after addition of FeCl_3 solution (21) by which deferriferrichrysine quickly changed to red ferrichrysine (Plate 7).

III. RESULTS OF OBSERVATION

Color of old cultures. The color of conidial heads changed with time from yellow, to yellow green, and green or olive in young cultures; and shifted day by day to brown, brown with green shades, and sometimes kept green and rarely white even in old cultures, which were found to be stable and characteristic to the strain. Thus, the color of molds was divided into two; green or green with brown shades, and brown without any green shades (including white). As previously demonstrated by SAKAGUCHI *et al.* (22), whether the colors show green or not seems to be one of the most fundamental characters of the koji molds.

Colonies. The koji molds usually had larger diameter when incubated at 34° than at 24° , and wrinkles on the reverse side and sclerotia were also produced abundantly at 34° .

Conidiophores. The roughened appearance of walls was neither pitted nor spiny, rather warty with many small processes of 0.08 to 0.24μ in height (23). The smooth walls were sometimes found in koji mold but none in the species *A. flavus*, and smooth walls seemed to change to rough walls by artificial mutation (24).

Conidial heads. Shape and size of heads were characteristic to some strains such as *A. sojae*, *A. tamarii*, and *A. parasiticus* (non-koji mold), showing loosely to irregularly radiate with chains divergent or adhering in loose thin columns. However, most of the koji mold showed the heads consisting of a mixture of different shapes and sizes (Plate 8).

Sterigmata. Ratio of the biseriate heads (heads including biseriate sterigmata) in the total heads of a strain ranged from zero to 20–30% in the

Plate 1. Segregation of sectors from the colony.

A. oryzae var. *brunneus* RIB 301 was incubated at 34° for 1 week. Left: Irregularly produced sectors by contamination with a different strain. Right: Regularly produced sectors by probable variation or that in progress.

Plate 2. Colony with several pieces removed with a cork borer.

Plate 3. Coloration of phenols.

Left: Dark red in hydroquinone and dark brown in pyrogallol.

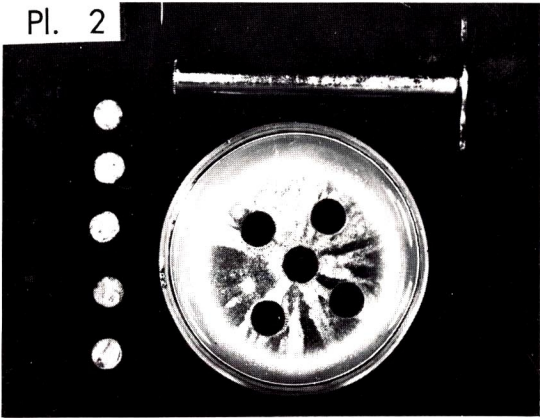
Middle: Pale red in hydroquinone and brown in pyrogallol.

Right: Czapek's agar without incubation. No coloring in hydroquinone, and pale brown in pyrogallol by its autoxidation.

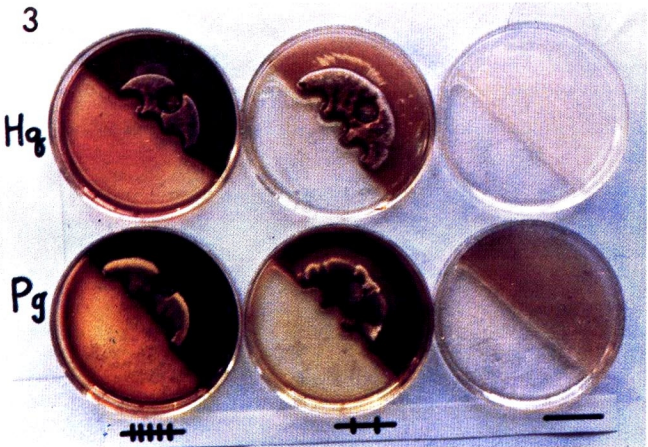
Pl. 1



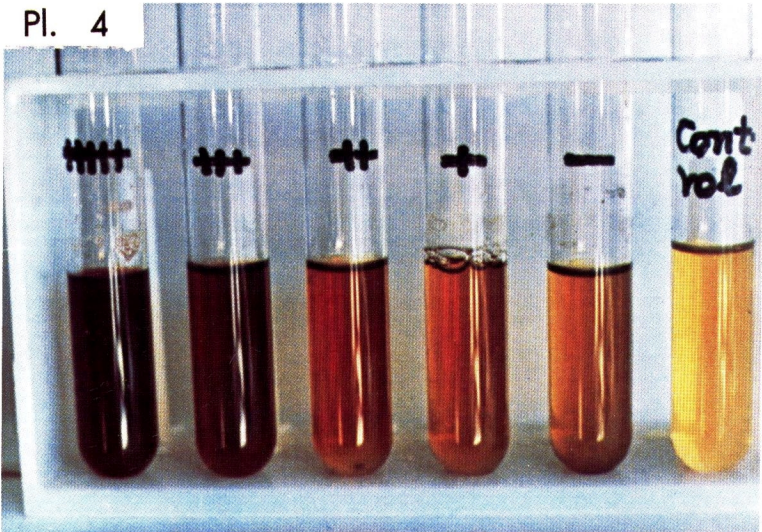
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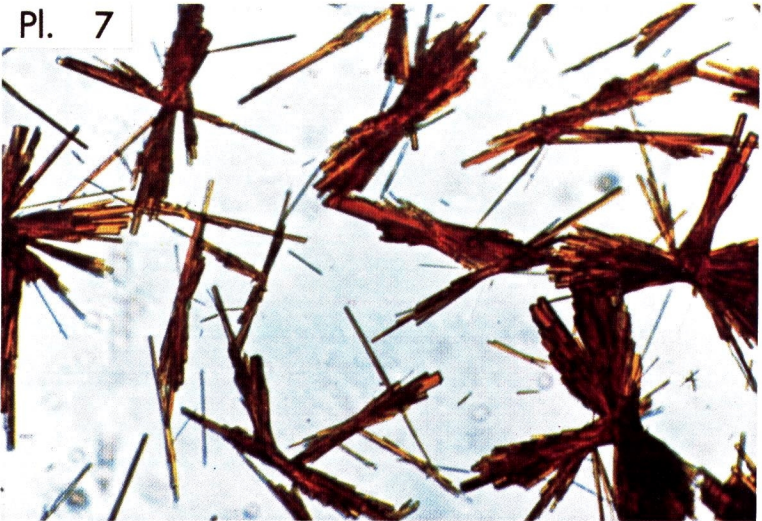
Pl. 3



Pl. 4



Pl. 7



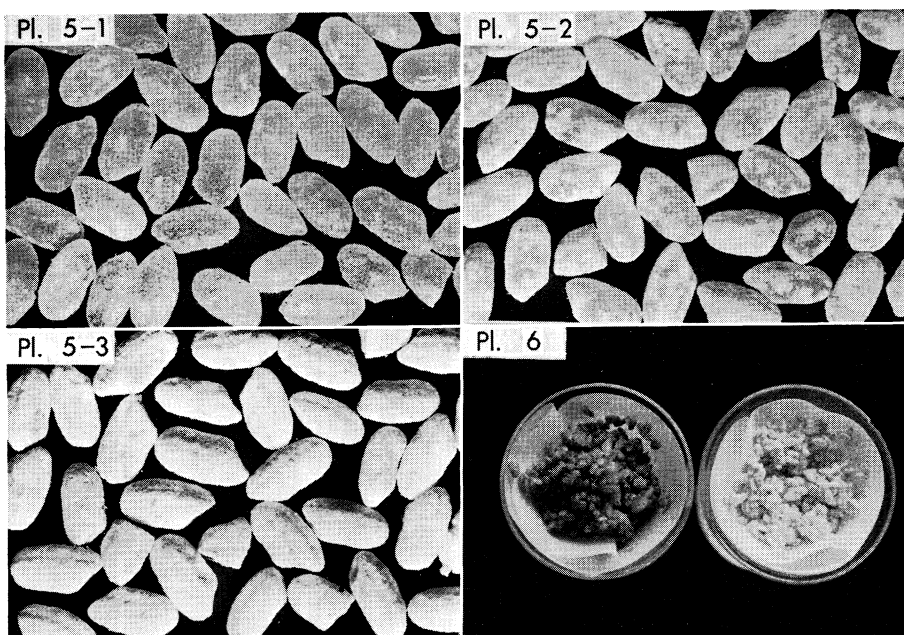


Plate 5. Change in appearance of rice grains during the koji making.

1. Growth of the mold negligible 10 hr after the inoculation.
2. Rice grains become white in localized area where the mycelia invade the grain tissue, 30 hr after the inoculation.
3. *Saké*-koji completed 40 hr after the inoculation, rice grains being covered with mycelial growth with further invasion of the tissue.

(Photograph by Dr. Teizo Inoue, engineer of *Saké* making in the city of Nishinomiya, Hyogo-ken, Japan)

Plate 6. Browning of rice koji.

White rice koji was soaked in water for a few hours, water removed by filtration, and kept at room temperature for a few days.

Left: Rice koji made by *A. oryzae* var. *viride* RIB 128 turned brown to sometimes black.

Right: Rice koji made by *A. oryzae* var. *brunneus* RIB 647 remained white.

Plate 4. Reaction of kojic acid by addition of FeCl_3 solution.

Production of kojic acid is characterized by appearance of specific red color in culture broth, and this production is parallel to the depth of color.

Plate 7. Ferrichrysine obtained from koji and *Saké*.

Colorless deferriferrichrysine easily changed to red and crystalline ferrichrysine.
(Photograph by Dr. M. Tadenuma and Dr. S. Sato, Research Institute of Brewing)

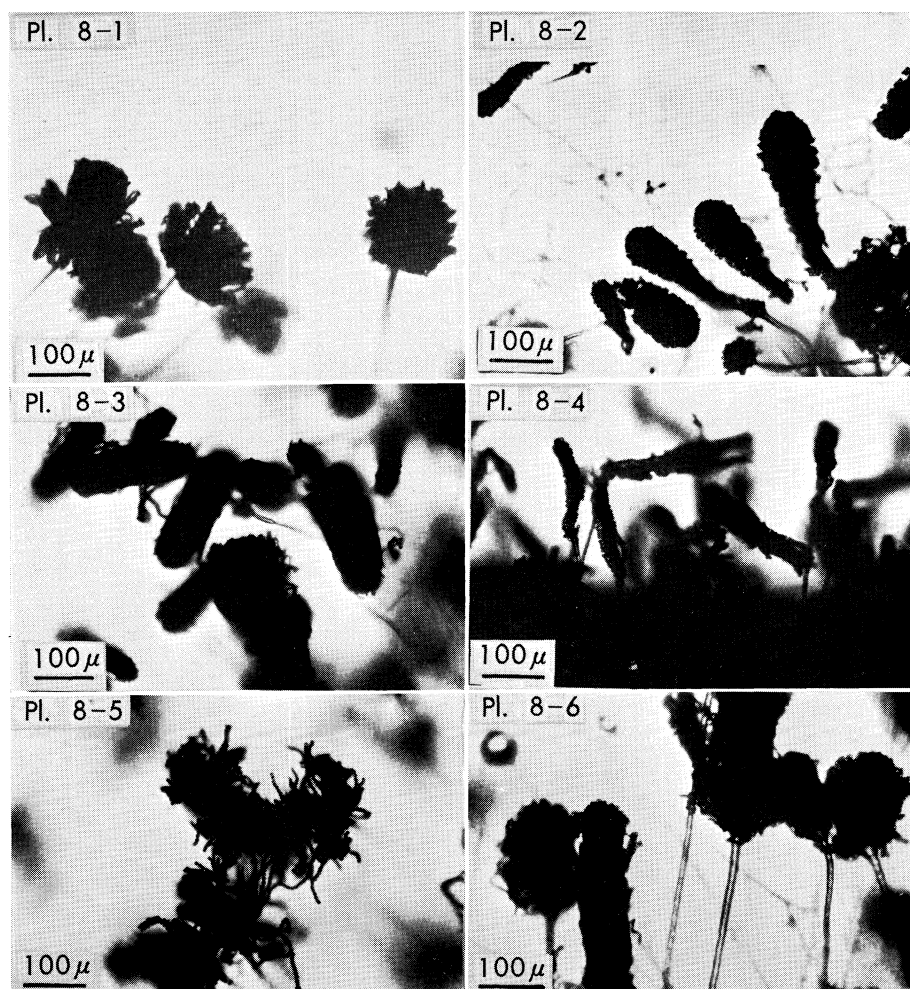


Plate 8. Various types of conidial heads.

1. *A. oryzae* var. *viride* RIB 207, radiate to globose.
2. *A. oryzae* RIB 537, broom like.
3. *A. oryzae* var. *viride* RIB 1133, broom-like.
4. *A. oryzae* var. *viride* RIB 402, columnar.
5. *A. sojae* RIB 1185, loosely to irregularly radiate.
6. *A. oryzae* var. *viride* RIB 1312, mixture of different type of head in the same culture.

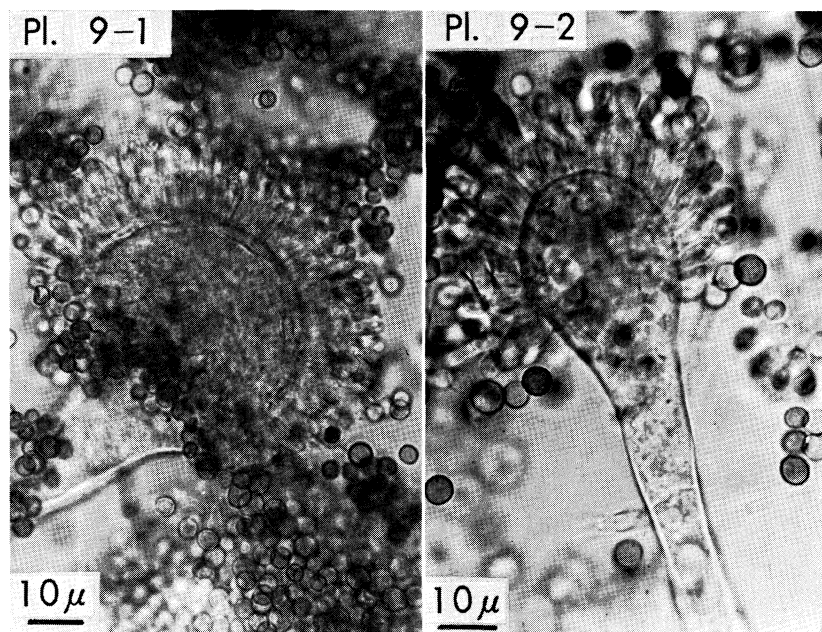


Plate 9. Biseriate sterigmata in large and small heads.

1. *A. oryzae* RIB 609, abundant sterigmata in a large head.
2. *A. oryzae* RIB 537, small number of sterigmata with predominance of uniseriate sterigmata in the small head.

koji mold. Strains having the biseriate heads more than several percent usually showed some common physiological characters such as weak amyolytic activity and much production of kojic acid. Therefore, the biseriate strain (strains having biseriate sterigmata) was regarded as that having biseriate heads of more than several percent (Plate 9).

Conidia. Although small and even-sized conidia are very stable, shape and size of conidia usually varied from strain to strain and with culture conditions, and also changed by natural or artificial mutation (24, 25). In contrast, the roughness of conidial walls was found to be very stable which was classified as tuberculate, echinulate, and rough, by phase-contrast microscope of 1,000 magnifications (Plate 10).

Pink coloration of conidia. The conidia of koji molds usually began to color pink within a few days after the inoculation, became deep pink for 1 week, and the color gradually faded to the original color of their own, though some kinds of mold did not color pink throughout the incubation (Plate 11). KUROSAWA (26) considered this coloration to be worthy for discrimination of *A. oryzae* and *A. flavus*, and the present author found that the strains having both echinulate conidia and uniseriate sterigmata can be divided into

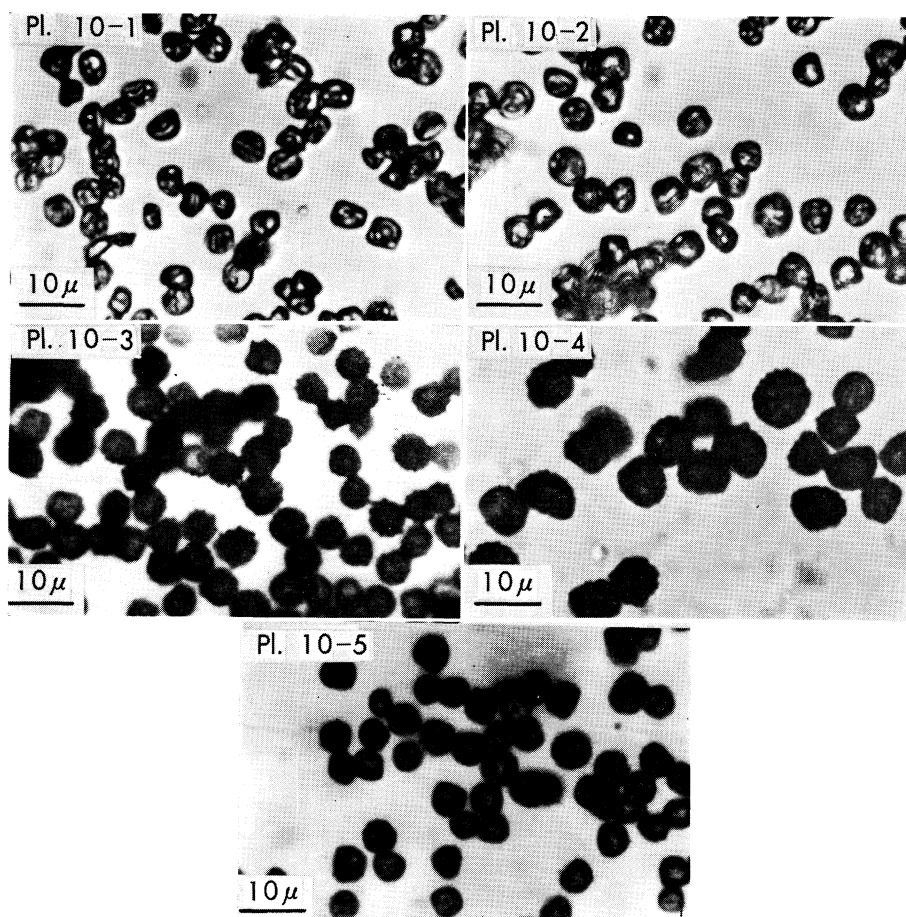


Plate 10. Character of conidial walls.

1. *A. oryzae* (Ahlburg) COHN WB 447 (Thom No. 113=RIB 1031), delicately rough.
2. *A. oryzae* RIB 1032(= *A. flavus* Link WB 482, Thom No. 108), delicately rough.
3. *A. sojae* RIB 1043, echinulate.
4. *A. tamarii* RIB 5002 (= *A. oryzae* var. *fulvus* IAM 2008), tuberculate.
5. *A. oryzae* var. *brunneus* RIB 1197, mixture of rough and some echinulate conidia in the same culture.

two different species from this coloration of conidia.

Enzyme activity. The amylolytic activity and the activity to reduce Methylene Blue seemed to be low and high, respectively, in the species *A. flavus* (14, 27, 28). The coloration of hydroquinone in the colony was the

most easily detected character of the molds, but the relation between this coloration and other mycological characters is still uncertain.

Kojic acid. Productivity of kojic acid sometimes depends on the kind of medium used, therefore, the rice-koji extract (Ballg. 6–8) or the filtrate of rice hydrolysate by Takadiastase (Ballg. 10) was used (29). The strain that produced kojic acid usually browned the rice koji, and produced *Saké* and *Saké* cake with deep yellow to brown and black, respectively.

Aflatoxins. Although many kinds of fluorescent substance were found in Japanese microbial products such as flavines (30), pterins (31), pyrazines (32), isocoumarins (33), ferulic acid, and harman (34, 35), aflatoxins have never been found and none of the koji molds examined were found to produce them.

Browning of rice koji. The browning absolutely needed the presence of air and proceeded rapidly even in temperatures lower than 10°, and the precursor of the brown pigments was found to be mainly L-DOPA (36). Over 90% of koji molds turned rice-koji brown while 50% of *A. flavus* did not.

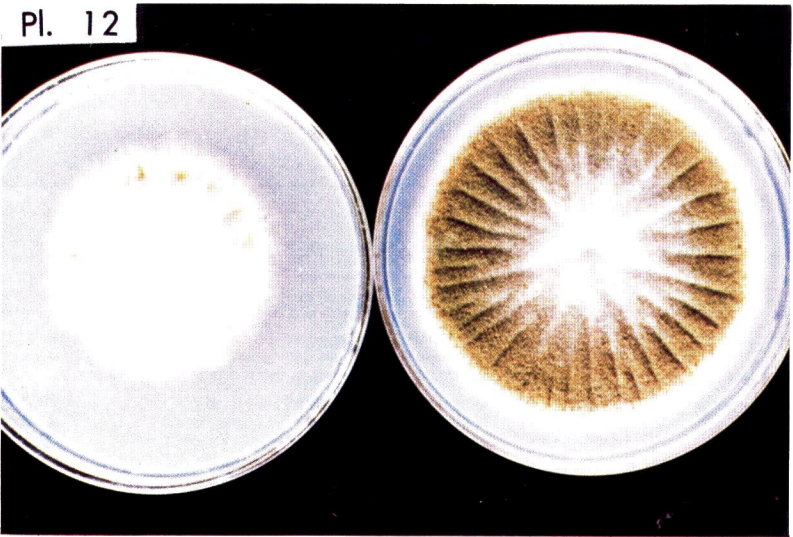
Deferriferrichrysine. In order to incorporate iron with respiratory enzymes during growth, molds seem to produce deferriferrichrysine. The amount of this substance in rice koji varied from 30 to 300 ppm with strain and culture conditions of koji, though the strains with echinulate conidia showed the smallest production (37, 38).

IV. A COMMENT ON *ASPERGILLUS ORYZAE* (AHLBURG) COHN

From the long history of classification of koji molds, it seems of importance to definitely discriminate the original isolates of AHLBURG-COHN's *A. oryzae* and LINK's *A. flavus*. The author has studied a strain WB (=NRRL) 447 (Thom No. 113, the author's RIB 1031) and a strain WB 482 (Thom No. 108, the author's RIB 1032) as respective representatives. Both were received from Prof. Kenneth B. Raper of the University of Wisconsin, in March 1966. However, these strains could not be discriminated by general morphology such as color of old cultures, seriation of sterigmata, and diameter of conidia which have long been taken as the significant discriminating characters. They were discriminated only from their physiology such as non-production of kojic acid in the rice-koji extract and browning of rice koji by *A. oryzae* but not by *A. flavus*.

WEHMER's *A. oryzae* (Thom No. 113) has long been used as an original strain, which had been isolated from koji sent from O. Kellner, Professor of the University of Tokyo in 1894, sent to the Centraalbureau voor Schimmelfcultures (CBS) at Baarn through Dr. Went in 1907, and then sent to THOM through Dr. Westerdijk of the CBS in 1909.

The strain RIB 430 had been marketed as *Tané*-koji (moldy starter for koji making) as long ago as 1910 from the factory of Shichiro FUKUDA in Tokyo, which was succeeded by the Nippon Jozo Kogyo Co., Ltd. of Tokyo,



in 1917, followed by extensive saling, and sent to the Research Institute of Brewing in 1918. The isolation of FUKUDA's strain dates back to 1890, before WEHMER or TAKAHASHI, and the fact suggests that it had been derived from AHLBURG's isolate because nobody isolated a mold from koji before AHLBURG who was a contemporary of FUKUDA and lived near him.

The koji used by AHLBURG and that sent to WEHMER from KELLNER seem to have been produced by FUKUDA, because the strains Thom No. 113 and RIB 430 showed similar morphological and physiological characters. Distribution of these strains is shown in Table 1. It is strange that Thom No. 113 has long been described as having uniseriate sterigmata by most of the workers though it has biseriate sterigmata. WEHMER described the arrangement of sterigmata as uniseriate (einfach in German) but did not refer to whether it mixes biseriate sterigmata or not, and the present author has found that more than 20% of heads in Thom No. 113 and RIB 430 are biseriate.

A. flavus LINK WB (=NRRL) 482 (Thom No. 108) is a BREFELD and WEHMER's *A. flavus* and not the original LINK's isolate, and it seems that the original LINK's strain cannot be obtained. Miss D.I. Fennell, in her letter of November 1970, regarded Thom No. 108 as *A. oryzae* NRRL 482 (Thom No. 108). Thus, the original isolate of *A. oryzae* was found to be RIB 430 as well as Thom No. 113, but no positive proof was obtained for LINK's original isolate.

V. RESULTS OF COMPUTER ANALYSIS

There seems to be several technical ways of using computer analysis for taxonomical classification. After arranging every strain in multiple dimensions by computing mycological vectors, one method is to determine a difference or similarity of a strain from the standard one, and the other is to cluster the strains by their similarity without reference to the standard strain. The component analysis was carried out because of the absence of the standard

Plate 11. Pink coloration of conidia and change in the color during growth.

Left 4 cultures: *A. oryzae* RIB 1025 was incubated for 1 week in Czapek's agar containing (from left to right) anise alcohol, anisaldehyde, anisic acid, and *p*-methoxyphenol. The conidia colored pink only in *p*-methoxyphenol.

Right 4 cultures: *A. oryzae* var. *viride* RIB 1116 was incubated in Czapek's agar containing anisic acid for 4, 10, 20 and 30 days (from left to right). The pink color gradually faded and the conidia returned to their own color.

Plate 12. Colony covered with central overgrowth of white mycelia.

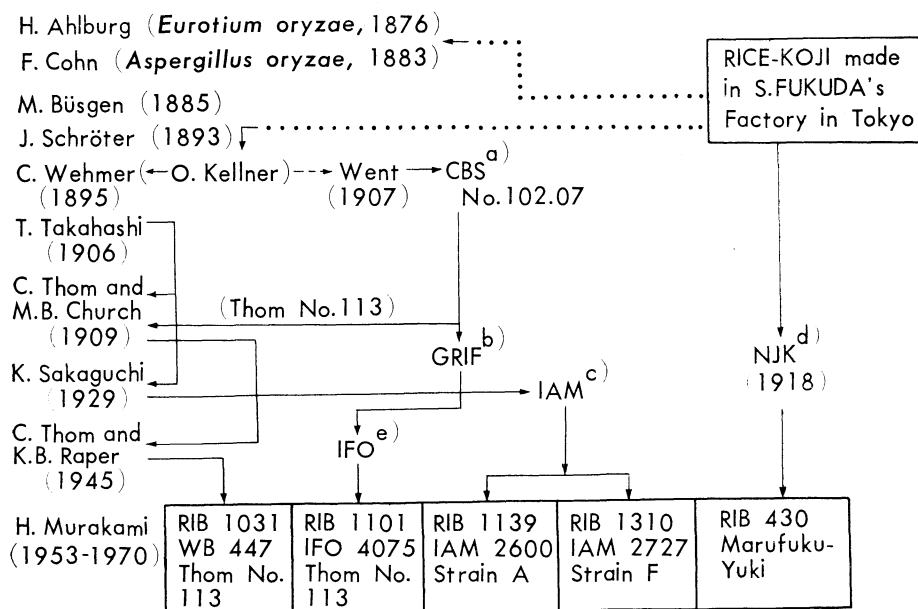
A. sojae RIB 1042 was incubated in Czapek's agar at 34° for 1 week, and it showed a snow-capped colony by mycelial overgrowth (right) but, when incubated at 24° it showed a wholly white appearance (left).

Table 1. Distribution of AHLBURG-COHN's original isolate.

RIB 1031 is the same as RIB 430 which is one of the oldest known koji molds in Japan, both producing biseriata sterigmata but not producing kojic acid in the rice-koji extract.

RIB 1101, 1139, and 1310 are considered to be similar to one another, all producing uniseriate sterigmata and kojic acid.

Abbreviations: (a) Centraalbureau voor Schimmelcultures, Baarn. (b) Government Research Institute of Formosa (defunct). (c) Institute of Applied Microbiology, University of Tokyo. (d) Nippon Jozo Kogyo Co., Ltd., a producer of *Tané*-koji in Tokyo. (e) Institute for Fermentation, Osaka.



Signs for process of distribution: — reliable, ---- probable, theoretically reliable.

strain of LINK's original isolate and this result has been reported in detail elsewhere (39, 40).

Molds tested. Four hundred and six strains were tested which included 361 koji-molds and 45 molds other than koji mold. Each of 20 mycological characters of a strain was classified into three grades from 1 to 3 as follows: (1) Color of old cultures in Czapek agar; brown 1, brownish green to greenish brown 2, green 3. (2) Diameter of colony; short 1, medium 2, long 3. (3) Wrinkling on the reverse side of colony; absence 1, medium 2, conspicuous 3. (4) Shade of the reverse side of colony; colorless 1, medium colored 2, deeply colored 3. (5) Production of sclerotia; none 1, little 2, much 3. (6) Conidiophore walls; smooth 1, rough 2, conspicuously rough 3. (7) Conidio-

Table 2. Normalized eigen vectors and contribution percentage of 5 components.

Kind of characters (variance) is shown by figures in parentheses as described in the text.

Component No.		1	2	3	4	5
Percentage (%)		19.5	10.6	10.4	7.4	5.9
Mean		0.0843	0.1356	0.0493	0.0341	0.0318
Variance		0.1816	0.2226	0.2254	0.2305	0.1460
Mycological characters	(1)	1.2784	-1.1055	0.5392	-0.1255	-0.0934
	(2)	0.2908	-0.9947	-1.4934	1.9570	-0.6651
	(3)	0.5030	-0.4263	0.9807	-1.1502	0.1811
	(4)	1.0718	-0.4760	0.5533	-0.8425	-0.9866
	(5)	1.1280	-0.0522	-1.5625	-1.1417	0.1891
	(6)	-0.0073	0.8321	0.5219	1.0282	1.6923
	(7)	-1.5301	1.0780	-1.0331	1.6705	0.2510
	(8)	0.3504	1.5888	0.1653	0.6410	1.1178
	(9)	-0.5025	-2.0932	0.1285	1.2789	0.6980
	(10)	-2.0014	-0.2173	0.0371	-0.4370	0.3350
	(11)	0.5017	0.4535	-1.7811	-0.9191	0.7984
	(12)	0.8571	-0.0076	0.2637	0.3190	-1.2333
	(13)	0.3532	0.9309	-0.4441	0.4939	-1.8265
	(14)	0.9994	-0.1695	-1.6627	-1.2259	0.6351
	(15)	-1.3538	0.9798	0.8071	-1.6250	-0.1999
	(16)	-1.8232	-0.3987	0.4400	-0.9208	1.5884
	(17)	0.5727	-1.6351	0.8950	0.5870	0.9173
	(18)	-0.4680	1.1858	1.4100	0.4256	-0.8980
	(19)	0.5931	-0.7689	1.5603	0.0973	-0.8172
	(20)	-0.8232	1.2949	-0.3233	-0.1138	-1.7105
Cumulative percentage		19.5	30.1	40.5	47.9	53.8

(Sample size=406, Variable=20)

phore length; short 1, medium 2, long 3. (8) Vesicle diameter; short 1, medium 2, long 3. (9) Conidial walls; slightly rough 1, rough 2, echinulate to tuberculate 3. (10) Conidia diameter; short 1, medium 2, long 3. (11) Ratio (%) of biserial heads; zero to less than several percent 1, 10-20% 2, over 30% 3. (12) Kojic acid; none 1, little 2, much 3. (13) Pigments; little 1, medium 2, much 3. (14) Aflatoxins; none 1, little 2, much 3. (15) Amylolytic activity; low 1, medium 2, high 3. (16) Coloration of hydroquinone; uncolored 1, medium colored 2, deeply colored 3. (17) Methylene-Blue reduction; very slow 1, medium 2, rapid 3. (18) Browning of rice koji; none

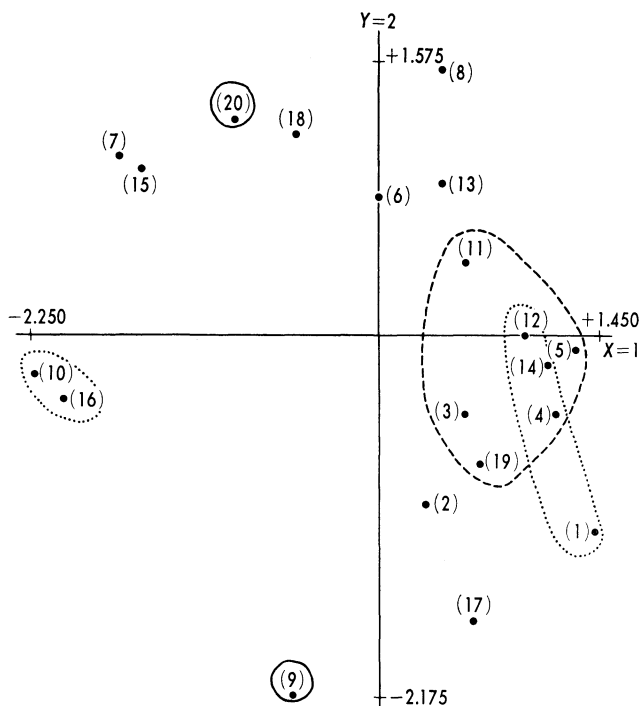


Fig. 1. Distribution of 20 mycological characters (Case I).

The normalized eigen vectors of the components 1 and 2 were plotted on X and Y, respectively. The characters are shown by numerals in parentheses as described in the text.

1, medium 2, high 3. (19) Pink coloration of conidia in anisic acid medium; uncolored 1, medium colored 2, deeply colored 3. (20) Pink coloration of conidia in anisaldehyde medium; the same as (19).

Distribution of characters. Normalized eigen vectors and contribution percentage of 5 components are shown in Table 2. From this table, the components 1, 2, and 3 seemed to be useful in distinguishing the characters and their distribution in three dimensions was compared on three planes (Cases), which were obtained by plotting the eigen vectors of two components on the axes X and Y as shown in Fig. 1 for Case I, Fig. 2 for Case II, and Fig. 3 for Case III. The relationship between the characters corresponded to the distance between plots, the shorter the distance, the closer the characters correlating one with another, and the longer the distance, the closer the characters correlating reversely, plots of which lay in the opposite side of the axis.

These graphs suggest that the characters are divided into three groups

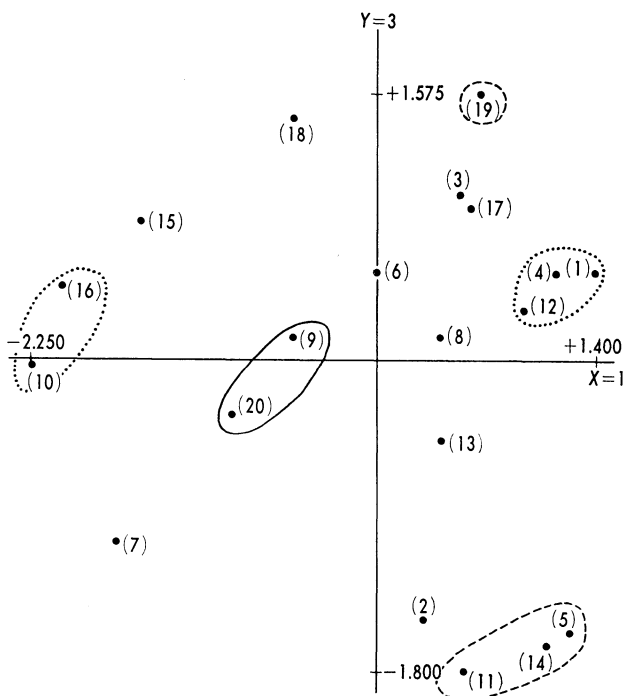


Fig. 2. Distribution of 20 mycological characters (Case II).

The normalized eigen vectors of the components 1 and 3 were plotted on X and Y, respectively. The characters are shown by numerals in parentheses as described in the text.

as follows: Group shown by a solid line; roughness of conidial walls and pink coloration of conidia in anisaldehyde medium (reverse correlation). Group shown by a dotted line; diameter of conidia, color of hydroquinone, color of old cultures, color on the reverse side of colony, and kojic acid production. Group shown by a broken line; arrangement of sterigmata, production of sclerotia and aflatoxins, and pink coloration of conidia in anisic acid medium.

Distribution of strains. Similar to mycological characters, distribution of the strains was obtained by plotting the normalized eigen vectors of the three components, 1, 2, and 3, of each strain on the plane. By the selection of significant mycological characters as above, the strains were clustered into several groups as shown in Figs. 4, 5, and 6, though the clusters overlap one another as seen in the cumulative contribution percentage (53.8%) of the components.

Results of component analysis. The strain RIB 1032 (=Thom No. 108) was always near the strain RIB 1031 (=Thom No. 113), which suggests that

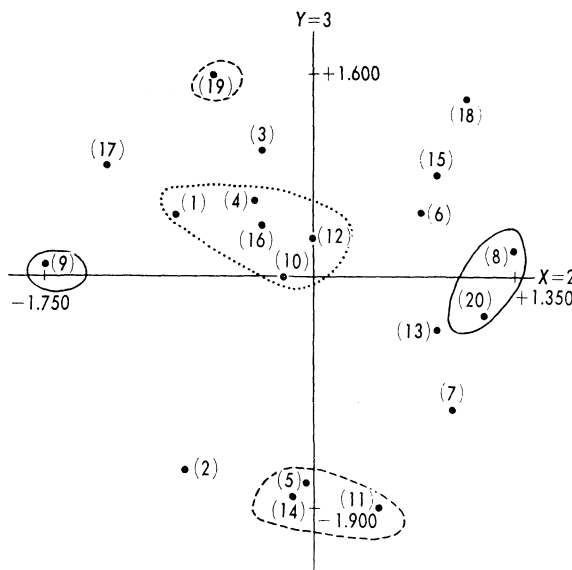


Fig. 3. Distribution of 20 mycological characters (Case III).

The normalized eigen vectors of the components 2 and 3 were plotted on X and Y, respectively. The characters are shown by numerals in parentheses as described in the text.

Thom No. 108 is not suitable for use as a standard strain of *A. flavus* LINK. The strains having both biserial sterigmata and conidia smaller than $4-5 \mu$ in diameter were clearly found to produce an independent cluster FF, mainly including *A. flavus*, while those having both biserial sterigmata and conidia larger than $5-6 \mu$ in diameter mainly consisted of *A. oryzae* (Ahlburg) COHN. The strains other than these were divided into two clusters, E and EE, by the color of old cultures; but the strains having echinulate conidia seemed to produce some independent cluster BB, mainly including *A. sojae*. Echinulate conidium in koji molds is so highly correlated to the noncoloration in anisaldehyde medium that any strain having both echinulate and pink conidia should be discriminated from the koji molds.

Among the strains added newly in the *A. flavus* group of RAPER-FENNELL, *A. subolivaceus* was included in the cluster of *A. flavus*, while *A. zonatus* and *A. avenaceus*, and *A. clavatoflavus*, lay outside and inside the cluster of *A. oryzae*, respectively.

VI. PROPOSAL OF A CLASSIFICATION SYSTEM

Key characters. Stability of each character was tested by long-term

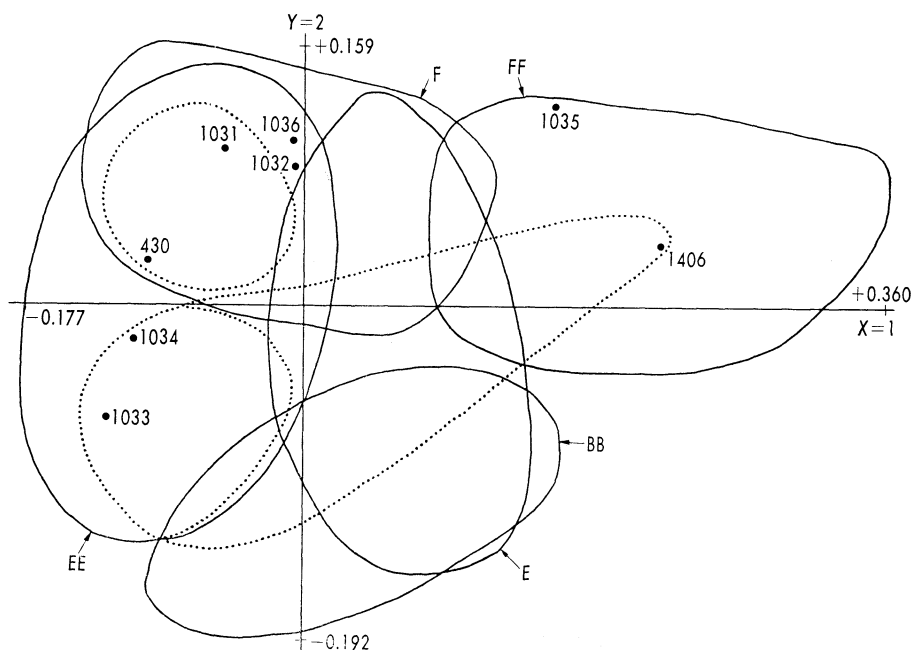


Fig. 4. Cluster of all strains examined (Case I).

The normalized eigen vectors of the components 1 and 2 of each strain were plotted on X and Y, respectively, and the strains were clustered according to their significant mycological characters. Numerals on X and Y and those in the clusters are the maximum eigen vectors and RIB-number of the strains, respectively.

BB Strains having prominently echinulate conidia with uniseriate sterigmata.

E Strains showing green in old cultures, with uniseriate sterigmata.

EE Strains showing brown in old cultures, with uniseriate sterigmata.

F Strains having both biseriate sterigmata and conidia larger than $5-6\mu$ in diameter.

FF Strains having both biseriate sterigmata and conidia smaller than $4-5\mu$ in diameter.

RIB 430, RIB 1031: *A. oryzae* (Ahlb.) COHN. RIB 1032: *A. flavus* LINK WB 482 (Thom No. 108). RIB 1033: *A. zonatus* KWON et FENNELL. RIB 1034: *A. clavato-flavus* RAPER et FENNELL. RIB 1035: *A. subolivaceus* RAPER et FENNELL. RIB 1033: *A. avenaceus* SMITH. RIB 1403: *A. flavus* LINK IFO 7600.

successive cultivation and/or by comparing some parent strains with their mutant strains, and sometimes by statistical relation of the characters in many strains. Among the stable characters, based on the result of component analysis, those suitable as keys for classification are listed below in the order of importance.

(1) Sclerotium: Presence and absence (with some lability in the koji mold).

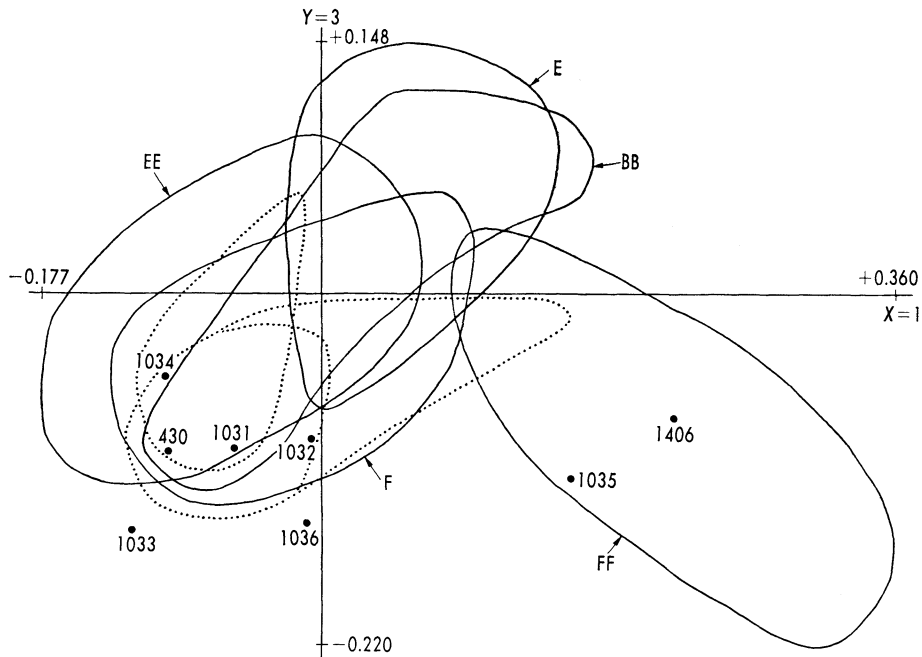


Fig. 5. Cluster of all strains examined (Case II).

The normalized eigen vectors of the components 1 and 3 of each strain were plotted on X and Y, respectively, and the strains were clustered according to their significant mycological characters. Numerals are the same as those in Fig. 4.

- (2) Seriation of sterigmata: Uniseriate, not having any biseriate head and/or having biseriate head less than several percent of total heads; and biseriate, having biseriate head more than several percent.
- (3) Roughness of conidial walls: Rough, echinulate, and tuberculate.
- (4) Color of old cultures: Green, including that with brownish shade; and brown without any green shades.
- (5) Diameter of conidium: Below $4-5\mu$ and above $5-6\mu$, limited to classification of the strains having biseriate sterigmata.
- (6) Pink coloration of conidium in anisaldehyde medium: Pink and non-pink, essentially useful for the strains having echinulate conidia with uniseriate sterigmata.
- (7) Wrinkling on the reverse side of colony: Wrinkling and plain (with some lability).
- (8) Production of kojic acid: Production and non-production, sometimes useful for some strains.

Species, varieties, and forms. The variety is considered as conspicuously different from the type species in important characters, and the form as more

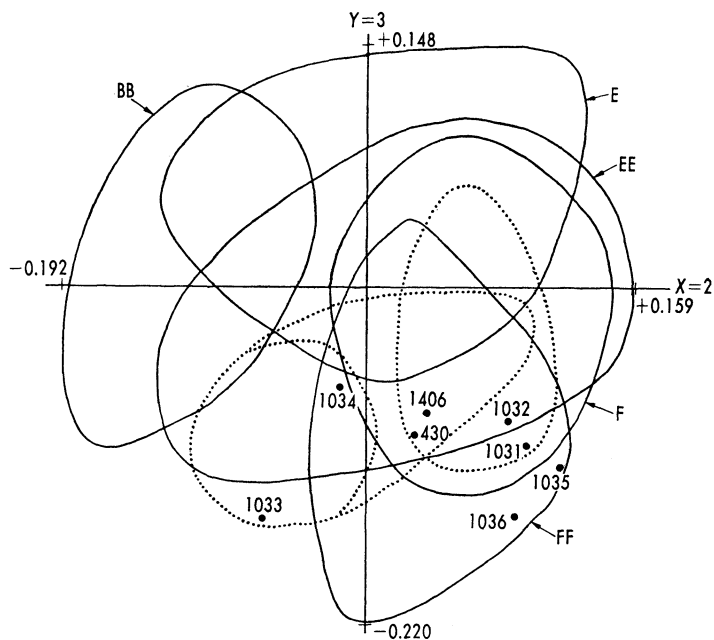


Fig. 6. Cluster of all strains examined (Case III).

The normalized eigen vectors of the components 2 and 3 of each strain were plotted on X and Y, respectively, and the strains were clustered according to their significant mycological characters. Numerals are the same as those in Fig. 4.

or less different from the type species or varieties in some significant characters for utilization of mold.

From the point of *Saké* making, amylolytic activity of molds is one of the most significant characters. This activity is usually correlated to the color of old cultures, wrinkling on the reverse side of the colony, and to the pink coloration of conidia in the anisaldehyde medium. The strong amylolytic strains show brown color, in addition to the pink conidia, and/or show with wrinkling, while strains with weak amylolytic activity show green without any wrinkling (28). Therefore, the afore-mentioned characters shown by (6), (7), and (8) can be used as keys for determination of the form.

Aspergillus oryzae group. It was not until 1921 that koji molds were grouped in *A. flavus-oryzae* series by THOM and CHURCH (5) who compared them with Thom No. 108 and Thom No. 113. However, this series or group name is clearly inadequate owing to the high similarity of Thom No. 108 to Thom No. 113. The koji molds were proved to belong to a quite different cluster from that of *A. flavus* by computer analysis. Thus, *Aspergillus oryzae* seems to be the most appropriate as a group name for koji molds.

Outstanding character of A. oryzae group. Conidial heads are globose to radiate or columnar, showing very light yellow-green, deep yellow-green, olive-brown, brown, or rarely white. Conidiophores are colorless, varying in length, usually roughened but varying from smooth or nearly so to coarsely roughened. Vesicles are subglobose to somewhat clavate or flask like. Arrangement of sterigmata is usually uniseriate but is sometimes biseriate. Conidia are globose, subglobose, or elliptical to pyriform; walls are usually delicately rough or rough but sometimes prominently echinulate to tuberculate; diameter varies from 4 to over 10 μ but, in the species with biseriate sterigmata, definitely larger than 5–6 μ . Sclerotia are seldom produced but few and globose, purple-brown or black at maturity.

Key to the A. oryzae group.

- A Conidia prominently echinulate, not pink colored in anisaldehyde medium*; sterigmata uniseriate; conidiophores sometimes smooth or nearly so *A. sojae* SAKAGUCHI et YAMADA
- AA Conidia not prominently echinulate
- B Conidia tuberculate with brown coloring matter
..... *A. tamarii* KITA (*A. sojae* series)
- BB Conidia not tuberculate
- C Conidial heads not including biseriate sterigmata
- D Slant cultures keeping green to green with brown shades in old
..... *A. oryzae* var. *viride*, var. nov.
- DD Slant cultures shifting to brown without any green shades, or sometimes keeping white, in old..... *A. oryzae* var. *brunneus*, var. nov.
- CC Conidial heads including biseriate sterigmata; conidia larger than 5–6 μ in diameter..... *A. oryzae* (Ahlburg) COHN (*A. oryzae* series)

VII. DESCRIPTION OF SPECIES

Aspergillus sojae SAKAGUCHI et YAMADA

Conidia valde echinulata, non colorata in 'anisaldehyd mediis'; sterigmata uniseriata.

Conidia definitely echinulate, not colored pink in anisaldehyde medium; sterigmata uniseriate; conidiophores sometimes smooth.

Note. The taxonomical significance of echinulate conidia was at first recognized by SAKAGUCHI in 1933 though they had been found in *A. parasiticus* by SPEARE without this recognition.

Color of old cultures brown, brown with some shades of green, and sometimes green. Colonies growing rapidly, usually wrinkling and coloring on the reverse side, and surface of colonies sometimes covered with over-

* Prepared by dissolving 0.05% anisaldehyde in Czapek's agar.

growth of long white mycelia at the center when incubated at 34° (Plate 12). *Conidial heads* loosely radiate to irregularly radiate with chains divergent or adhering in loose thin columns; *conidiophores* roughened but sometimes smooth; *vesicles* subglobose to clavate, ranging from 10 to 30 μ in diameter; *sterigmata* definitely uniseriate; *conidia* globose, prominently echinulate, usually 5 to 6 μ in diameter, often 4 μ and rarely reaching 7 to 8 μ , and not colored pink in the anisaldehyde medium.

Amylolytic activity usually low but proteolytic one sometimes high.

The species description is based on 33 koji molds.

The type culture, RIB 1045=*A. sojae* IAM 2669, was isolated from koji for *Shoyu* making as a strain SH-10-6 by SAKAGUCHI in 1930.

Mycological differences among *A. flavus*, *A. sojae*, and *A. parasiticus* have been somewhat ambiguous by the conidial walls described as conspicuously echinulate for *A. flavus* and coarsely echinulate for *A. parasiticus* by RAPER-FENNELL. However, the difference between *A. flavus* and *A. parasiticus* or *A. sojae* became clear by recognition of the roughness of conidial walls, and that between *A. sojae* and *A. parasiticus* by respective non-coloration and coloration of conidia in the anisaldehyde medium.

Aspergillus tamaraii KITA

Mycological characters have been described in The Genus *Aspergillus* by RAPER-FENNELL in 1965.

Four strains of the koji mold, rarely isolated now from koji and *Tané-koji*, were placed in this species by the present author from their mycological similarities to *A. tamaraii* KITA WB 429 received from Prof. Raper in 1967, including *A. oryzae* var. *fulvus* YAMAMOTO.

Comparison of the type culture WB 429, in addition to *A. flavo-furcatis* BATISTA et MAIA WB 4911, with the koji molds of this species indicated that the koji molds always had uniseriate sterigmata, sometimes plain reverse-side of colony, colored hydroquinone, and browned the rice koji, contrary to the WB 429 and WB 4911.

The strains placed in *A. sojae* and *A. tamaraii* were found in the ratio of 9.3% in the koji mold, and they were placed in *A. sojae* series.

Aspergillus oryzae (Ahlburg) COHN var. *viride*, var. nov.

Conidia acervulata viridula etiam in culturis vetere; sterigmata uniseriata.

Color of old cultures persisting green or green with brown shades; sterigmata uniseriate.

Color of old cultures persisting green or that with brown shades. *Conidial heads* usually globose to radiate, but sometimes broom-like to columnar, and sometimes their mixture; *sterigmata* uniseriate, biseriate heads less than several percent rarely produced; *conidia* usually 5 to 6 μ in diameter but

sometimes 3 to 4 μ , and rarely 7 to 8 μ , usually not colored pink in the anisaldehyde medium.

Physiological characters seem to depend on the conidial color; weak amyolytic activities were shown either by strains showing green color alone in old, not coloring hydroquinone, or by strains with vesicles smaller than 15–20 μ in diameter and coarsely roughened conidia. Especially, none of strong amyolytic strains were found in those having plain reverse side of colony.

The strains showing deep green color alone in old usually bear many conidia on shorter conidiophores, rapidly reduce Methylene Blue, and brown the rice koji, and they were frequently found in koji for *Shoyu* making and sometimes *Miso* making, while the strains showing green color with brown shades sometimes showed high amyolytic activity and were found in the koji for *Saké* making.

The varietal description is based on 126 koji molds, including 99 wrinkling strains and 27 non-wrinkling ones.

The type culture, RIB 128, wrinkling strain, was frequently found in *Tané*-koji for *Saké* making.

Included among this variety are *A. oryzae* var. *variabilis* IFO 5768, *A. oryzae* var. *sporoflavus* IFO 5785, *A. oryzae* var. *pseudoflavus* IAM 2779, *A. flavus* var. *asper* IFO 5324, and *A. flavus* var. *columnaris* NRRL 4818.

Aspergillus oryzae (Ahlburg) COHN var. *brunneus*, var. nov.

Conidia viridula sed in culturis vetere conidia acervulata brunnea, vel semper alba; sterigmata uniseriata.

Color of old cultures shifting to brown without any green shades in old, and sometimes persisting white even in old; sterigmata uniseriate.

Color of old cultures shifting to brown without any green shades in old, and sometimes persisting white even in old. *Conidial heads* usually globose to radiate, but sometimes broom-like to columnar, and sometimes with their mixture; *conidiophores* roughened, occasionally smooth to less roughened, varying from 0.2 to 2.0 mm in length, sometimes over 5 mm; *sterigmata* uniseriate, biseriate heads less than several percent rarely produced; *conidia* infrequently mixed with some echinulate ones even in the same culture, usually larger than 4 to 5 μ in diameter, often reaching 9 to 10 μ , variable in pink coloration in the anisaldehyde medium.

Amyolytic activity usually high and sometimes the highest among koji molds, depending on the ability of the conidia to color pink in the anisaldehyde medium; most of the high amyolytic strains colored pink and the low to medium amyolytic strains did not, and the former was frequently found in the koji for *Saké* making. Reduction of Methylene Blue was usually slow and sometimes nil.

The varietal description is based on 190 koji molds, including 99 pink strains and 91 non-pink ones.

The type culture, RIB 1172 = *A. oryzae* IAM 2648, showing pink conidia, was isolated from koji for *Saké* making as SAKAGUCHI's strain S-3-8 in 1930.

Included among this variety are *A. oryzae* var. *pseudoflavus* IFO 4083 and OUT 5054 (both pink strains), and *A. oryzae* var. *microvesiculosus* IFO 4261 (non-pink strain). *A. oryzae* var. *effusus* NRRL 506 seems also to be included in this variety.

Aspergillus oryzae (Ahlburg) COHN

Mycological characters have been described in The Genus *Aspergillus* by RAPER-FENNELL in 1965, and were compared with those of the koji mold by the present author using the strains *A. oryzae* WB 447 (Thom No. 113 = RIB 1031) and RIB 430 as control.

The species is characterized by having both biseriate sterigmata and conidia larger than $5-6\ \mu$ in diameter. The diameter of conidia, however, usually varies even in the same culture so that the species should be carefully decided by further considering the following characters in addition to the diameter: (1) Color of old culture either brown or green, mostly brown in this species; (2) sclerotia either absent or present, mostly absent in this species; (3) conidiophores either long or short, mostly longer than 1 mm in this species; (4) conidiophore walls either smooth or rough, sometimes smooth in this species; (5) kojic acid either not produced or produced, sometimes not produced in this species; and (6) reduction of Methylene Blue either slow or rapid, usually slow in this species.

Sterigmata biseriate, and biseriate heads usually less than 20–30%; *conidia* commonly larger than $5-6\ \mu$ in diameter and sometimes up to $9\ \mu$ with a mixture of 3.6 to $3.8\ \mu$ even in the same culture.

The species description is based on 46 koji molds, including 8 strains not producing kojic acid and 38 strains producing it. The type cultures RIB 430 and RIB 1031 are non-producer of kojic acid when cultured in the rice-koji extract.

Included among this species are *A. oryzae* var. *wehmerii* IFO 5770 and *A. oryzae* var. *tenuis* IFO 4134 (both producers of kojic acid), and also *A. flavus* LINK Thom No. 108 in which diameter of conidia was mostly $4.8-5.4\ \mu$, including those of $4.2-4.6\ \mu$ and $6-7\ \mu$ (Plate 10).

The strains placed in *A. oryzae* var. *viride*, *A. oryzae* var. *brunneus*, and *A. oryzae* (Ahlburg) COHN were found in the ratio of 90.7% in the koji mold, and they were placed in *A. oryzae* series.

VIII. MOLDS OTHER THAN KOJI MOLD

The great majority of *Aspergilli* in nature have long been distinguished

as wild molds, never being used in the Japanese fermentation industries, while some of them have been used as koji molds selected throughout the long history of the industry. This chapter deals with these wild molds in addition to some aflatoxin-producing molds which were placed in the *A. flavus* group by the present author.

Outstanding character of A. flavus group. Conidial heads are globose to radiate or columnar, showing yellow-green, deep yellow-green, or olive-brown. Conidiophores are colorless, usually short, definitely roughened to coarsely roughened. Vesicles are globose or subglobose at maturity, fertile over most of their surface. Sterigmata are arranged either uniseriate or biseriate according to species; in the biseriate species, mixing rate of the biseriate heads ranged from more than several percent to 100%. Conidia are globose or subglobose with rough to prominently echinulate walls; definitely smaller than $4-5\ \mu$ in diameter in the species with non-echinulate conidia, but varying from 3 to $8\ \mu$ in the species with prominently echinulate conidia. Sclerotia are frequently produced, many and globose, purple-brown or black at maturity.

Key to the A. flavus group.

- A Conidia prominently echinulate
- B Sterigmata uniseriate; conidia colored pink in anisaldehyde medium* *A. parasiticus* SPEARE
- BB Sterigmata biseriate *A. toxicarius*, sp. nov.
- AA Conidia not prominently echinulate, smaller than $4-5\ \mu$ in diameter; conidial heads including biseriate sterigmata *A. flavus* LINK

Description of species.

A. flavus LINK

The species is characterized by having both biseriate sterigmata and conidia smaller than $4-5\ \mu$ in diameter, which produced an independent cluster of strains by component analysis. When there is some doubt in diameter of conidia, the species should be carefully decided from the description of *A. oryzae* (Ahlburg) COHN in Chapter VII.

Sclerotia usually produced, sometimes dominating the colony. *Vesicles* globose to subglobose and sometimes oval, larger than 30 to $40\ \mu$ in diameter and often reaching $60\ \mu$; *conidia* roughened but not quite echinulate, smaller than 4 to $5\ \mu$ in diameter, mostly less than 3 to $4\ \mu$ but sometimes mixed with those of 4.8 to $5.2\ \mu$.

Kojic acid usually produced; activities to produce amylase, to color hydroquinone, and to brown the rice koji usually low, but Methylene Blue reduced

* Prepared by dissolving anisaldehyde (0.05%) in Czapek's agar.

rapidly. Aflatoxins or somewhat aflatoxin-like substances sometimes produced in some strains.

The species description is based on 37 strains including 30 strains from abroad and 7 strains isolated in Japan from koji during the past 60 years as contaminants.

The type culture, RIB 1406 = *A. flavus* LINK IFO 7600, was received from the Institute for Fermentation, Osaka, in 1968. RIB 1427 (= *A. flavus* NRRL 1957) received from Miss D.I. Fennell in 1970 is also representative of this species.

Included among this species are *A. oryzae* var. *wehmerii* IAM 2776 and IAM 2778, *A. oryzae* var. *sporoflavus* IAM 2780, and 3 strains of the *A. flavus* group of RAPER-FENNELL, *A. zonatus*, *A. subolivaceus*, and *A. avenaceus*.

A. parasiticus Speare

This species was characterized by RAPER and FENNELL (15) as not shifting to brown in old, having radiate heads, uniseriate sterigmata, and prominently echinulate conidia. The present author's recognition of the species, however, is based on the uniseriate sterigmata and pink coloration of the prominently echinulate conidia in the anisaldehyde medium by which the species was discriminated from the *A. sojae* species.

Included among this species are 3 strains of *A. parasiticus*, WB (=NRRL) 465, IFO 4082, and IFO 4301, all producing a large amount of aflatoxins.

Aspergillus toxicarius, sp. nov.

Sterigmata biseriata, conidia valde echinulata. Vesiculis globosis, 30–40 μ in diameter.

Sterigmata biseriate, conidia prominently echinulate. Vesicles globose, 30–40 μ in diameter.

Holotype. August 3, 1966. London (preserved in the Research Institute of Brewing)

Note. The species was formerly identified by the present author, in 1966, with *A. parasiticus* SPEARE var. *globosus* (17), but was now found to be quite different from *A. parasiticus* by the fact that the sterigmata were arranged in two series and the conidia did not color pink in the anisaldehyde medium. None of such strains have been reported in taxonomy which led to the establishment of a new species, *A. toxicarius*, owing to its high production of aflatoxins.

Color of old cultures showing green and sometimes green with some shades of brown. Colonies growing rapidly, attaining a diameter of 60 to 70 mm and often 80 mm at 34° for 1 week incubation, conspicuously wrinkling and deeply coloring on the reverse side. Sclerotia usually produced. *Conidial*

heads loosely radiate to irregularly radiate; *conidiophores* roughened, usually less than 1 mm in length; *vesicles* globose to subglobose, 30 to 40 μ in diameter; *sterigmata* definitely biseriate; *conidia* globose, prominently echinulate, usually 4 to 5 μ in diameter and sometimes up to 6 μ , not colored pink in the anisaldehyde medium. Kojic acid produced abundantly; aflatoxins and pigments produced abundantly; hydroquinone scarcely colored but Methylene Blue rapidly reduced, and amylolytic activity commonly weak.

The species description is based on 6 strains received from abroad as follows: ATCC 15517, CMI 89717, NRRL 2999, NRRL 3145, NRRL A11613, and NRRL A12353.

The type culture, RIB 4002=CMI 89717=TPI M039, was received in August, 1966, from the Tropical Products Institute in London.

Many cultures were received from a total of 12 institutions, here and abroad, which are gratefully acknowledged. The author is particularly indebted to Dr. Kin'ichiro Sakaguchi, Emeritus Professor of the University of Tokyo, for his kind guidance and valuable suggestions throughout the course of this study. The author benefited greatly by the kind advices of Dr. K. Matsumoto, former chief scientist of this Institute, Dr. T. Hasegawa, Director of the Institute for Fermentation, Osaka, Prof. H. Iizuka, University of Tokyo, and Dr. Y. Kurosawa, Teikoku Hormone Mfg. Co., Ltd., Kawasaki.

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The culture strains examined were deposited in the organizations described, and those isolated from koji by the present author are preserved in the Research Institute of Brewing, Takinogawa 2-5, Kita-ku, Tokyo 114, Japan.

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