

## 原 著

## Isolation of Yeasts from Japanese and Italian Fruits and their Analysis on Chromosomal Electrophoretic Karyotype

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## &lt;Abstract&gt;

Pure isolation of the yeast from Japanese and Italian fruits cultivated locally was tried. As a result, for the domestic fruit, six yeast strains were isolated from five fruit samples which were selected by the existence of viable yeasts out of 40 fruit samples cultivated in 17 prefectures. Three genus classes of six isolated yeasts were identified by the electrophoretic karyotype analysis of those chromosomes. The isolation efficiency from the number of the fruit samples was 12.5%. Considering the fact the isolation efficiency was 100% 20 years ago, it was presumed domestic fruit that the yeast which had been present previously had decreased sharply. The cause of the sudden decrease is not clear, but the influence of the kind of sprayed pesticide having changed is thought to be a likely cause. This result corresponds to the recent report that the *Bacillus natto* had been almost exterminated from a rice field in Fukuoka prefecture. There is concern about microflorae in natural environments changing suddenly during these past dozens of years. This should be studied at the long-term span. About the Italian fruit, 21 yeast strains were isolated from 15 fruit samples which were selected by the existence of viable yeasts out of 30 fruit samples cultivated in the suburbs of three cities. Six genus classes of 15 isolated yeasts were identified by the electrophoretic karyotype analysis of the chromosome. The isolation efficiency from the number of the fruit samples was 50%.

Keywords: Japanese fruits, Italian fruits, isolation of yeast, karyotype analysis

## INTRODUCTION

There are some reasons why we have begun to try isolation of the yeast from the fruit of various countries. One reason is that we aim at finding out novel yeast for bread to produce which can bring on unprecedented taste and flavor, and further we aim at bringing it up as industrial use yeast.

Another reason is to find out *Candida humilis* which contributes to fermentation of Italian panettone dough. In northern Italy, panettone is sweet bread made in each family in a Christmas season for more than 200 years, and it features unique sweet-sour flavor. It is presumed that the

dough was made by mixing fruit or vegetables, or horse droppings with wheat flour in the old times and when succeeded in dough fermentation, the original dough was kept for next bread making. The dough kept for next bread making is called as mother dough. Each family have been inherited it for more than 100 years from generation to generation. In that point, it is just like Japanese salted rice-bran paste (nukadoko), and the mother dough was caught as a family treasure<sup>1)</sup>. As same as Japanese nukadoko, it was rare to hand over to another person. The mother dough is also used to a bread product of the high sugar content, such as pandoro, danish pastry and croissant that are eaten through the

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year in the northern part of Italy. The unique flavor is received favorably in Japan and the several kinds of bread made with panettone mother dough are sold. However, probably, as for reproducing the traditional manufacturing method in Japan, it is said that it is difficult because it is not easy to maintain a lactic acid bacterium and symbiosis of special yeast<sup>2)</sup>. Therefore we are making effort to find out yeast secreting organic acids<sup>3)</sup> like a lactic acid and aim at producing panettone-like bread with the use of one kind of microbe.

In addition as a different purpose, we investigate yeast florae having habitation to fruit how it varies according to a country. The reason why fruit was chosen is because the yeast which ferments sugar is living on the surface of fruit and such a sugar fermenting yeast is mostly in the case suitable for bread dough fermentation. In this report, we reported the result that investigated Japanese and Italian fruit, but are going to report the fruit which is to be sampled sequentially in various countries. Because it was revealed that number of yeast in itself having habitation to fruit in a process is in the decreasing situation by this research, we are going to observe a change of number of in the specific area of some of Japan the yeast and yeast flora in future.

## MATERIALS AND METHODS

### *Japanese fruit*

14 kinds of fruit cultivated with 17 prefectures, 40 samples in total were used for an experiment. A table in the text showed which fruit was cultivated at which production area. 14 kinds of fruit are as follows: grape, cherry, loquat, apple, la france, kiwi, mandarin orange, citrus orange, persimmon, papaya, passion fruit, peach, melon and pear.

### *Italian fruit*

Eight kinds of fruit cultivated with suburbs of three cities (Rome, Florence, Milan), 30

samples in total were used for an experiment. A table in the text showed which fruit was cultivated at which production area. Eight kinds of fruit are as follows: grape, fruit of the cactus, plum, pear, apricot, blue apple, red apple and prune.

### *Isolation of yeast*

With the sterilized knife, the small fragment of fruit which was prepared in a state with the peel and it was put it into 15 ml Falcon tube aseptically. The tube was restored in refrigerator at approximately 4°C in darkness for approximately two years. Meanwhile, a convenient condition for isolating the yeast would be set because the mold which disturb in isolation of the yeast perished, and yeast multiplied. A loop was inserted into the Falcon tube and dipped it into the liquid which collected at the bottom. At this time, the yeast which propagated will attach to the tip of the loop. The tip of the loop must be careful not to contact with mold when mold spreads on the surface of the fruit. The loop tip was streaked on the surface of YPD agar medium (1% yeast extract, 2% peptone, 2% glucose, 1% agar, pH5.8) which was prepared to a circular type dish. The static culture was performed for 2-5 days at 30°C until the yeast colony grew. Judging from the shape of the colony, the tip of the toothpick which sterilized was ranked a single colony thought to be the yeast and planted it on new YPD agar medium. On one circle dish, 20-30 colonies were planted then. The electrophoretic karyotype analysis was performed about each colony and performed the identification that was yeast.

### *Contour-clamped homogenous electric field (CHEF) gel electrophoresis.*

CHEF gel electrophoresis was performed as described elsewhere<sup>4)</sup>, using a CHEF-DR II system (Nippon Bio-Rad Laboratories, Fukuoka, Japan). The 1% agarose gels in 0.5×TBE (45mM Tris-borate, 45mM boric acid, 1mM pH8.0EDTA) were prepared by pouring 100 ml

agarose (Agarose NA, Pharmacia AB, Uppsala, Sweden) into a 12×12cm frame. Electrophoresis was carried out in 0.5×TBE as running buffer at a constant temperature at 14°C, which was maintained by re-circulation of the buffer through a heat exchanger, at a constant voltage of 170V for 20 hrs. The stepped switching interval was 60sec for the first 12hr and 90sec for the next 8hrs. The gel was stained with 0.0025% SYBR Green solution (TAKARA BIO Inc., Tokyo, Japan) for 1hr with moderate shaking in darkness. The resulting gel images were captured by Chemi-Imager4400 (Alpha innotech corporation, San Leandro, CA, USA).

#### ***Preparation of agarose embedded yeast DNA***

A single colony on YPD (1% yeast extract, 2% peptone, 2% glucose, pH5.8) agar plate was inoculated into 2 ml YPD broth. After grown for 20hrs to stationary growth phase, the cells were collected by centrifugation at 5,000×g, 5min, at 25°C. The supernatant was decanted and the cells were re-suspended in 1ml SE (75mM NaCl, 25mM pH7.4EDTA). Cells were collected again by centrifugation at 5,000×g, 5min, at 25°C. 50 μl of SE and 150 μl of agarose suspension buffer (ASB) which was equilibrated to 55°C in a water bath were added. ASB was freshly prepared in each experiment. 200 μl of ASB was mixed with following four solutions, 500 μl of 3.5% low melt agarose (BRL, Gaithersburg, MD, USA) which was equilibrated to 55°C in a water bath after autoclaved in SE for 1min at 121°C, 20 μl of 1M DTT (filter-sterilized after dissolved in water), 10 μl of 1mg/ml Zymolyase (Kirin 100T, Kirin Brewery Co. Ltd, Tokyo, Japan) suspended in sterile water, and 470 μl of SE. After immediately combining the cell mixture, transfer the mixture plug mold and solidify for 8min at -20°C. Each solidified agarose plug was pushed into a well of 20 well micro plate and 80 μl of 1M DTT (filter-sterilized after dissolved in water), 40 μl of 1mg/ml Zymolyase (Kirin 100T, Kirin Brewery Co. Ltd, Tokyo, Japan) suspended in sterile water, 1880 μl of SE were added into each well. After incubated for

1hr at 37°C, each agarose plug was washed with ES (0.5M pH9.5EDTA, 1% sodium sarcosinate) twice for 10min. 1ml ES and 50 μl of proteinase K (Merk, Darmstadt, FRG) solution (10mg/ml in 50% glycerol) was added to each well and incubated at 55°C for 20 hrs. After proteinase K treatment, each agarose plugs were washed with 1×TE (20mM Tris-HCl, 1mM EDTA, pH7.4) twice for 20min and stored at 4°C until use.

## RESULTS AND DISCUSSION

### ***Japanese fruit***

Yeast was grown on YPD agar plate from five fruits out of 40 fruits experimented. The isolation efficiency was 12.5%. Though a judgment is difficult how this efficiency should be evaluated because there are not documents data, 12.5% is thought to be very low regarding that the efficiency was 100% when the writer carried out a similar experiment in a Japanese company laboratory 20 years ago (the data not shown).

Table 1 summarized the kind of the fruit experimented and their production area, and the samples that yeast is isolated. Yeast was isolated from the grape (the brand name is Berry A) cultivated in Fukuoka prefecture, from the cherry cultivated in Yamagata prefecture, from the loquat cultivated in Kagoshima prefecture, from the la france cultivated in Yamagata prefecture and from the pear (the brand name is atagonashi) cultivated in Fukuoka prefecture. About the fruit that yeast was isolated, association is found in neither a kind of the fruit nor the cultivated area.

Figure 1 shows the result of CHEF analysis on the isolated yeast. The karyotype demonstrated that five different yeast strains were successfully isolated finally. The obtained karyotype was compared with literatures data<sup>5-9)</sup> and the genus classes of three strains were estimated as follows. That is, the yeast isolated from Berry A (lane 4, Fukuoka prefecture) is

*Candida atmosphaerica*, the yeast isolated from the pear (lane 40, Atagonashi, Fukuoka prefecture) is *Pichia membranaefaciens* and the yeast isolated from the cherry (lane 7, Yamagata prefecture) is *Candida atmosphaerica*. In the past study, *Pichia kudravzevii* and *Saccharomyces cerevisiae* were able to be gathered from Japanese fruit widely.<sup>10</sup> This is a fact in unison with the experience of the writer. As for the isolated five yeasts, it is very clear from the karyotype that such general genus was not found at all. This is mysterious, and yeast flora of the domestic fruit might have changed dramatically.

Table 1. The cultivated prefecture in Japan and isolated yeast of experimented fruit.

NO.	Kinds	Brand Name	Prefecture	Yeast	
1	Grape	Kyoho	Fukuoka	×	
2		Alexandria	Okayama	×	
3		Pione	Hiroshima	×	
4		BerryA	Fukuoka	○	
5		Delaware	Shimane	×	
6		Delaware	Yamanashi	×	
7	Cherry	—	Yamagata	○	
8	Loquat	—	Kagoshima	○	
9	Apple	Sekaiichi	Aomori	×	
10		Jhonagold	Aomori	×	
11		Ourin	Aomori	×	
12		Sunfuji	Aomori	×	
13		Sunfuji	Aomori	×	
14		Koutoku	Aomori	×	
15		Kougyku	Aomori	×	
16		Mutsu	Aomori	×	
17		Kinseiringo	Aomori	×	
18		—	Aomori	×	
19		Sunjhonagold	Aomori	×	
20		Sunfuji	Nagano	×	
21		Ourin	Nagano	×	
22		Fuji	Nagano	×	
23		Jhonagold	Nagano	×	
24		Ourin	Fukushima	×	
25		Sunfuji	Iwate	×	
26		Sunfuji	Ishikawa	×	
27		Sunfuji	Fukuoka	×	
28		Sunfuji	Fukuoka	×	
29		La France	—	Yamagata	○
32		Kiwi	—	Fukuoka	×
33		Mandarin Orange	—	Fukuoka	×
34		Persimmon	—	Fukuoka	×
35		Papaya	—	Okinawa	×
36		Passion Fruit	—	Okinawa	×
37		Peach	—	Yamaguchi	×
38		Melon	—	Yamaguchi	×
39	Pear	Kousuinashi	Kumamoto	×	
40		Atagonashi	Fukuoka	○	
41		bansankichi	Oita	×	
42		Japanese Orange	Hyuganatsu	Miyazaki	×

※ — indicates the brand name is unknown or is not set.  
 ※ ○ indicates yeast was found and × indicates yeast was not found.

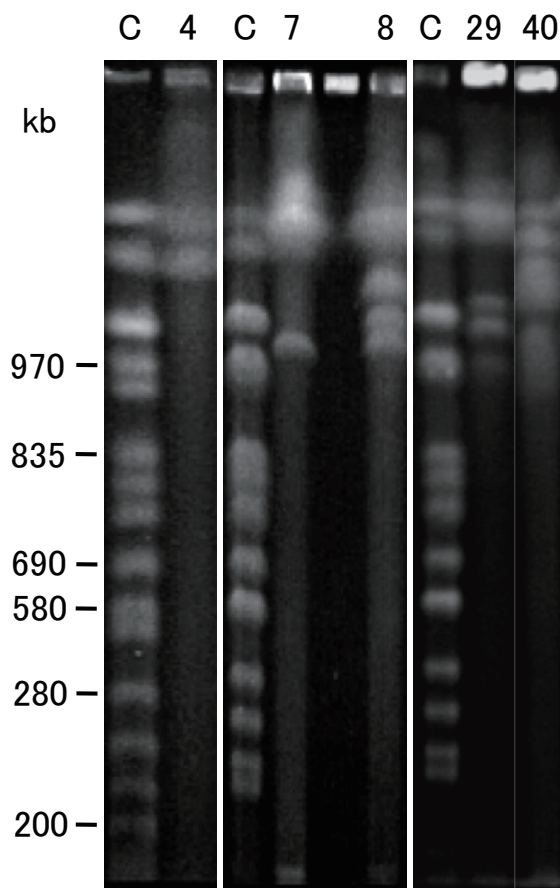


Figure 1. The result of CHEF analysis. Lane C is yeast type culture, *Saccharomyces cerevisiae* S288C used as a size marker of chromosomes. Lane 4 is a yeast's karyotype from Berry A (Fukuoka prefecture), lane 29 is one from the la france (Yamagata prefecture), lane 40 is one from the pear (Atagonashi, Fukuoka prefecture), lane 7 is one from the cherry (Yamagata prefecture) and lane 8 is one from the loquat (Kagoshima prefecture). The lane number corresponds to the number shown in Table 1.

### Italian fruit

Yeast was grown on YPD agar plate from 15 fruits out of 30 fruits experimented. The isolation efficiency was 50%. It can be said that 50% is fairly high efficiency compared with the data obtained from Japanese fruit mentioned above.

Table 2 summarized the kind of the fruit experimented and their production area, and the samples that yeast is isolated. Yeast was isolated from fruit of the cactus, the plum, the blue apple, the red apple cultivated in suburbs of Rome, from the grape, the red apple, the blue apple, the pear and the plum cultivated in

suburbs of Florence, and from the two grapes, the red apple, the blue apple, the two pears cultivated in suburbs of Milan. As same as the result obtained from Japanese fruit, the isolated yeast was not associated with neither a kind of the fruit nor the cultivated area.

Figure 2 shows the result of CHEF analysis on the isolated yeast. The karyotype demonstrated that six different yeast strains were successfully isolated finally. The obtained karyotype was compared with literatures data<sup>5-9)</sup> and the genus classes of three strains were estimated as follows. That is, the yeast isolated from the grape (lane 11, the fruit grown around Florence) is *Candida tenuis*, the yeast isolated from the red apple (lane 15, the fruit grown around Florence) is *Pichia amethiona*, the yeast isolated from the grape (lane 23, the fruit grown around Milan) is *Pichia segobiensis*, the yeast isolated from the red apple (lane 25, the fruit grown around Milan) is *Candida blankii*, the yeast isolated from the blue apple (lane 26, the fruit grown around Milan) is *Pichia bimundalis*, and the yeast isolated from the pear (lane 27, the fruit grown around Milan) is *Candida blankii*. *Pichia kudravzevii* and *Saccharomyces cerevisiae* were not found either here as same the result as Japanese fruit.

Table 2. The cultivated area in Italy and isolated yeast of experimented fruit.

NO.	Kinds	Area	Yeast
1	Grape	Rome	×
2	Grape		×
3	Fruit of the cactus		○
4	Plum		○
5	Pear		×
6	Pear		×
7	Apricot		×
8	Blue Apple		○
9	Red Apple		×
10	Red Apple		○
11	Grape	Florence	○
12	Grape		×
13	Grape		×
14	Grape		×
15	Red Apple		○
16	Red Apple		×
17	Blue Apple		○
18	Pear		○
19	Pear		×
20	Pear		×
21	Prune	×	
22	Plum	○	
23	Grape	Milan	○
24	Grape		○
25	Red Apple		○
26	Blue Apple		○
27	Pear		○
28	Pear		○
29	Plum		×
30	Plum		×

※ ○ indicates yeast was found and × indicates yeast was not found.

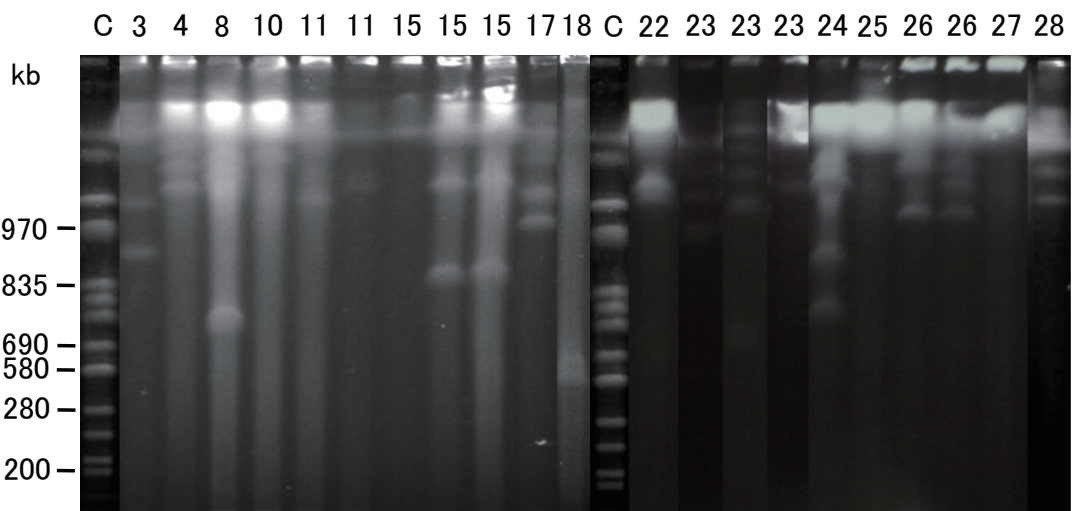


Figure 2. The result of CHEF analysis. Lane C is yeast type culture, *Saccharomyces cerevisiae* S288C used as a size marker of chromosomes. The lane number corresponds to the number shown in Table 2.

### General consideration

From Japan and Italian fruit, the yeast of the same genus class was not isolated. However, the species were different, but yeast of the same genus was isolated about the *Pichia* genus and the *Candida* genus. About *Pichia kudravzevii* and *Saccharomyces cerevisiae* where it had been said to that they were isolated from fruit generally, they were not found from the fruit of the two countries. The isolation efficiency may have been decreased probably in these about 20 years, that is to say, numbers of yeast alive within or on the fruit has fairly decreased and yeast flora has changed remarkably. It is thought that yeast flora have changed by some kind of environmental factor during several decades and these decreased by the absolute number of yeast. A similar thing is observed about the *Bacillus natto*. Around 20 years ago, so many types of *Bacillus natto* were isolated by most rice fields or post-harvest rice straw in Fukuoka prefecture, but is almost destroyed now.<sup>11)</sup> A pesticide is considered as one of the factor considered most. If a pesticide assumes it a cause, the difference with Japan and Italy provided in this study is explicable. In the meantime, it is estimated that the microbial flora of the natural world changes in every moment before we notice. It is unknown whether it is a bad happening whether it is a thing having good it for the human or natural world itself at this stage. It will be necessary to perform continuous research like this time in future and the study would be accurate by squeezing the study in some specific areas. And it will be necessary to pursue it to know what a cause is or whether the change is desirable for our environment.

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原 著

## 日本とイタリアの果物からの酵母の分離および その染色体の電気泳動核型分析

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### <要 旨>

現地で栽培された日本とイタリアの果物から酵母の純粋分離を試みた。その結果、国内産の果物については、17県で栽培された果物40試料のうち5試料から6株の酵母が分離され、染色体の電気泳動核型分析によって、そのうち3株の属種が同定された。果物試料数からの分離効率は12.5%であり、20年前の分離効率100%に比べて国産果物に棲息する酵母が激減していることが推定された。激減した原因は明確ではないが、散布されている農薬の種類が変遷してきたことによる影響が考えられる。これは、福岡県の水田から納豆菌がほぼ全滅したという報告と一致した結果であり、自然環境中のマイクロフローラがここ数十年の間に激変していることが懸念され、これについては長期的スパンでの研究が望まれるところである。イタリアの果物については、3都市の近郊で栽培されている果物30試料のうち15試料から21株の酵母が分離され、染色体の電気泳動核型分析によって、そのうち6株の属種が同定された。果物試料数からの分離効率は50%であった。

キーワード：日本の果物、イタリアの果物、酵母の分離、核型分析

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