

NUMBER 18

INSTITUTE FOR FERMENTATION, OSAKA

# RESEARCH COMMUNICATIONS

(1995-1996)

**1997**

財団法人 発酵研究所

# RESEARCH COMMUNICATIONS

No. 18

(1995–1996)



1 9 9 7

**INSTITUTE FOR FERMENTATION, OSAKA (IFO)**

Published by  
INSTITUTE FOR FERMENTATION, OSAKA  
17-85, JUSO-HONMACHI 2-CHOME  
YODOGAWA-KU, OSAKA 532, JAPAN  
Copyright 1997

## INSTITUTE FOR FERMENTATION, OSAKA (IFO)

*CHAIRMAN* Katsura MORITA

### *BOARD OF TRUSTEES*

|                |                |
|----------------|----------------|
| Teruhiko BEPPU | Saburo FUKUI   |
| Osamu HAYAISHI | Teiji IIJIMA   |
| Yoshio OKADA   | Yoshiro OKAMI  |
| Yukio SUGINO   | Masao TAKEUCHI |
| Hideaki YAMADA |                |

### *AUDITORS*

|               |                 |
|---------------|-----------------|
| Ryohei KIZAKI | Toshio MIWATANI |
|---------------|-----------------|

### *COUNCILORS*

|                    |                    |
|--------------------|--------------------|
| Masahiko FUJINO    | Shinsaku HAYASHIDA |
| Tsuguo HONGO       | Masao ISONO        |
| Akira KIMURA       | Kazuo KOMAGATA     |
| Ken-ichi MATSUBARA | Einosuke OHMURA    |
| Yasuji OSHIMA      | Rokuro TAKEDA      |
| Gakuzo TAMURA      | Fusao TOMITA       |
| Kumao TOYOSHIMA    | Keisuke TUBAKI     |

### *HONORARY MEMBERS*

|                |                 |
|----------------|-----------------|
| Shigeyasu AKAI | Takezi HASEGAWA |
| Yuji SASAKI    |                 |

### *IFO STAFF*

Director Masao TAKEUCHI

#### Research Staff

Fungi: Tadayoshi ITO, Akira NAKAGIRI, Izumi OKANE

Yeasts: Kozaburo MIKATA, Kumiko UEDA

Bacteria, Bacteriophages, Actinomycetes:

Kazunori HATANO, Tadashi NISHII, Mariko TAKEUCHI,  
Yasuyoshi NAKAGAWA, Tomohiko TAMURA

Animal Cells: Touho YOSHIDA, Motonobu SATOH

Business Staff Masayoshi MORIWAKI, Kinuko SHIOYAMA,  
Takayoshi MORITA, Kuniko SATOH

## CONTENTS

|   |     |
|---|-----|
| REPORT OF THE DIRECTOR: M. TAKEUCHI .....   | 1   |
| ORIGINAL REPORTS  |     |
| Detection of human papillomavirus types 16, 18, and 33 in cell lines<br>derived from human genital organs by polymerase chain reaction:<br>H. KUNO and T. YOSHIDA .....                             | 6   |
| Enhancement of neuronal progenitor cell divisions by fibroblast growth<br>factor-2 and leukemia inhibitory factor in organ culture of murine<br>olfactory epithelium: M. SATOH and T. YOSHIDA ..... | 13  |
| Detection of heterogeneity of 18S rRNA inter-genes and mutation arising<br>during PCR amplification: K. UEDA and K. MIKATA .....  | 20  |
| Actinomycete populations in mangrove rhizospheres: K. HATANO .....  | 26  |
| A mycofloral study on mangrove mud in Okinawa, Japan:<br>T. ITO and A. NAKAGIRI .....   | 32  |
| Mycoflora of the rhizospheres of mangrove trees: T. ITO and A. NAKAGIRI .....   | 40  |
| Preliminary study of endophytic fungi in evergreen plants from<br>Ishigaki and Iriomote islands: I. OKANE, A. NAKAGIRI and T. ITO .....   | 45  |
| Amplification and sequencing of mitochondrial cytochrome c oxidase<br>subunit II gene for phylogenetic analysis of yeast:<br>Y. NAKAGAWA and K. MIKATA .....  | 52  |
| Conidium development of an aero-aquatic hyphomycete, <i>Peyronelina</i><br><i>glomerulata</i> : A. NAKAGIRI and T. ITO .....  | 57  |
| DESCRIPTIVE CATALOGUES  |     |
| Descriptive catalogue of IFO fungus collection XV .....   | 63  |
| Descriptive catalogue of IFO actinomycetes collection III .....   | 70  |
| ANNOUNCEMENTS   |     |
| Catalogue of newly accepted strains .....   | 73  |
| Scientific papers, 1995-1996 .....  | 93  |
| Presentation of papers at scientific meetings, 1995-1996 .....  | 100 |
| Miscellaneous scientific papers, 1995-1996 .....  | 106 |
| Distributive regulations .....  | 107 |
| Corrections .....   | 108 |



## Report of the Director

Masao TAKEUCHI

Since its establishment in 1944, the Institute for Fermentation, Osaka (IFO) has strongly endeavored to maintain culture collection for over 50 years with the total support of Takeda Chemical Ind. Ltd. The IFO is an affiliate of approximately 800 culture collection centers around the world, that are listed in the World Directory of Collections of Cultures of Microorganisms (WDCM). The number of strains stored in IFO and its activities rank it as a leading center among the culture collections in the world. An important role of the IFO will be not variable for biological researchers as a nonprofit organization of culture collection. The IFO expects to continue its work through continued support by generous donors.

Dr. Masao Takeuchi took office as the director of the IFO April 1995, after the previous director retired. The financial situation of the IFO has deteriorated, since 1991 as interests obtained from endowments decreased. To improve economic situation of the IFO, the management of the institute was reorganized. To avoid a qualitative change in services to clients, the IFO was compelled to decrease personnel expenses and furthermore, to increase the income of the IFO, has entered into cooperation with the Japan Health Sciences Foundation beginning in 1995 by managing a research resources bank for the Ministry of Health and Welfare.

In the past two years, the IFO organized the following two meetings: The 3rd Annual Meeting of the Japan Society for Culture Collections on 27 and 28 June 1996, and The 16th Meeting for Classification of Microorganisms on 15 and 16 November 1996. These two meetings were both excellent meetings related to culture collection organization in Japan, and successfully highlighted the activities of the IFO.

Dr. Akira Nakagiri was awarded a Scientific Study Award for ecological research on marine fungi in 1995 by the Mycological Society of Japan, and Mr. Tomohiko Tamura was awarded the Hamada Award (Formerly SAJ Encouragement Award) for study on the classification of actinomycetes in 1995 by the Society for Actinomycetes Japan.

### Personnel Affairs

In April 1995 Dr. Toru Hasegawa moved to Hiroshima University and was resigned from the service of the director of the IFO. He was succeeded by Dr. Masao Takeuchi,

the deputy director of the IFO. At the 100th meeting of the Board of Trustees in March 1995, Dr. Masao Takeuchi was nominated as a member of the Board of Trustees, Mr. Ryohei Kizaki was nominated as auditor, and Dr. Toru Hasegawa were nominated as councilor.

At the 103rd meeting of the Board of Trustees in May 1996, Dr. Masahiko Fujino of Takeda Chemical Industries Ltd., was nominated as a councilor from June 1996.

In December 1995 Syunji Ietuka, treasurer of the institute, retired; and in January 1997 Hisayasu Suzuki retired and in May 1996 Dr. Ko Imai moved to the Japan Science and Technology Corporation. In April 1996 Mr. Tateo Hasegawa; in July 1996 Mr. Takeshi Sakane, Mr. Ken-ichi Kuroshima, and Ms. Etsuko Imanishi; and in October 1996 Mr. Haruhiko Kuno and Ms. Yumiko Nakagaito moved to Takeda Chemical Industries Ltd. The IFO is grateful to these persons for their dedication while working in the institute.

The new treasurer of the institute, Mr. Masayoshi Moriwaki, joined the IFO in November 1995 and was appointed as treasurer on 1 December 1995.

### **International Meetings**

The stuffs of the IFO attended the international meetings during these two years, and presented scientific papers at the meetings and promoted mutual cooperation between collections in the world.

Dr. Akira Nakagiri, attended the International Marine Mycology Symposium, held in England in July 1995, and presented papers on "Growth and reproduction of *Halophytophthora* species in mangrove ecosystems" and "Morphology and taxonomy of *spathulosporaceous* fungi" and he also attended the Diversities in Western Pacific and Asia International Workshop on Biodiversity and the Dynamics of Ecosystems, held in Singapore in December 1995, and presented a paper on "Biodiversity and ecology of *Halophytophthora*."

Dr. Akira Nakagiri and Mr. T. Ito attended the Asia International Mycological Congress in Chiba in July 1996, and they presented papers on "Ecology of a versatile fungus, *Dactylella iridis*" and "Preservation and maintenance of fungus cultures at the Institute for Fermentation, Osaka (IFO)," respectively.

Dr. Masao Takeuchi attended the 8th International Congress for Culture Collection, held in the Netherlands in August 1996 and presented papers on "Recent Activities of the IFO culture collection" and "Detection of reverse transcriptase in cell cultures by polymerase chain reaction." Mr. Tadayosi Ito attended the same congress and presented papers on "Preservation of fungal cultures at the Institute for Fermentation, Osaka (IFO), and "Preservation of fungus cultures by L-drying at the Institute for Fermentation, Osaka (IFO)."

### **Collection and Publications**

The total number of cultures stored in the IFO culture collection reached 15,309 at the

end of 1995 and 15,600 at the end of 1996. The newly accepted strains during each year are listed in the present issue of the IFO Research Communications. The total number of cultures distributed from the IFO culture collection reached 8,000 in 1995 and 8,500 in 1996.

IFO Research Communications No. 17 was published as a commemorative issue on the 50th anniversary of the IFO in March 1995, and the IFO List of Cultures, 10th edition, Microorganisms, was published in March 1996. The List of Animal Cell Lines was published in 1994. These catalogues involve about 10,800 strains altogether, that is, 5,800 fungi, 2,800 bacteria, 2,000 yeasts, 60 bacteriophages and 170 animal cell lines. Manuscript for these catalogues were prepared and edited from the database of the IFO culture collection on an IBM /36 computer. Data was transferred to an NEC 9800 computer and printed through a Macintosh computer. A new pamphlet was published in June 1995 describing the activities of the IFO in that year.

### Finances

On the establishment of a cell line section in 1984, Takeda Chemical Industries, Ltd. decided to give ¥45 million to the IFO as additional support to promote its activities every year from the fiscal year 1986. The donor also helped the IFO by increasing of total ¥300 million foundation in 1986 and 1987. Through this generosity, the IFO staff have been able to show their real abilities in the international activities of culture collection study. The IFO staff would like to express their profound gratitude to Takeda Chemical Industries, Ltd. for over 50 years of continuous support. The financial standing of the IFO in recent years is summarized in Table 1, which shows the annual income and expenses of the IFO for each fiscal year from 1991 to 1995.

Table 1 CLOSING ACCOUNTS OF IFO

|                               | (100 thousand yen) |       |       |       |              |
|-------------------------------|--------------------|-------|-------|-------|--------------|
| Year                          | 1991               | 1992  | 1993  | 1994  | 1995         |
| <b>INCOME:</b>                |                    |       |       |       |              |
| Donations                     | 1,750              | 1,750 | 1,750 | 1,750 | 1,950 (57%)  |
| Interest & Dividends          | 1,032              | 923   | 778   | 635   | 550 (16%)    |
| Grants & Contracts            | 0                  | 0     | 0     | 0     | 170 (5%)     |
| Cultures                      | 366                | 377   | 387   | 487   | 537 (15%)    |
| Other Services                | 99                 | 89    | 99    | 96    | 234 (7%)     |
| Total                         | 3,247              | 3,139 | 3,014 | 2,968 | 3,441 (100%) |
| <b>EXPENSES:</b>              |                    |       |       |       |              |
| Personnel Expenses            | 2,135              | 2,120 | 2,296 | 2,521 | 2,439 (71%)  |
| Supplies                      | 401                | 441   | 435   | 246   | 316 (9%)     |
| Facility Maintenance Expenses | 329                | 369   | 395   | 390   | 398 (12%)    |
| Other                         | 183                | 222   | 181   | 249   | 267 (8%)     |
| Total                         | 3,040              | 3,152 | 3,307 | 3,406 | 3,420 (100%) |
| Balance                       | 199                | -13   | -293  | -438  | 21           |

Takeda Chemical Industries Ltd. has continued to support us with annual donations

of ¥175 million (¥195 million in 1995). "Interest and Dividends" is income acquired from the ¥10 billion endowment. "Cultures" indicates income acquired from distribution fees for cultures. "Other services" includes income from commission of patent deposits or safety deposits. "Grants and Contracts" refers to a contract with the Japan Health Sciences Foundation for management of the HSRRB bank. "Supplies" means expenses for purchase of expendable supplies and equipment. "Facility Maintenance Expenses" is composed of expenses related to rent of facilities used by the IFO. This table shows that annual income decreased from 1991 to 1994. The main cause of this is a falloff of the interest acquired from the endowment of ¥10 billion due to reduction of official rate. As a consequence of an increase in donations, contracts and other services (for example, commission of patents and safety deposits), total income increased in 1995.

On the other hand, IFO's expenses continued to increase by ¥10-15 million annually from 1991 to 1994. Increasing personnel expenses were the major contributory factor. The balance showed a deficit from 1992, which reached ¥43.8 million in 1994. In fiscal year 1995, this was turned to a ¥2.1 million surplus as a result of increased income and decreased expenses, but it is difficult to predict the outcome of the following year's account. Judging from the fiscal data, the IFO should continue to consider ways to cut expenses in order to rebuild its finances.

### Visitors

The IFO has welcomed a number of foreign visitors in the past two years:

Dr. W. Potacharoen, Curator, TISTR Culture Collection, Bangkok, Thailand, on 20 March 1995.

Dr. S. S. Miyazaki, Investigator UBA-CONICET, Capital Federal, Argentina, on 10 May 1995.

Dr. D.-H. Lee, Forestry Research Institute, Seoul, Korea, on 11 July 1995.

Dr. N.B. Lantican, National Institute of Molecular Biology and Biotechnology, Laguna, Philippines, on 23 October 1995

Dr. K.-S., Chung, Director of Food Biotechnology Division, Republic of Korea, on 28 November 1995.

Dr. J.-H. Yu, Yonsei University, Seoul, Korea, on 15 February 1996.

Dr. V. Arunpairojana, TISTR Culture Collection, Bangkok, Thailand, on 29 March 1996.

Dr. S.-K. Kim, Soosan National University, Pusan, Korea, on 3 April 1996.

Dr. T. Myoda, Prof. Microbiology, Rockland, USA, on 18 April 1996.

Dr. I. Gandjar and M. Asturi, University of Indonesia, Indonesia, on 19 June 1996.

Dr. T.B. Thuan, Hanoi University, Hanoi, Vietnam, on 18 September 1996.

Dr. P. Atthasampunna and Dr. A. Mahakhant, Thailand Institute of Scientific and Technological Research, on 29 October 1996.

Lectures or seminars were given by the following guests. Their useful speeches created a great impression for us.



Dr. R.J. Hay, ATCC, on March 10, 1995: Management of ATCC Cell Bank.

Dr. P. Atthasampunna, Thailand Institute of Scientific and Technological Research, on October 29 1996: Culture collections in South East Asia.

---

Professor emeritus Dr. Tokuya Harada, councilor of the institute, passed away on 29th August 1996. He made great contributions to the development of the Institute for Fermentation, Osaka. Our heartfelt condolence is extended to the bereaved.

---

## **Detection of Human Papillomavirus Types 16, 18, and 33 in Cell Lines Derived from Human Genital Organs by Polymerase Chain Reaction**

Haruhiko KUNO and Touho YOSHIDA

### **Summary**

Human papillomavirus (HPV) types 16, 18, and 33 play a causative role in the development of uterine cervix cancer. Fourteen cell lines derived from human genital organs are maintained at the Institute for Fermentation, Osaka (IFO). To know whether these cell lines were infected by HPV types 16, 18, and 33, we tried to detect these viral DNAs by polymerase chain reaction (PCR) with specific primers homologous to the E6 open reading frame. All seven cell lines derived from uterine cervix carcinoma possessed the HPV types 16 or 18 DNA, whereas none of seven cell lines derived from uterus body cancer or ovarian cancer possessed the HPV types 16, 18, and 33 DNA. In addition, this PCR was applicable to detect the cross-contamination of cell lines by HeLa cells.

**Keywords:** human papillomavirus, polymerase chain reaction, cell lines.

To authenticate the quality of cell lines maintained at the Institute for Fermentation, Osaka (IFO), we routinely check bacterial and fungal contaminations (16) and cross-contaminations (11). In recent years, there has been an increasing awareness of bio-hazards associated with viral contaminations in cell lines, e.g., hepatitis B virus and human immunodeficiency virus. Like mycoplasmas, viruses usually remain undetected in cell lines as latent contaminants and can cause serious complications in the interpretation of experimental results. At the American Type Culture Collection, virus detection has been conducted as part of the quality control of cell lines using a cytopathogenic test, an inoculation test to chick embryos, and a biochemical test for reverse transcriptase (7). Recently, we have developed a method for detecting retroviruses in cell lines by polymerase chain reaction (PCR) (9). We are now examining methods for detecting specifically each type of virus.

Human papillomaviruses (HPVs) are recognized as human pathogens of major importance involved in the pathology of benign and malignant lesions of cutaneous and

mucosal epithelium. HPV types 16, 18, and 33 are closely associated with malignant progression of the uterine cervix and their DNA has frequently been found in biopsies and in cell lines derived from uterine cervix cancer (3, 12). These so-called high-risk HPV types are found to integrate their DNA into the host genome. Some regions of the HPV DNA are missing in the host genome, but the E6 and E7 regions are preferentially conserved (15). It has been shown that the E6 gene product of these high-risk HPVs binds and degrades the p53 tumor suppressor gene protein (19), while the E7 gene product binds the Rb tumor suppressor gene protein (4). We maintain 14 cell lines derived from human genital organs. In this study, we examined these cell lines for the presence of E6 gene of HPV types 16, 18, and 33 by PCR. In addition, we tried to apply this PCR method for the detection of cross-contamination of cell lines by HeLa cells.

### Materials and Methods

**Cell lines.** Seventeen human cell lines that have been maintained at IFO were used in this study. The establishment and characterization of each cell line are described in the IFO catalogue of animal cell lines (17). CaSki (IFO 50007), SKG-IIIa (IFO 50310), SKG-IIIb (IFO 50311), BOKU (IFO 50323), SKG-I (IFO50308), SKG-II (IFO50309), and HeLaS3 (IFO 50011) are cell lines derived from uterine cervix carcinoma. SKN (IFO 50314), NJG (IFO 50322), SNG-II (IFO 50312), and SNG-M (IFO 50313) are cell lines derived from uterus body cancer. RMG-I (IFO 50315), RMUG-S (IFO 50320), and RMUG-L (IFO 50319) are cell lines derived from ovarian cancer. The original sources of WISH (IFO 50004), KB, and Chang Liver (IFO 50016) were described in the papers of their establishments as the amnion (8), oral epidermoid carcinoma (5), and the liver (2), respectively. HeLaS3, WISH, Chang Liver, and KB were cultured in Eagle's minimum essential medium supplemented with 10% fetal calf serum. Other cell lines were cultured

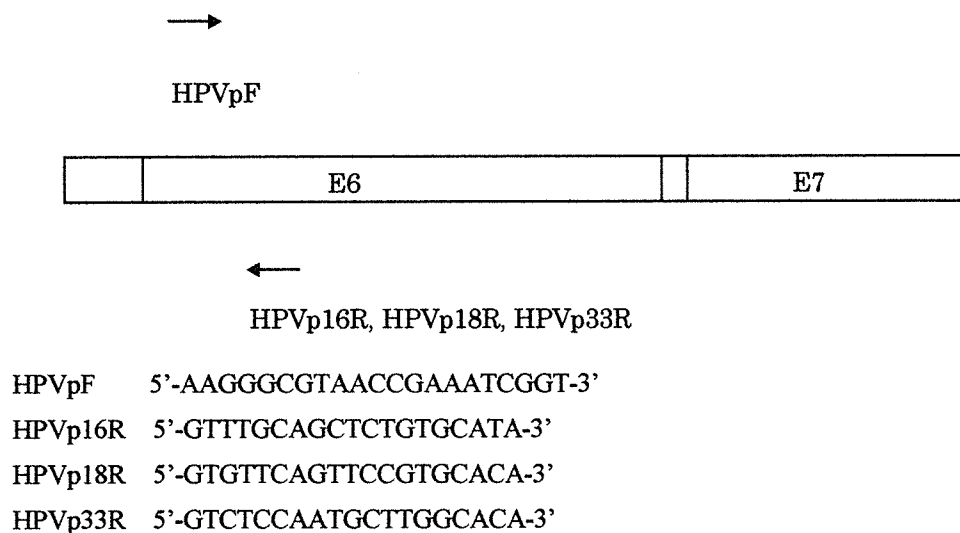


Fig. 1. Sequences of specific oligonucleotide primers used in the PCR. These primers correspond to sequences homologous to the E6 open reading frame of HPV types 16, 18, or 33.

in Ham's F12 medium supplemented with 10% fetal calf serum.

**PCR.** High-molecular-weight DNA from cell lines was prepared by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. PCR was performed by the method of Shimada *et al.* (14). The PCR primers were purchased from Takara Shuzo Co. Ltd. and their sequences are shown in Fig. 1. The primers correspond to the sequences homologous to the E6 open reading frame of HPV types 16, 18, or 33. The forward primer HPVpF was used as the common primer of HPV types 16, 18, and 33. The reverse primers, HPVp16R, HPVp18R, and HPVp33R, were used as the specific primers for the antisense sequences of HPV types 16, 18, and 33, respectively. The reaction mixture (50  $\mu$ l) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 2.5 mM deoxynucleotide triphosphate (each), 1  $\mu$ g of genome DNA, 1.25U of Taq polymerase, and 25 pmol of primers (each). The mixture was overlaid with mineral oil, then the reaction was cycled 30 times with denaturation at 94 °C for 0.5 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min. After the amplification, aliquots (10  $\mu$ l) of the PCR products were electrophoresed in 2.0% agarose gels and stained with ethidium bromide. Only samples with a clearly visible band of the correct size, namely, 140-bp, for the primer pairs in the PCR were considered to be positive. For a control of the PCR, a 262-bp sequence of human  $\beta$ -globin gene was amplified by use of the primers GGTGGCCAATCTACTCCCAGG (forward) and TGGTCTCCTTAAACCTGTC-TTG (reverse).

### Results and Discussion

A total of 14 cell lines derived from human genital organs were examined for the presence of DNA sequences of HPV types 16, 18, and 33. High-molecular-weight DNA was isolated from each cell line and analysed by PCR. We employed a sensitive and HPV type-specific PCR that was developed by Shimada *et al.* (14). Figure 2 shows the profiles of PCR products on agarose gel electrophoresis. Using the primer pair of HPVpF and HPVp16R, the predominant 140-bp band of HPV type 16 DNA was amplified in DNA of CaSki, which has been reported to possess HPV type 16 (13), but not amplified in DNA of HeLaS3 containing HPV type 18 (1). In the case of HPVpF and HPVp18R, the predominant 140-bp band of HPV type 18 DNA was amplified in DNA of HeLaS3, but not amplified in DNA of CaSki. No 140-bp band of HPV type 33 was amplified by HPVpF and HPVp33R in DNA of CaSki and HeLaS3, whereas many HPV-unrelated extra bands appeared. The results in Fig. 2 indicate that these primers are useful for the specific detection of E6 gene of HPV types 16, 18, or 33.

Table 1 summarizes the results for 14 cell lines derived from human genital organs. The DNA sequences of HPV types 16 or 18 were detected in all seven cell lines derived from uterine cervix cancer. Among them, four cell lines possessed the HPV type 16 DNA sequence and three cell lines possessed the HPV type 18 DNA sequence. Tsunokawa *et al.* (18) and Shirasawa *et al.* (15) have previously shown by Southern blot analysis that SKG-I and SKG-II possess the HPV type 18 DNA sequence and SKG-IIIa and SKG-IIIb possess the HPV type 16 DNA sequence. We confirmed their findings by PCR.



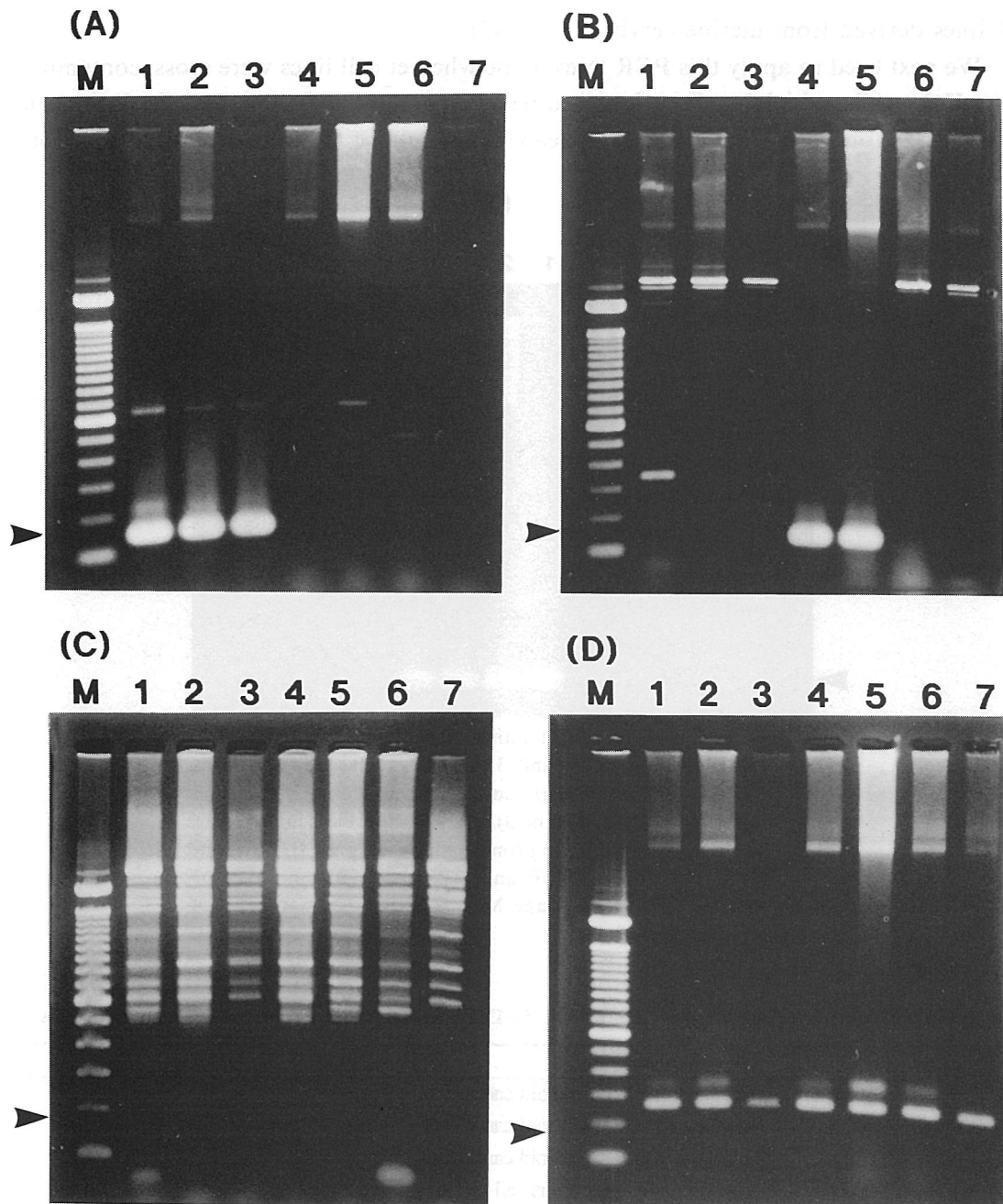


Fig. 2. Agarose gel electrophoresis of HPV type 16, 18, or 33 DNA sequences in seven cell lines. Cellular DNAs ( $1 \mu\text{g}$ ) isolated from CaSki (lane 1), SKG-IIIa (lane 2), BOKU (lane 3), HeLaS3 (lane 4), SKG-I (lane 5), RMG-I (lane 6), and NJG (lane 7) were subjected to PCR. The primer pairs used were HPVpF and HPVp16R (A), HPVpF and HPVp18R (B), and HPVpF and HPVp33R (C). The primers for the amplification of  $\beta$ -globin (D) are described in Materials and Methods. Lane M, molecular size markers. The arrowhead, 140-bp.

None of the seven cell lines derived from uterus body cancer or ovarian cancer possessed the DNA sequences of HPV types 16, 18, and 33. These results on IFO cell lines are in agreement with the findings of high incidence of HPV types 16 and 18 in biopsies and in

cell lines derived from uterine cervix cancer (12).

We next tried to apply this PCR to examine whether cell lines were cross-contaminated by HeLa cells, which has been found to be a contaminant of a number of cell lines (6). Characterization performed at the American Type Culture Collection showed that some

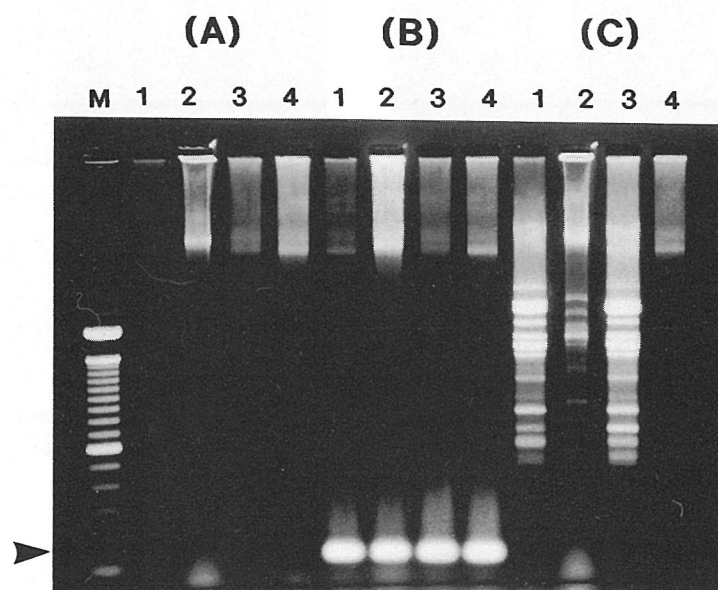


Fig. 3. Detection of cross-contamination by HeLa cells by amplifying HPV type 16, 18 and 33 DNA sequences in cell lines. Cellular DNAs ( $1 \mu\text{g}$ ) isolated from HeLaS3 (lane 1), WISH (lane 2), KB (lane 3), and Chang Liver (lane 4) were subjected to PCR. The primer pairs used were HPVpF and HPVp16R (A), HPVpF and HPVp18R (B), and HPVpF and HPVp33R (C). Lane M, molecular size markers. The arrowhead, 140-bp.

Table 1. Detection of HPV types 16, 18, and 33 DNA in cell lines derived from genital organs.

| Cell lines                     | IFO No. | Origins of cell lines                   | type 16 | type 18 | type 33 |
|--------------------------------|---------|---|---------|---------|---------|
| CaSki                          | 50007   | uterine cervix, epidermoid carcinoma    | +       | -       | -       |
| SKG-IIIa                       | 50310   | uterine cervix, epidermoid carcinoma    | +       | -       | -       |
| SKG-IIIb                       | 50311   | uterine cervix, epidermoid carcinoma    | +       | -       | -       |
| BOKU                           | 50323   | uterine cervix, squamous cell carcinoma | +       | -       | -       |
| SKG-I                          | 50308   | uterine cervix, epidermoid carcinoma    | -       | +       | -       |
| SKG-II                         | 50309   | uterine cervix, squamous cell carcinoma | -       | +       | -       |
| HeLaS3                         | 50011   | uterine cervix, epidermoid carcinoma    | -       | +       | -       |
| SKN                            | 50314   | uterus, leiomyosarcoma                  | -       | -       | -       |
| NJG                            | 50322   | uterus, choriocarcinoma                 | -       | -       | -       |
| RMG-I                          | 50315   | ovary, clear cell carcinoma             | -       | -       | -       |
| RMUG-S                         | 50320   | ovary, mucinous cystadenocarcinoma      | -       | -       | -       |
| RMUG-L                         | 50319   | ovary, mucinous cystadenocarcinoma      | -       | -       | -       |
| SNG-II                         | 50312   | uterine endometrium, adenocarcinoma     | -       | -       | -       |
| SNG-M                          | 50313   | uterine endometrium, adenocarcinoma     | -       | -       | -       |
| +, detected    -, not detected |         |   |         |         |         |

human cell lines have two or more HeLa marker chromosomes and type A isozyme for glucose-6-phosphate dehydrogenase, indicating that these cell lines may be contaminated with or replaced by HeLa cells (10). We examined three IFO cell lines, WISH (IFO 50004), KB, and Chang Liver (IFO 50016), which were shown to have HeLa cell markers, for the cross-contamination by HeLa cells using the PCR. These three cell lines possessed the HPV type 18 DNA sequence as well as HeLaS3 (Fig. 3). The original sources of WISH, KB, and Chang Liver are described as the amnion, oral epidermoid carcinoma, and the liver, respectively, in which HPV is usually absent. These results indicated the strong possibility that these cell lines are cross-contaminated by HeLa cells. This PCR is expected to be applicable for diagnosing and defining cross-contamination, together with the classical glucose-6-phosphate dehydrogenase isozyme test and chromosomal analysis.

The results of this study provided important information on the cell lines. First, we can note that the cell lines possessing HPV types 16, 18, and 33 present potential biohazards. Second, the presence of HPV genes in cell lines is informative data for the characterization of cell lines, since the products of the E6 and E7 genes exert a great influence on the growth and differentiation of the host cells (4, 19). Third, the presence of the HPV type 18 DNA sequence in cell lines derived from sources other than the uterine cervix revealed the strong possibility of the cross-contamination of these cell lines by HeLa cells. This simple PCR should become one of quality control methods for human cell lines.

### References

- 1) Boshart, M., L. Gissmann, H. Ikenberg, A. Kleinheinz, W. Scheurlen, and H.A. zur Hausen. 1984. New type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J.* 3: 1151-1157.
- 2) Chang, R.S. 1954. Continuous subcultivation of epithelial-like cells from normal human tissues. *Proc. Soc. Exp. Biol. Med.* 87: 440-443.
- 3) Durst, M., L. Gissmann, H. Ikenberg, and H.A. zur Hausen. 1983. A papillomavirus DNA from a cerevical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc. Natl. Acad. Sci. USA*, 80: 3812-3815.
- 4) Dyson, N., P.M. Howley, K. Munger, and E. Harlow. 1989. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science*, 243: 934-937.
- 5) Eagle, H. 1955. Propagation in a fluid medium of a human epidermoid carcinoma, strain KB. *Proc. Soc. Exp. Biol. Med.* 89: 362-364.
- 6) Gartler, S.M. 1966. Apparent HeLa cell contamination of human heteroploid cell lines. *Nature*, 217: 750-751.
- 7) Hay, R.J., Y.A. Reid, P.R. McClintock, T.R. Chen, and M.L. Macy. 1996. Cell line banks and their role in cancer research. *J. Cell. Biochem.* 24: 107-130.
- 8) Hayflick, L. 1961. The establishment of a line (WISH) of human amnion cells in continuous cultivation. *Exp. Cell Res.* 23: 14-20.
- 9) Kuno, H., H. Ikeda, M. Takeuchi, and T. Yoshida. A simple and sensitive method for the detection of reverse transcriptase activity in cell lines by nested polymerase chain reaction. (submitted).
- 10) Lavappa K.S., M.L. Macy, and J.E. Shannon. 1976. Examination of ATCC stocks for

- HeLa marker chromosomes in human cell lines. *Nature*, 259: 211-213.
- 11) Satoh, M. and M. Takeuchi. 1993. Cross-contamination of cell lines as revealed by DNA fingerprinting in IFO animal cell bank. *IFO Res. Commun.* 16: 18-23.
  - 12) Schiffman, M.H. 1994. Epidemiology of cervical human papillomavirus infections. In H. Hausen (ed.) *Human pathogenic papillomaviruses*. p. 55-82. Springer-Verlag. Berlin.
  - 13) Seedorf, K., T. Oltersdorf, G. Krammer, and W. Rowekamp. 1987. Identification of early proteins of the human papillomaviruses type 16 (HPV 16) and type 18 (HPV 18) in cervical carcinoma cells. *EMBO J.* 6: 139-144.
  - 14) Shimada, M., M. Fukushima, H. Mukai, I.Kato, A. Nishikawa, and K. Fujinaga. 1990. Amplification and specific detection of transforming gene region of human papillomavirus 16, 18 and 33 in cervical carcinoma by means of the polymerase chain reaction. *Jpn. J. Cancer Res.*, 81: 1-5.
  - 15) Shirasawa, H., Y. Tomita, S. Sekiya, H. Takamizawa, and B. Simizu. 1987. Integration and transcription of human papillomavirus type 16 and 18 sequences in cell lines derived from cervical carcinomas. *J. Gen. Virol.* 68: 583-591.
  - 16) Takeuchi, M., T. Yoshida, M. Satoh, H. Kuno, H. Mizusawa, and T. Ohno. 1993. Survey of mycoplasmal contamination in animal cell lines collected by three cell banks in Japan. *Bull. J.F.C.C.* 9: 13-18.
  - 17) Takeuchi, M., T. Yoshida, M. Satoh, Y. Nakagaito, and H. Kuno. 1994. Institute for Fermentation, Osaka (IFO) list of cultures, Animal cell lines, fourth edition.
  - 18) Tsunokawa, Y., N. Takebe, and S. Nozawa. 1986. Presence of human papillomavirus type -16 and type-18 DNA sequences and their expression in cervical cancers and cell lines from Japanese patients. *Int. J. Cancer* 37: 499-503.
  - 19) Werness, B.A., A.J. Levine, and P.M. Howley. 1990. Association of human papillomavirus type16 and 18 E6 protein with p53. *Science*. 248: 76-79.



# Enhancement of Neuronal Progenitor Cell Divisions by Fibroblast Growth Factor-2 and Leukemia Inhibitory Factor in Organ Culture of Murine Olfactory Epithelium

Motonobu SATOH and Touho YOSHIDA

## Summary

A serum-free, organ culture system of olfactory epithelium from embryonic 14.5-day mouse was developed. In this culture system, olfactory neuronal progenitor cells continued to divide as assessed by bromodeoxyuridine (BrdU) incorporation into proliferating cells that later differentiated into neurons. Fibroblast growth factor-2 (FGF-2) and leukemia inhibitory factor (LIF) increased the proportion of BrdU-labeled neurons. This result suggests FGF-2 and LIF act on the olfactory epithelium in total to promote the proliferation of neuronal progenitor cells in the organ culture.

Keywords: olfactory epithelium, neuronal progenitor cell, organ culture, fibroblast growth factor-2, leukemia inhibitory factor

Most types of mammalian neurons are generated in the embryonic stage and are not replaced thereafter. One of the exceptions is sensory neurons in olfactory epithelium, which are continually generated from proliferative progenitor cells even in adult animals (11). The olfactory epithelium consists of multiple cell types that are arranged in an ordered form (8). Horizontal basal cells (also referred to as dark basal cells or basal cells proper) lie along the basal lamina that partitions the underlying connective tissue and olfactory epithelium. Somewhat superficial to the horizontal basal cell layer are located globose basal cells (also referred to as light basal cells). More superficially is an olfactory sensory neuron layer, in which young neurons are situated basally and mature neurons move toward the luminal surface. The most superficial layer facing the luminal surface is that of supporting cells (also referred to as sustentacular cells). The globose basal cells now prove to belong to the neuronal lineage in heterogeneous developmental stages, most being the immediate neuronal progenitor cells that can rapidly proliferate (1, 2, 20, 21), and a few being more immature precursors expressing neuronal determination-related gene *Mash-1* (10, 12). Antigenic heterogeneity of globose basal cells is also

known but the implication of this remains unclear (9). Among the basal cell populations, the presence of true neuronal stem cells that slowly turn over and give rise to neurons *via* a globose basal cell population (defined as transit amplifying cells) has been assumed (5, 15), but until now they remain unidentified.

Olfactory neurogenesis actively occurs under such experimental conditions as the removal of the olfactory bulb (20), the target tissue of olfactory sensory neurons, or the excision of the olfactory axon (22). These conditions first induce the gross retrograde degeneration of preexisting neuron layer and the reduction of epithelial thickness. Thereafter, the neuronal progenitor cells proliferate rapidly in basal region of the epithelium, and they differentiate into new neurons in the prospective neuron layer. Most basally, the horizontal basal cells change from flat to elongated morphology during the active neurogenesis (22, 23). Thus, the olfactory neurogenesis may be regulated by a cascade of tissue interactions in a spatially restricted manner, in which case the local tissue environments must play central roles in the process (7).

To analyze the molecular mechanisms of olfactory neurogenesis, the factors that affect olfactory neurogenesis have been surveyed *in vitro*, and the involvement of several cytokines has recently been reported (3, 5, 13, 16, 19). To further evaluate the precise roles of these cytokines in living animals, however, it is important to reproduce the local environments that arise from three-dimensional tissue organization of olfactory epithelium consisting of heterogeneous cell populations. Organ culture is suitable for this purpose, since the integration of tissues is well retained. In this paper we report a serum-free, organ culture system of olfactory epithelium and the effect of fibroblast growth factor-2 (FGF-2) and leukemia inhibitory factor (LIF) on the proliferation of olfactory neuronal progenitor cells in this system.

### Materials and Methods

**Reagents.** Reagents were purchased from Sigma unless otherwise noted.

**Cultures.** Olfactory epithelia from the turbinal region of day-14.5 C3H/He mouse embryos were isolated as described previously (18). The isolated epithelia were rinsed three times with Hanks' balanced salt solution (HBSS, pH 7.2-7.4; Nissui) containing 50  $\mu$ g/ml kanamycin and vitally stained with 5  $\mu$ g/ml neutral red (Wako) in HBSS for 5 min, which made it easy to find their position on a filter (see below). They were rinsed three times with HBSS and once with a chemically defined basal medium consisting of MCDB153 (Kyokuto Seiyaku), 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 5  $\mu$ g/ml insulin, 0.1% bovine serum albumin (BSA), 0.3 mM  $\text{CaCl}_2$ , and 50  $\mu$ g/ml kanamycin, then put on a cellulose acetate/nitrocellulose filter (pore size; 0.45  $\mu$ m, diameter; 25 mm, Millipore HA filter). The epithelia from 6-7 embryos were collected together on the filter and were covered with small amount of 1% methylcellulose in basal medium to maintain moist conditions during the organ culture. The filter with the explants was set on the support of a stainless steel grid in a 35-mm culture dish containing 2-3 ml of basal medium, in which explants were situated at the interface between humidified air/5%  $\text{CO}_2$  and the medium (Fig. 1). The explants were either

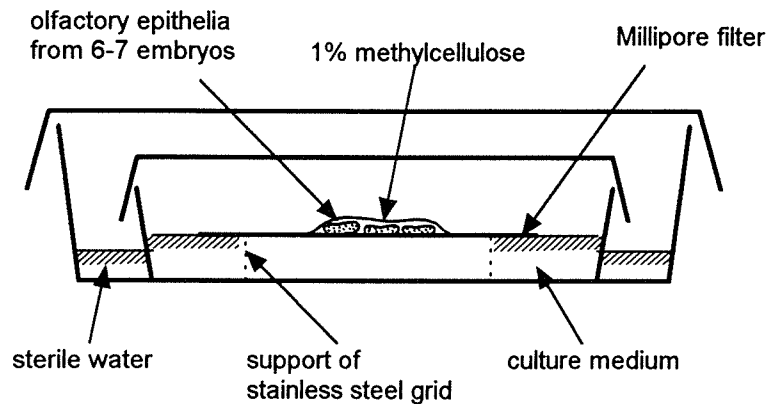


Fig. 1. Schematic illustration of the organ culture method of olfactory epithelium in this study.

cultured with 5 ng/ml recombinant human FGF-2 (Boehringer Mannheim), with  $10^4$  units/ml recombinant murine LIF (ca 100 ng/ml; ESGRO, GIBCO BRL) or without added growth factor during the organ culture period of 48 hr. To label proliferating cells, the cultures were treated with 10  $\mu$ M bromodeoxyuridine (BrdU) and 1  $\mu$ M fluorodeoxyuridine from 24 hr to 48 hr of culture period. After organ culture, the explants were recovered from filters, washed three times with HBSS, and dissociated into single cells by incubation with 0.1% crystalline trypsin (Wako) and 20 units/ml deoxyribonuclease I in phosphate-buffered saline (PBS; pH 7.2-7.4) for 15 min at 37°C. The cells were collected by a brief centrifugation (900 rpm, 3 min) after stopping the action of trypsin with soybean trypsin inhibitor (final concentration; 10 BAEE units/ml), resuspended in basal medium and were cultured on coverslips coated with poly-L-ornithine in a 24-well plate for 1 day. Under the dissociated cell culture conditions in the chemically defined medium, neuronal progenitor cells have been reported mostly to differentiate into neurons within 1 day (2, 19).

**Immunohistochemistry.** The cultures were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and processed for immunohistochemistry for BrdU and class-III  $\beta$ -tubulin. After denaturing DNA with 2N HCl, the cultures were incubated with anti-BrdU monoclonal antibody (Progen Biotechnik; 1:100 dilution in blocking solution consisted of 0.2% Triton X-100, 1% BSA in PBS) overnight at 4°C, and BrdU-incorporated nuclei were visualized by incubation with FITC-conjugated anti-mouse IgG (Cappel; 1:200 dilution). Then they were treated with mouse monoclonal anti-class-III  $\beta$ -tubulin (BioMakor; 1:200 dilution) followed by incubation with Texas red-conjugated anti-mouse IgG (Amersham; 1:200 dilution), each for 30 min, and mounted in 90% glycerin in PBS. The proportion of BrdU-incorporated neurons was determined by counting BrdU- and class-III  $\beta$ -tubulin-positive cells for independent 10 fields under a phase-contrast/fluorescent microscope (Olympus BH2).

## Results

The olfactory epithelia from nasal turbinate were explanted as organ cultures and treated with BrdU during 24–48 hr of the culture period to label the cells synthesizing DNA including the proliferative neuronal progenitor cells. Because no specific marker for the neuronal progenitor cells has been developed to date, they have been characterized as the cells that later give rise to neurons (2, 5). So we set up the additional 1-day dissociated cell culture in which most of them proved to differentiate into neurons immediately (2, 19), and the neurons that derived from the progenitor cells that incorporated BrdU were detected by the double immunostaining for BrdU and for neuron-specific tubulin isoform (class-III  $\beta$ -tubulin) (14, 17). Among the class-III  $\beta$ -tubulin<sup>+</sup> neurons, a certain portion of neurons was recognized as also BrdU<sup>+</sup> ( $10.4 \pm 1.4\%$ ; mean  $\pm$  S. E. M. ( $n = 10$ )), demonstrating that the progenitor cell divisions occur in the organ culture. We next added FGF-2 (5 ng/ml) or LIF (100 ng/ml) to the organ culture, and the effect of these cytokines on the proliferation of neuronal progenitor cells was estimated using the proportion of BrdU-labeled neurons after dissociated cell culture as the criterion. FGF-

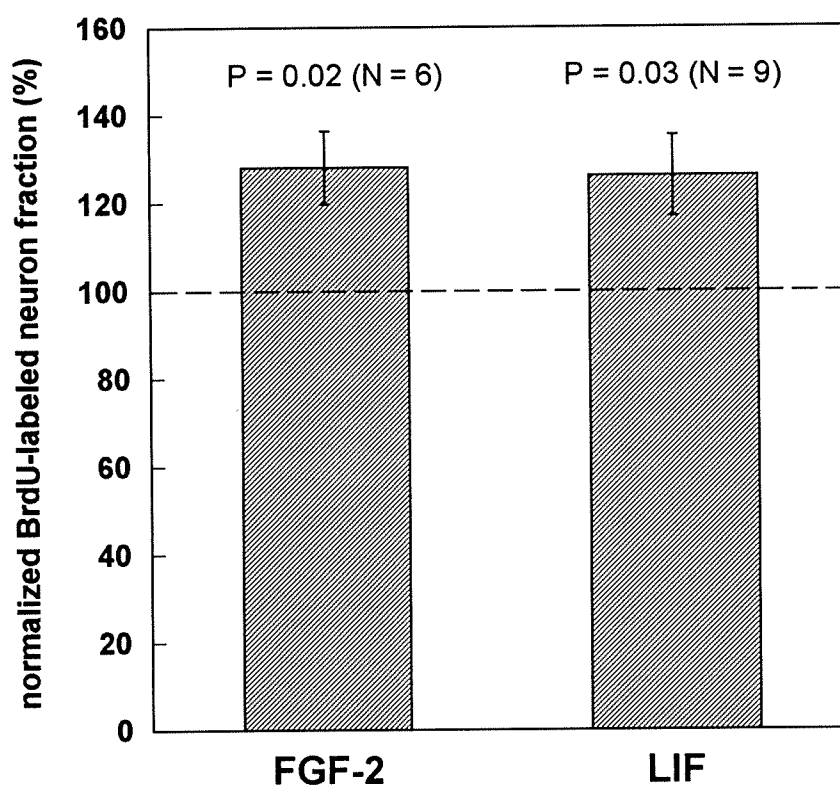


Fig. 2. Effect of FGF-2 and LIF on the proliferation of olfactory neuronal progenitor cells in organ culture. Values represent mean proportion of BrdU<sup>+</sup> neurons (%) in total number of neurons  $\pm$  S. E. M., normalized against the corresponding control cultures as 100%. N indicates number of independent experiments. Significance (P) was determined by double-sided Student's *t*-test.



2 and LIF increased the BrdU-labeled neuron fraction each by about 1.3-fold compared to the control (Fig. 2). Thus it was suggested that FGF-2 and LIF have a proliferation-promoting effect on the neuronal progenitor cells when administrated to the whole olfactory epithelium in organ culture.

### Discussion

Hitherto, *in vitro* studies on the effect of cytokines on olfactory neurogenesis have been conducted using two-dimensional culture systems (3, 5, 13, 16) and purified cell culture systems (5, 19). These culture systems are simple and, therefore, suitable for the analysis of the direct function of cytokines on the cells of interest. In such systems, however, the tissue order of olfactory epithelium was considerably disorganized or completely missing. In tissues of living organisms, the cytokines may function on certain cell types indirectly, as well as directly, by affecting neighboring cells of other types. Moreover, their messages may be modified by the local tissue environments. The organ culture system can overcome these disadvantages of the conventional culture systems because it is three-dimensional and the tissue organization is relatively conserved, and it is therefore expected to reproduce the local tissue environments as *in vivo*. The organ cultures of olfactory tissues, including the connective tissues underlying the olfactory epithelium, which were accompanied by neuronal differentiation/maturation, have been reported (4, 6). We here established an organ culture system of pure olfactory epithelium in which neuronal progenitor cell divisions occur. In addition, the culture medium is chemically defined, which excludes the unknown extrinsic factors in natural supplements such as serum. Thus our culture system provides a tool suitable for investigation of the functions of cytokines in neurogenesis in the whole olfactory epithelium environment.

FGF-2 has been reported to promote the proliferation of olfactory neuronal progenitor cells in the explant culture of olfactory epithelium or in the dissociated culture of partially purified neurons/neuronal progenitor cells (5). We recently found that LIF also promotes cell division in a culture of partially purified olfactory neuronal progenitor cells (19). These reports emphasize the direct effects of FGF-2 or LIF on the progenitor cell divisions. In the present paper we demonstrated that the olfactory epithelium also exhibits enhanced progenitor cell division in response to FGF-2 or LIF in the organ culture. Thus, FGF-2 and LIF seem to promote, whether directly or indirectly, the proliferation of neuronal progenitor cells even in the organized olfactory tissue environments, which further suggests the roles of these cytokines *in vivo*. Our organ culture system may provide a bridge between the simple monolayer culture systems and the complex living organisms.

One limitation of our organ culture system related to the fact that olfactory epithelium *in vivo* continually produces neurons from the proliferating progenitor cells (11). In the present organ culture system, the proportion of labeled neurons declined to 1-2 % when BrdU was administrated during 48-72 hr in the organ culture (not shown), suggesting that the proliferative progenitor cells are rapidly lost under the conditions used here. *In vivo*, actively proliferating-differentiating progenitor cells may be recruited by true stem cells

that slowly turn over (5, 15). If this is the case, our culture conditions may be insufficient for the survival or growth of stem cells. In turn, the regulation of survival and division of the stem cell is the fundamental mechanism of continual olfactory neurogenesis. Therefore, the search must continue for the conditions responsible for the maintenance of stem cells.

### References

- 1) Caggiano, M., Kauer, J. S., and D. D. Hunter. 1994. Globose basal cells are neuronal progenitors in the olfactory epithelium: a lineage analysis using a replication-incompetent retrovirus. *Neuron* 13: 339-352.
- 2) Calof, A. L. and D. M. Chikaraishi. 1989. Analysis of neurogenesis in a mammalian neuroepithelium: proliferation and differentiation of an olfactory neuron precursor in vitro. *Neuron* 3: 115-127.
- 3) Calof, A. L., Lander, A. D., and D. M. Chikaraishi. 1991. Regulation of neurogenesis and neuronal differentiation in primary and immortalized cells from mouse olfactory epithelium. In G.R. Bock and J. Whelan (eds.) *Regeneration of Vertebrate Sensory Receptor Cells*, Ciba Foundation Symposium 160, Wiley, Chichester, p. 249-276.
- 4) Chuah, M. I. and A. L. Ferbman. 1983. Olfactory bulb increases marker protein in olfactory receptor cells. *J. Neurosci.* 11: 2197-2205.
- 5) Dehamer, M. K., Guevara, J. L., Hannon, K. H., Olwin, B. B., and A. L. Calof. 1994. Genesis of olfactory receptor neurons in vitro: regulation of progenitor cell divisions by fibroblast growth factors. *Neuron* 13: 1083-1097.
- 6) Ferbman, A. I. 1977. Differentiation of olfactory receptor cells in organ culture. *Anat. Rec.* 189: 187-200.
- 7) Ferbman, A. I. 1990. Olfactory neurogenesis: genetic or environmental controls?, *Trends Neurosci.* 13: 362-365.
- 8) Ferbman, A. I. 1992. *Cell Biology of Olfaction*. Cambridge University Press, New York.
- 9) Goldstein B. J. and J. E. Schwob. 1996. Analysis of the globose basal cell compartment in rat olfactory epithelium using GBC-1, a new monoclonal antibody against globose basal cells. *J. Neurosci.* 16: 4005-4016.
- 10) Gordon, M. K., Mumm, J. S., Davis, R. A., Holcomb, J. D., and A. L. Calof. 1995. Dynamics of MASH1 expression *in vitro* and *in vivo* suggest a non-stem cell site of MASH1 action in the olfactory receptor neuron lineage. *Mol. Cell. Neurosci.* 6: 363-379.
- 11) Gradiadei, P. P. C. and G. A. Monti Gradiadei. 1978. Continuous nerve cell renewal in the olfactory system. In M. Jacobson (ed.) *Handbook of Sensory Physiology, Volume IX: Development of Sensory Systems*, Springer-Verlag, New York, p. 55-83.
- 12) Guillemot, F., Lo, L.-C., Johnson, J. E., Auerbach, A., Anderson, D. J., and A. L. Joyner. 1993. Mammalian *achaete-scute* homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* 75: 463-476.
- 13) Holcomb, J. D., Mumm, J. S. and A. L. Calof. 1995. Apoptosis in the neuronal lineage of the mouse olfactory epithelium: regulation *in vivo* and in vitro, *Develop. Biol.* 172: 307-323.
- 14) Lee, V. M. and S. K. Pixley. 1994. Age and differentiation-related differences in neuron-specific tubulin immunostaining of olfactory sensory neurons, *Dev. Brain Res.* 83: 209-215.
- 15) Mackay-Sim, A. and P. Kittel. 1991. Cell dynamics in the adult mouse olfactory epithelium: a quantitative autoradiographic study. *J. Neurosci.* 11: 979-984.
- 16) Mahanthappa, N. K. and G. A. Schwarting. 1991. Peptide growth factor control of olfactory neurogenesis and neuron survival in vitro: roles of EGF and TGF- $\beta$ s. *Neuron*

- 10: 293-305.
- 17) Pixley, S. K. 1992. CNS glial cells support *in vitro* survival, division, and differentiation of dissociated olfactory neuronal progenitor cells. *Neuron* 8: 1191-1204.
  - 18) Satoh, M. and M. Takeuchi. 1995. Induction of NCAM expression in mouse olfactory keratin-positive basal cells *in vitro*. *Dev. Brain Res.* 87: 111-119.
  - 19) Satoh, M., Takeuchi, M., and T. Yoshida. Promotion of neurogenesis of mouse olfactory neuronal progenitor cells by leukemia inhibitory factor *in vitro*. (submitted).
  - 20) Schwartz Levey, M., Chikaraishi, D. M. and J. S. Kauer. 1991. Characterization of potential precursor population in the mouse olfactory epithelium using immunocytochemistry and autoradiography. *J. Neurosci.* 11: 3556-3564.
  - 21) Schwob, J. E., Huard, J. M., Luskin M. B., and S. L. Youngentob. 1994. Retroviral lineage studies of the rat olfactory epithelium. *Chem. Senses* 19: 671-682.
  - 22) Suzuki, Y. and M. Takeda. 1991. Basal cells in the mouse olfactory epithelium after axotomy: immunohistochemical and electronmicroscopic studies. *Cell Tissue Res.* 266: 239- 245.
  - 23) Suzuki, Y. and M. Takeda. 1993. Basal cell in the mouse olfactory epithelium during development: immunohistochemical and electron-microscopic studies. *Dev. Brain Res.* 73: 107-113.

## Detection of Heterogeneity of 18S rRNA Inter-genes and Mutation Arising during PCR Amplification

Kumiko UEDA and Kozaburo MIKATA

### Summary

Direct sequencing revealed sequence heterogeneity among ribosomal RNA gene (rDNA) operons, consisting of 8 base heterogeneous sites on the 18S rDNA of *Galactomyces citri-aurantii* IFO 10822, and 6 base heterogeneous sites in the same region on the 18S rDNA of *G. citri-aurantii* IFO 10821. Sequence analysis of the cloned 18S rRNA genes of 14 species (19 strains) of ascomycetous yeast-like fungi detected a total of 32 substitutions between two cloned sequences from each of 10 strains. Eight substitutions came from heterogeneity of *G. citri-aurantii* IFO 10822, and 24 substitutions were predicted to be due to misincorporation by the *Taq* DNA polymerase. A low frequency of random substitution, estimated to occur in PCR at approximately 1 in 2690 nucleotides, was detected; and transitions occurred 7 times more frequently than transversions.

Keywords: 18S rDNA, heterogeneity, misincorporation during PCR

It has been shown that mutations are produced by misincorporation of nucleotides in DNA synthesis by PCR (2, 5, 6, 8, 11). If mutations occur at an early step of PCR, mutants may become a significant portion of the reaction product.

The number of rDNA copies in different organisms varies greatly: bacteria possess from 1 to 14 rDNA operons per genome (13), while there are hundreds or even thousands of ribosomal transcription units per eukaryotic genome. *Saccharomyces cerevisiae* possesses about 140 rDNA (7). It has been commonly assumed that these multiple gene copies are functionally identical. In the last decade, the phylogenetic relationships of organisms have been estimated by the comparison of rRNA sequences (12), but it has been shown that some rDNA operons contain sequence heterogeneity (4).

Sequence data obtained from cloned PCR products may represent a mutation that occurred during PCR or a minority among multiple gene copies. Direct sequencing of PCR products produces a consensus sequence in which mutations become hidden or a mixed sequence as heterogeneous sequences. In this study, some misincorporations (0.04%) were detected in the sequence analysis of two clones each of PCR-amplified 18S rDNA obtained from genomic DNA, and heterogeneous sequences among the rDNA

operons were detected by direct sequencing.

### Materials and Methods

**Strains used** The strains of ascomycetous yeast-like fungi studied are listed in Table 1. The strains were grown in YM broth at 24°C for DNA extraction.

**Isolation of genomic DNA** DNA isolation for the polymerase chain reaction (PCR) was performed as described below. Logarithmically growing cells were harvested by centrifugation, resuspended in water, and centrifuged again. The pellet was resuspended in CPES buffer (20 mM citrate-phosphate (pH 5.6), 50 mM EDTA (pH 7.5), 900 mM sorbitol) and centrifuged again. The pellet was resuspended in CPES buffer with Zymolyase and Novozyme (Zymolyase-100T 0.2 mg/ml, Novozyme 234 4 mg/ml in CPES), and resuspended cells were incubated at 37°C until spheroplasting was complete. The spheroplasted cells were centrifuged, and the pellet was resuspended in breaking buffer (100 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 1% sodium dodecyl sulfate (SDS)). The resuspended cells were incubated at 65°C with occasional swirling. Phenol/chloroform extraction, nucleic acids precipitation with ethanol and RNA digestion by

Table 1. Strains used in this study and sequenced base number.

| Species                            | Strain    | Sequence size (base) | Heterogeneous site* |
|------------------------------------|-----------|----------------------|---------------------|
| <i>Dipodascus aggregatus</i>       | IFO 10816 | 1744                 | ND                  |
| <i>Dipodascus ambrosiae</i>        | IFO 10801 | 1596                 | ND                  |
| <i>Dipodascus australiensis</i>    | IFO 10805 | 1748                 | 1                   |
| <i>Dipodascus capitatus</i>        | IFO 10819 | 1595                 | 5                   |
| <i>Dipodascus capitatus</i>        | IFO 10820 | 1595                 | 1                   |
| <i>Dipodascus geniculatus</i>      | IFO 10806 | 1750                 | ND                  |
| <i>Dipodascus macrosporus</i>      | IFO 10807 | 1752                 | ND                  |
| <i>Dipodascus magnusii</i>         | IFO 10808 | 1594                 | ND                  |
| <i>Dipodascus spicifer</i>         | IFO 10809 | 1595                 | ND                  |
| <i>Dipodascus tetrasperma</i>      | IFO 10810 | 1594                 | ND                  |
| <i>Dipodascus armillariae</i>      | IFO 10802 | 1753                 | ND                  |
| <i>Dipodascus armillariae</i>      | IFO 10803 | 1753                 | 3                   |
| <i>Dipodascus armillariae</i>      | IFO 10804 | 1753                 | ND                  |
| <i>Dipodascus armillariae</i>      | IFO 10818 | 1753                 | 2                   |
| <i>Galactomyces citri-aurantii</i> | IFO 10821 | 1737                 | 2                   |
| <i>Galactomyces citri-aurantii</i> | IFO 10822 | 1737                 | 12                  |
| <i>Galactomyces geotrichum</i>     | IFO 9541  | 1737                 | 1                   |
| <i>Galactomyces reessii</i>        | IFO 10823 | 1738                 | 2                   |
| <i>Arxula terrestris</i>           | IFO 10828 | 1755                 | 3                   |
| Total                              |           | 32279                | 32                  |

\*Heterogenous base number between two sequenced clones.

RNaseA were carried out as described by Sambrook et al. (9).

**PCR amplification** The sequences of the 20-mer primers used for PCR amplification of the 18S rDNA were 18-F (5'-ATCTGGTTGATCCTGCCAGT-3') and 18-R (5'-GATCCTTCCGCAGGTTTACC-3'). PCR amplification with a thermostable TaKaRa Ex Taq (TaKaRa) was conducted for 30 cycles with 10 pmol of primers and 1  $\mu$ g of genomic DNA (3). Amplification consisted of melting at 94°C for 30 sec, annealing at 55°C for 30 sec, and primer extension at 72°C for 2 min.

**DNA sequencing** PCR-amplified 18S rDNA cloned by using standard procedures (9) and diluted unpurified PCR products were used as a template for sequencing. DNA sequencing was carried out by the dideoxy sequencing method (10) using a Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham LIFE SCIENCE) and an A.L.F. DNA sequencer II (Pharmacia) according to the manufacturers' instructions.

## Results and Discussion

18S rDNA of two clones per strain was sequenced. In ten strains, a total of 32 base-substitution sites between two clones were detected (Table 1). In nine strains, both

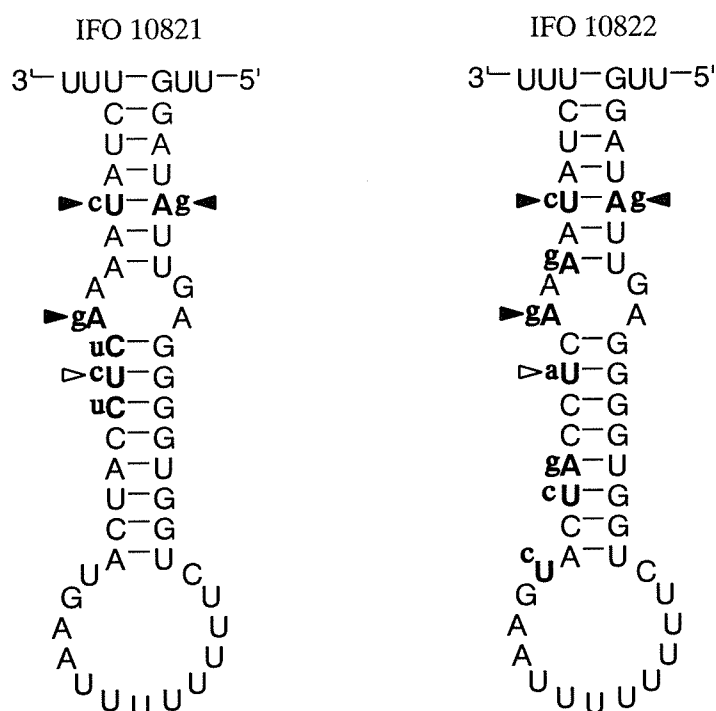


Fig. 1. Secondary structure model for *Galactomyces citri-aurantii* 18S rRNA helix E21-1. Helix numbering is according to de Rijk et.al. (1). Bold letters show heterogeneity sequences and capital letters show consensus sequences. Black arrows show consensus heterogeneous sequence and white arrows show consensus positions and different heterogeneous sequences between IFO 10821 and IFO 10822.

clone sequences matched each other completely. The source of nucleotide substitutions is supposed to be either sequence heterogeneity among multiple gene copies or misincorporation of nucleotides in DNA synthesis of PCR. Mutation during PCR will occur at random. On the other hand, sequence heterogeneity among gene copies is expected to occur in variable regions rather than evolutionarily conserved regions, which are functionally more important. The major 18S rDNA sequences among rDNA operons were

Table 2. Base substitutions.

| Species                            | Strain    | Clone no. | Mutation |              | Site* |
|------------------------------------|-----------|-----------|----------|--------------|-------|
| <i>Dipodascus armillariae</i>      | IFO 10803 | 3-4       | T→C      | transition   | C     |
|                                    |           |           | A→G      | transition   | C     |
|                                    |           |           | G→A      | transition   | C     |
| <i>Dipodascus armillariae</i>      | IFO 10818 | 18-14     | C→T      | transition   | C     |
|                                    |           | 18-15     | A→G      | transition   | V     |
| <i>Dipodascus australiensis</i>    | IFO 10805 | 5-1       | A→G      | transition   | C     |
| <i>Dipodascus capitatus</i>        | IFO 10819 | 19-3      | C→T      | transition   | C     |
|                                    |           |           | T→C      | transition   | C     |
|                                    |           |           | T→C      | transition   | C     |
|                                    |           |           | T→C      | transition   | C     |
|                                    |           |           | G→A      | transition   | C     |
| <i>Dipodascus capitatus</i>        | IFO 10820 | 20-5      | A→G      | transition   | C     |
| <i>Galactomyces citri-aurantii</i> | IFO 10821 | 21-1      | T→C      | transition   | V     |
|                                    |           |           | T→C      | transition   | C     |
| <i>Galactomyces citri-aurantii</i> | IFO 10822 | 22-1      | A→C      | transversion | C     |
|                                    |           |           | A→G      | transition   | C     |
|                                    |           | 22-2      | T→C      | transition   | C     |
|                                    |           |           | G→A      | transition   | C     |
| <i>Galactomyces geotrichum</i>     | IFO 9541  | 41-1      | G→C      | transversion | V     |
| <i>Galactomyces reessii</i>        | IFO 10823 | 23-2      | A→G      | transition   | C     |
|                                    |           |           | T→A      | transversion | C     |
| <i>Arxula terrestris</i>           | IFO 10828 | 28-21     | A→G      | transition   | C     |
|                                    |           |           | G→A      | transition   | C     |
|                                    |           | 28-22     | A→G      | transition   | C     |

\* C: substitutions at evolutionarily conserved inter-species sites.; V: substitutions at variable inter-species site.



determined by direct sequencing. Among 32 base-substitution sites, 8 base-substitution sites were detected as mixed sequences, which indicated heterogeneity among multiple gene copies. And each heterogeneous sequence was supposed to exist in high population in the genome.

Heterogeneous 8 bases of *G. citri-aurantii* IFO 10822 were located in the variable helix E21-1 (1). In spite of the identity sequences of both clones from *G. citri-aurantii* IFO 10821, heterogeneous 6 bases located in helix E21-1 were again detected by direct sequencing. The secondary structures of both heterogeneous sequences were identical (Fig. 1). Sequences of two clones of IFO 10821 and clone 22-3 of IFO 10822 were identical. Between the two strains of *G. citri-aurantii*, three pairs of heterogeneous sequences were common and the other heterogeneous sites were different. Since heterogeneous sites were found in an unalignable variable region, the heterogeneous sequences could not be used in phylogenic analysis.

Though 18S rDNA heterogeneity could be thought normal for multi-copy gene's, none of the analyzed strains other than *G. citri-aurantii* were found to have heterogeneity by direct sequencing. It is interesting that the two strains of *G. citri-aurantii* have different heterogeneous sequences in the same region helix E21-1. In most genomes having identical 18S rDNA by direct sequencing, it is predicted that identity of 18S rDNA genes is due to coevolution of multi 18S rDNA genes. In the case of the *G. citri-aurantii* strains, it is expected that the genes are coevolving to unity now, or that the species lacked some factors for coevolution.

Twenty-four base substitutions between two clones were randomly distributed as single nucleotide substitutions (Table 2). Heterogeneity was expected to exist in variable regions which are functionally less important. Most of the 24 substitutions were expected to be due to misincorporation of PCR. Deletions or insertions were not observed in this study. One random misincorporation has been found in cloned 2690-nucleotide 18S rDNA sequences following 30 cycles of PCR with the *Taq* DNA polymerase (Table 3). Transitions were found seven times more frequently than transversions. AT → GC transitions were the predominant mutational change (15/24) (Table 2). These data were consistent with earlier reports (8, 11).

Because the target sequence is amplified exponentially during PCR, the mutation frequency in a population of amplified sequences depends upon how early in the amplification process the error occurs. In this study, approximately 35% (13/38) of the sequenced clones possessed mutations due to PCR, but the mutation frequency was so low that

Table 3. Template DNA specificity of mutations and mutation frequency following 30 cycles PCR using *Taq* DNA polymerase.

|                                    | Template DNA |         |       |
|------------------------------------|--------------|---------|-------|
|                                    | A and T      | G and C | Total |
| Total base number of template      | 35868        | 28690   | 64558 |
| Total base number of mutation site | 17           | 7       | 24    |
| Mutation frequency (base/base)     | 0.05%        | 0.02%   | 0.04% |

the mutations could not greatly affect the result of phylogenetic analysis using mutated sequences.

It was reconfirmed that direct sequence analysis of PCR-amplified DNA was effective to minimize or eliminate the effects of mutation arising during PCR amplification and of minor heterogeneous sequences, and to detect major heterogeneous sequences.

### References

- 1) De Rijk, P., J.-M. Neefs, Y. van de Peer, and R. de Wachter. 1992. Complication of small ribosomal subunit RNA sequences. *Nucleic Acids Res.* 20: 2075-2089.
- 2) Doh-ura, K., J. Tateishi, H. Sasaki, T. Kitamoto, and Y. Sakaki. 1989. Pro → Leu change at position 102 of prion protein is the most common but not the sole mutation related to gerstmann-sträussler syndrome. *Biochem. Biophys. Res. Commun.* 163: 974-979.
- 3) Ehrlich, H.A., D.H. Gelfand, and R.S. Saiki. 1988. Specific DNA amplification. *Nature* 331: 461-462.
- 4) Gunderson, J.H., M.L. Slogin, G. Wollett, M. Hollingdale, V.F. de la Cruz, A.P. Waters, and T.F. McCutchan. 1987. Structurally distinct, stage-specific ribosomes occur in *Plasmodium*. *Science* 238: 933-937.
- 5) Ho, S.N., H.D. Hunt, R.M. Horton, J.K. Pullen, and L.R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77: 51-59.
- 6) Horton, R.M., H.D. Hunt, S.N. Ho, J.K. Pullen, and L.R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* 77: 61-68.
- 7) Long, E.O. and I.B. Dawid. 1980. Repeated genes in eukaryotes. *Annu. Rev. Biochem.* 49: 727-764.
- 8) Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.
- 9) Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed., Cold Spring Harbor Laboratory Press. New York.
- 10) Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain termination inhibitors. *Proc. Natl. Acad. Sci. USA* 74: 5463-5467.
- 11) Tindall, K.R. and T.A. Kunkel. 1988. Fidelity of DNA synthesis by the *Thermus aquaticus* DNA Polymerase. *Biochemistry* 27: 6008-6013.
- 12) Wilmotte, A., Y. van de Peer, A. Goris, S. Chapelle, R. de Baere, B. Nelissen, J.-M. Neefs, G.L. Hennebert, and R. de Wachter. 1993. Evolutionary relationships among higher fungi inferred from small ribosomal subunit RNA sequence analysis. *System. Appl. Microbiol.* 16: 436-444.
- 13) Young, M. and S.T. Cole. 1993. *Clostridium*. In A.L. Sonenshein, J.A. Hoch and R. Losick (ed.) *Bacillus subtilis* and Other Gram-Positive Organisms, p. 35-52. American Society for Microbiology. Washington, DC.

## Actinomycete Populations in Mangrove Rhizospheres

Kazunori HATANO

### Summary

Actinomycete populations in rhizosphere soils of six kinds of mangrove forest in Iriomote and Ishigaki islands were examined by an agar plate method. Actinomycete populations ranged from  $10^3$  to  $10^4$  colony-forming units (cfu)/g of soil (dry weight), except for that ( $10^6$  cfu/g) of the sample taken from the Nagura River in Ishigaki. Actinomycete diversity was represented by about 55% of *Micromonospora*, 33% of *Streptomyces*, 4% of actinobacteria, 2% of the *Nocardia* - *Rhodococcus* group and 6% of other genera among the total isolates. In addition to isolated strains of *Actinomadura*, *Microtetraspora*, *Catellatospora*, *Microbispora*, *Streptosporangium*, *Actinoplanes*, and *Thermoactinomyces*, two unidentified strains were detected. The diversity of actinomycetes in mangrove forests suggested that the mangrove area is a good source for isolation of diverse actinomycetes.

Keywords: Ecology, Actinomycetes, Mangrove rhizosphere, Actinomycete diversity in mangrove rhizosphere.

Mangrove forests are located in subtropical and tropical regions, such as Iriomote and Ishigaki islands in Japan, and have formed complex environments under the influence of tidal ebb and flow, the influx of fresh water, and high temperature and humidity. Soils in the environments are muddy and in anoxic states due to permanent or intermittent inundation, although it is known that mangrove roots supply oxygen to rhizosphere soil [8], and in addition soils are imposed the stress of salinity. Many ecological studies have been conducted on plants and animals in mangrove forest, but almost none have addressed the actinomycete community, except for the study of Nakagaito and Hasegawa [9], who briefly reported the isolation of several strains of the genus *Streptomyces* and a strain of the genus *Promicromonospora* from fallen leaves.

The objectives of the present study are to investigate the ecology of actinomycetes in mangrove rhizosphere soils and to evaluate their potential as sources for isolating new actinomycetes.

## Materials and Methods

*Soil samples and treatments.* Samples were collected on 24–26 October 1995 from the mangrove rhizosphere in the estuaries of the rivers listed in Table 1. All samples were dried in air at room temperature for 4 days, and then dried soil and fine roots were separated by use of a sieve (60 mesh) to be weighed.

*Isolation and enumeration of actinomycetes.* Dried soil (1 g) was suspended in 9 ml of YE-SDS solution (6% yeast extract and 0.05% sodium dodecyl sulfate, pH 7.3) and treated with an Ultrasonic Cleaner (38 KHz, KAIJOU DENKI Co. Ltd., Tokyo, Japan) for 20 min at 40°C, as recommended by Hayakawa and Nonomura [2, 3]. A diluted aliquot (0.1 ml) of soil suspension was spread on HV agar plate [2] and starch-casein agar plate [6]. Plates were incubated for 2 to 3 weeks at 28°C. All colonies appearing on agar plates were enumerated, picked up and transferred to HV agar and half-strength yeast extract-malt extract agar (ISP medium 2) [10] plates, which were incubated at 28°C for 2 weeks to observe morphology and phenotypic characteristics.

To determine actinomycete populations on the surface of fine roots of mangrove, dried root samples (about 0.3 g) were rinsed thrice with 10 ml of distilled sterilized water, and then rinsed root samples were treated by the same procedure as soil samples.

*Classification of isolates.* Isolates were classified on the basis of their morphological and chemotaxonomic characteristics. Morphological observation of isolates grown on HV agar and ISP media 2, 3, 4 and 5 agar plates was carried out according to the method of Shirling and Gottlieb [10]. Chemical compositions of cells were analyzed by the methods of Lechevalier and Lechevalier [7] and Kroppenstedt [5].

## Results and Discussion

### *Actinomycete populations in mangrove rhizospheres.*

Table 1 shows the total number of actinomycetes in mangrove rhizosphere soils and on the surface of fine roots of mangrove collected at seven sampling sites. Actinomycete populations in rhizosphere soils and on the surface of the fine roots were generally  $10^3$  to  $10^4$  cfu/g of dry weight, except for those of sample No. 6 collected at a bank near the Nagura River. In rhizosphere soils, actinomycete populations were 1000 to 10,000 times smaller than those of arable lands [1]. Results suggest that even if oxygen is supplied to the mangrove rhizospheres through fine roots, these environments are not favorable for the survival of actinomycetes. The population of actinomycetes at site No. 6 was about  $10^6$  cfu/g, being similar to that of arable lands [1], which suggests that this site is not highly anoxic, probably due to the absence of tidal influence.

### *Diversity of actinomycete isolates in genus level.*

The genus diversity of isolates differed according to sampling sites as shown in Table 2. In general, *Micromonospora* and *Streptomyces* were the major genera of actinomycetes detected from the rhizosphere soils and fine roots, except for the case of sample No. 6. This result was very similar to those of Jiang et al. [4] and Cross [1]; *Micromonospora*

Table 1. Actinomycete populations in mangrove rhizosphere soils and on the surface of mangrove roots growing at seven sampling sites.

| Sample No. |      | Population of actinomycetes, cfu/g <sup>1)</sup> | Water content of soil (%) <sup>2)</sup> | Sampling site               | Species of mangrove          |
|------------|------|--|---|-----------------------------|------------------------------|
| 1          | Soil | $1.1 \times 10^4$                                | 54.5                                    | Maira River <sup>3)</sup>   | <i>Avicennia marina</i>      |
|            | Root | $4.1 \times 10^3$                                |   |                             |                              |
| 2          | Soil | $3.0 \times 10^3$                                | 22.4                                    | Maira River <sup>3)</sup>   | <i>Rhizophora stylosa</i>    |
|            | Root | $0.7 \times 10^3$                                |   |                             |                              |
| 3          | Soil | $0.6 \times 10^5$                                | 24.1                                    | Shiira River <sup>3)</sup>  | <i>Sonneratia alba</i>       |
|            | Root | $1.5 \times 10^4$                                |   |                             |                              |
| 4          | Soil | $2.6 \times 10^4$                                | 27.9                                    | Shiira River <sup>3)</sup>  | <i>Bruguiera gymnorrhiza</i> |
|            | Root | $3.6 \times 10^3$                                |   |                             |                              |
| 5          | Soil | $0.9 \times 10^4$                                | 27.3                                    | Urauchi River <sup>3)</sup> | <i>Kandelia candel</i>       |
|            | Root | $4.1 \times 10^3$                                |   |                             |                              |
| 6          | Soil | $2.0 \times 10^6$                                | 38.1                                    | Nagura River <sup>4)</sup>  | <i>Lummitzera racemosa</i>   |
|            | Root | $0.6 \times 10^6$                                |   |                             |                              |
| 7          | Soil | $1.7 \times 10^4$                                | 35.3                                    | Fukidou River <sup>4)</sup> | <i>Rhizophora stylosa</i>    |
|            | Root | $0.6 \times 10^4$                                |   |                             |                              |

<sup>1)</sup> : colony-forming unit/ g of dry weight

<sup>2)</sup> : calculated by loss of sample weight after drying

<sup>3)</sup> : Iriomote island

<sup>4)</sup> : Ishigaki island

Table 2. Diversity of actinomycetes isolated from mangrove rhizospheres in seven sampling sites.

| Sampling site | Strain number identified as |                       |   |                       |     |         | Unidentified | Total |
|---------------|-----------------------------|-----------------------|---|-----------------------|-----|---------|--------------|-------|
|               | <i>Streptomyces</i>         | <i>Micromonospora</i> | <i>Nocardia</i> -<br><i>Rhodococcus</i> | <i>Actinobacteria</i> |     | Others* |              |       |
| 1             | 16                          | 243                   | 0                                       | 3                     | 0   | 13      | 3            | 278   |
| 2             | 22                          | 49                    | 4                                       | 1                     | 0   | 8       | 1            | 85    |
| 3             | 8                           | 0                     | 0                                       | 0                     | 0   | 2       | 2            | 12    |
| 4             | 83                          | 7                     | 4                                       | 7                     | 4   | 3       | 4            | 112   |
| 5             | 6                           | 1                     | 1                                       | 2                     | 1   | 0       | 0            | 11    |
| 6             | 37                          | 0                     | 0                                       | 0                     | 0   | 0       | 0            | 37    |
| 7             | 28                          | 24                    | 1                                       | 5                     | 0   | 4       | 3            | 65    |
| Total         | 200                         | 324                   | 10                                      | 18                    | 5   | 30      | 13           | 600   |
| (%)           | 33                          | 54                    | 1.7                                     | 3                     | 0.8 | 5       | 2.2          | 100   |

\* : *Actinomadura*, *Microbispora*, *Dactylosporangium*, *Microtetraspora*, *Actinoplanes*, *Streptosporangium*, *Catellatospora*, *Promicromonospora*, *Thermoactinomyces*

and *Streptomyces* were the major genera in isolates from soggy soil. Actinobacteria such as *Cellulomonas*, *Aureobacterium* and *Gordona* [11], nocardioform bacteria, seven other genera of actinomycetes and two unidentified strains were also detected. Almost all actinomycetes isolated from sample No. 6 were classified to the genus *Streptomyces*.

Table 3. Diversity of *Streptomyces* isolated from mangrove rhizospheres.

| Aerial mass<br>color | Morphology | Melanin<br>formation | Sampling site <sup>1)</sup> |     |    |    |    |     |    | Total<br>number |
|----------------------|------------|----------------------|-----------------------------|-----|----|----|----|-----|----|-----------------|
|                      |            |                      | 1                           | 2   | 3  | 4  | 5  | 6   | 7  |                 |
| Gray                 | RF         | —                    | 2 <sup>2)</sup>             | 7   | 2  | 1  | 5  | 16  | 2  | 33              |
|                      |            | +                    | 3                           | 29  | 1  | 0  | 2  | 9   | 1  | 45              |
|                      | Spira      | —                    | 100                         | 45  | 24 | 18 | 2  | 2   | 26 | 217             |
|                      |            | +                    | 1                           | 7   | 1  | 3  | 0  | 242 | 24 | 278             |
| Red                  | RF         | —                    | 0                           | 0   | 0  | 0  | 0  | 1   | 1  | 2               |
|                      |            | +                    | 0                           | 0   | 1  | 0  | 0  | 41  | 0  | 42              |
|                      | Spira      | —                    | 0                           | 0   | 4  | 1  | 0  | 0   | 1  | 6               |
|                      |            | +                    | 0                           | 2   | 1  | 0  | 0  | 0   | 5  | 8               |
| Yellow               | RF         | —                    | 1                           | 5   | 0  | 0  | 0  | 5   | 2  | 13              |
|                      |            | +                    | 0                           | 4   | 3  | 1  | 0  | 11  | 0  | 19              |
|                      | Spira      | —                    | 0                           | 1   | 2  | 2  | 0  | 9   | 1  | 15              |
|                      |            | +                    | 0                           | 0   | 1  | 1  | 0  | 0   | 1  | 3               |
| White                | RF         | —                    | 3                           | 3   | 2  | 3  | 2  | 0   | 2  | 15              |
|                      |            | +                    | 2                           | 1   | 0  | 0  | 0  | 0   | 0  | 3               |
|                      | Spira      | —                    | 4                           | 2   | 2  | 2  | 1  | 0   | 2  | 13              |
|                      |            | +                    | 3                           | 0   | 0  | 0  | 0  | 0   | 0  | 3               |
| Blue                 | RF         | —                    | 0                           | 0   | 0  | 0  | 0  | 0   | 0  | 0               |
|                      |            | +                    | 0                           | 0   | 0  | 0  | 0  | 0   | 0  | 0               |
|                      | Spira      | —                    | 0                           | 1   | 0  | 0  | 0  | 29  | 0  | 30              |
|                      |            | +                    | 0                           | 1   | 0  | 0  | 0  | 0   | 0  | 1               |
| Total                |            |                      | 117                         | 108 | 44 | 32 | 12 | 365 | 68 | 746             |

Morphology, aerial mass color and melanin formation are defined by the method of Shirling and Gottlieb [10].

<sup>1)</sup> : Same as in Table 1.

<sup>2)</sup> : Number of colonies appeared.

#### Diversity of *Streptomyces* isolates.

Table 3 shows the diversity of the genus *Streptomyces* on the basis of morphology of spore-bearing hyphae, aerial mass color and melanin formation. About 77% of isolates belonged to the Gray series according to be defined of Shirling and Gottlieb [10], and about 80% of this series were of the *Spira* type which spore-bearing hyphae form spirals. From sample No. 6, forty one strains (about 11%) resembling *Streptomyces lavendulae* belonging to the Red series, eleven strains (about 3%) resembling *Streptomyces griseus* in the Yellow series and twenty nine strains (8%) resembling *Streptomyces viridochromogenes* in the blue series were isolated, but they were barely isolated from other samples. Generally, the range of diversity in species level of the genus *Streptomyces*

Table 4. Chemotaxonomic properties of unique strains.

| Strain | Cell wall<br>Chemotype <sup>1)</sup> | Major menaquinone   | Fatty acid type <sup>2)</sup> | Presumed genus name               |
|--------|--------------------------------------|---|-------------------------------|-----------------------------------|
| 25     | III/C                                | MK-7  | 2c or 2b                      | <i>Thermoactinomyces</i>          |
| 43     | IV/A                                 | MK-9(H <sub>2</sub> )   | 1b                            | <i>Nocardia-Rhodococcus</i> group |
| 79     | III/B                                | MK-9(H <sub>2</sub> , H <sub>4</sub> ), MK-8(H <sub>4</sub> ) | 3b                            | Unidentified                      |
| 118    | III/B                                | MK-9(H <sub>6</sub> , H <sub>8</sub> , H <sub>2</sub> )       | 3a                            | <i>Actinomadura viridis</i>       |
| 122    | IV/A                                 | MK-9(H <sub>4</sub> ), MK-10(H <sub>4</sub> )                 | 2c                            | Unidentified                      |
| 131    | IV/A                                 | MK-9(H <sub>2</sub> , H <sub>4</sub> )                        | 1b                            | <i>Nocardia-Rhodococcus</i> group |
| 149    | IV/A                                 | MK-9(H <sub>2</sub> )   | 1b                            | <i>Nocardia-Rhodococcus</i> group |
| 156    | IV/A                                 | MK-8(H <sub>2</sub> ), MK-9(H <sub>2</sub> )                  | 1b                            | <i>Nocardia-Rhodococcus</i> group |

<sup>1)</sup> : defined by Lechevalier and Lechevalier [7].

<sup>2)</sup> : defined by Kroppenstedt [5].

appeared to be narrow.

#### Unidentifiable isolates.

Thirteen isolates were difficult to classify by the morphology and phenotypic characters because none of them except for the isolate No. 118 bore aerial hyphae. These isolates were cultivated in liquid medium, harvested, and their cell compositions of menaquinone and fatty acids in cell were analyzed as a means for the genus identification. Table 4 summarizes the results including their chemotype of cell wall. Isolate No. 25 had off-white substrate mycelia growing on yeast extract-malt extract agar (ISP 2 medium), seven isoprenoid units of menaquinone, and cell wall chemotype III/C. These results indicate that the isolate No. 25 belongs to the genus *Thermoactinomyces*. Isolates No. 43, 131, 149 and 156, which penetrated into HV agar with fragmentation were classified as *Nocardia-Rhodococcus* group on the basis of cell wall chemotype and cellular fatty acid patterns. Isolate No. 118 had dark green aerial mycelia and hyphae bore irregularly two to four spores. Judging from its cell wall chemotype (III/B) and MK-9 (H<sub>6</sub>, H<sub>8</sub> and H<sub>2</sub>), it was identified as *Actinomadura viridis*. Isolates No. 79 and 122 could not be classified into known genera and further analyses are required.

This preliminary study on actinomycete ecology in the mangrove rhizosphere has proven that a number of aerobic actinomycetes such as *Streptomyces* and other genera such as *Micromonospora* are in mangrove rhizosphere soils where are considered to be in anaerobic. In addition, several rare actinomycetes were isolated. We therefore conclude that mangrove rhizosphere is a good source for isolating new and diverse actinomycetes.

#### References

- 1) Cross, T. 1981. Aquatic actinomycetes: a critical survey of the occurrence, growth and role of actinomycetes in aquatic habitats. J. Appl. Bacteriol. 50: 397-423.
- 2) Hayakawa, M. and H. Nonomura. 1987. Efficacy of artificial humic acid as a selective nutrient in HV agar used for the isolation of soil actinomycetes. J. Ferment. Technol. 65: 609-616.



- 3) Hayakawa, M. and H. Nonomura. 1989. A new method for the intensive isolation of actinomycetes from soil. *Actinomycetologica* 3: 95-104.
- 4) Jiang, C.-L. and L.-H. Xu. 1996. Diversity of aquatic actinomycetes in lakes of the middle plateau, Yunnan, China. *Appl. Environ. Microbiol.* 62: 249-253.
- 5) Kroppenstedt, R.M. 1985. Fatty acid and menaquinone analysis of actinomycetes and related organisms. p 173-199. In Goodfellow, M. & Minnikin, D. E. (ed.). *Chemical methods in bacterial systematics*. Academic Press, London, Orlando, San Diego, New York, Austin, Boston, Sydney, Tokyo, Toronto.
- 6) Küster, E., and S.T. Williams. 1964. Selection of media for isolation of *Streptomyces*. *Nature* 202: 928-929.
- 7) Lechevalier, M.P. and H.A. Lechevalier. 1980. The Chemotaxonomy of Actinomycetes. p. 225-291. In A.T. Dietz (ed.). *Actinomycete Taxonomy*. Arlington.
- 8) Mitsch, W.J. and J.G. Gosselink. 1993. *Wetlands*. p 173 and p 303 Van Nostrand Reinhold. New York.
- 9) Nakagaito, Y. and T. Hasegawa. 1991. Actinomycete strains isolated from fallen leaves in mangrove areas. *IFO Res. Comm.* 15: 52-56.
- 10) Shirling, E.B. and D. Gottlieb. 1966. Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* 16: 313-340.
- 11) Takeuchi, M and K. Hatano. Unpublished data.

## A Mycofloral Study on Mangrove Mud in Okinawa, Japan

Tadayoshi ITO and Akira NAKAGIRI

### Summary

The fungal flora of mangrove mud in Okinawa, Japan was investigated by four isolation methods. From 36 mud samples, 36 genera of fungi representing 11 Ascomycotina, 21 Deuteromycotina, 2 Zygomycotina, and 2 unidentified Basidiomycotina were detected. The most dominant species were, in order, *Penicillium purpurogenum*, *Aspergillus terreus*, *Trichoderma harzianum*, *Penicillium crustosum*, *Acremonium alabamense*, *Talaromyces flavus* var. *flavus* and *Phialophora fastigiata*.

No significant differences were found in the numbers of species and isolates detected in the estuaries of six rivers. Fewer fungal populations were detected in mangrove mud than in agricultural soils. Tolerance to sodium chloride was tested for some isolates.

Keywords: fungal flora, mangrove mud, number of fungi.

Mangrove forest in Japan is mainly distributed in Okinawa prefecture, which lies in the subtropical zone. The salinity and pH of the mud in mangrove forest in Japan were reported to be 2.6-5.1‰ and 6.1-8.1 at 20 cm depth, respectively. Mangrove mud is also reported to be deficient in dissolved oxygen and to show accumulation of heavy metals (8). Mangrove trees are known to adapt to these conditions (8). The fungal flora in mud of mangrove forest, however, has not been surveyed in Japan, and only a few surveys have been reported from other countries (5, 6, 7).

### Materials and Methods

Thirty-six samples of mangrove mud were collected from Okinawa prefecture on 25-27 January 1994 (Fig. 1). The collection sites are estuaries in three areas of Okinawa prefecture: Urauchi, Maira and Shiira rivers in Iriomote Is.; Nagura river, Ishigaki Is.; and Kesaji and Ooura rivers, Okinawa Is. The vegetation of these estuaries is dominated by plants such as *Rhizophora stylosa* Griff., *Avicennia marina* Vierh., *Bruguiera gymnorhiza* Lamk., *Kandelia candel* Druce and *Sonneratia alba* J. E. Sm.

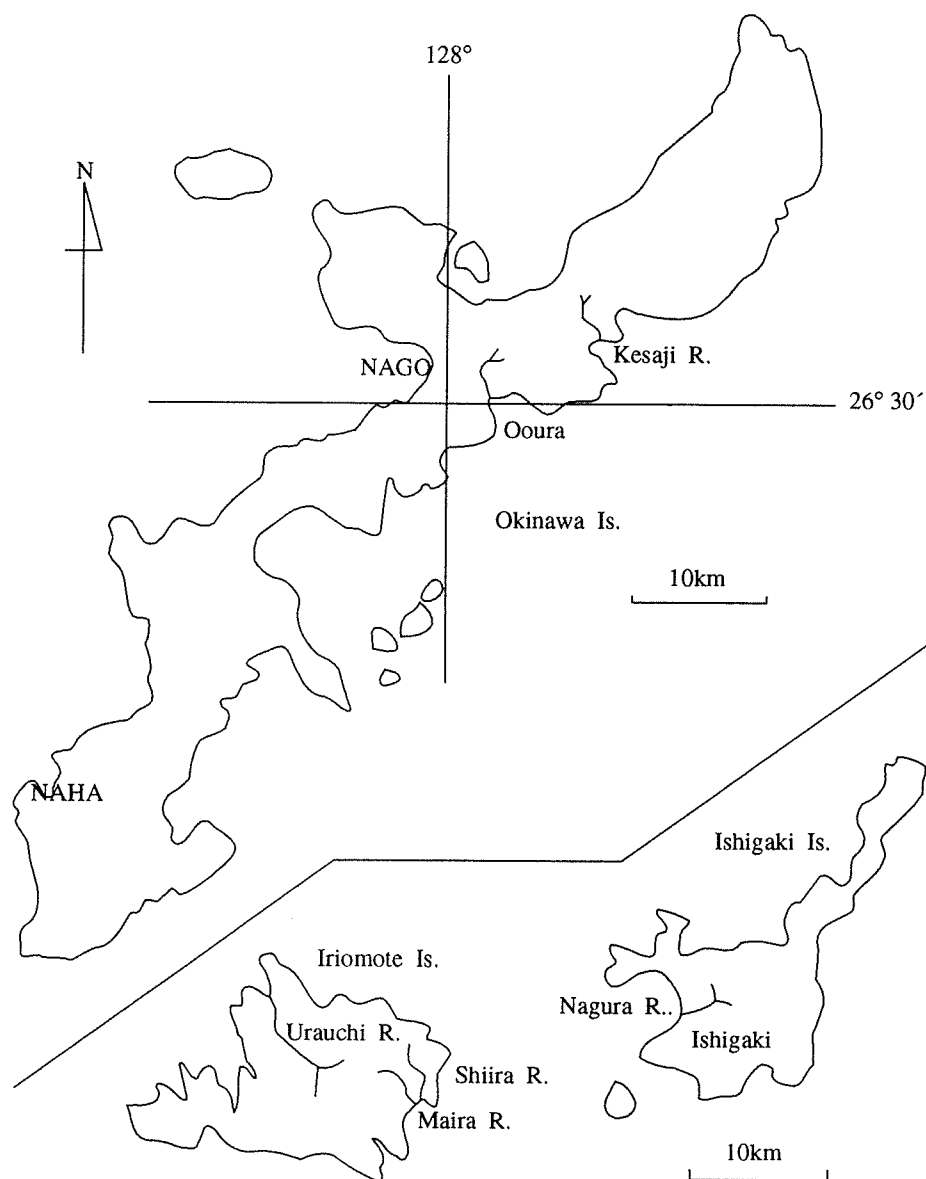


Fig. 1. Map of Okinawa prefecture showing the study sites.

The mud samples were collected from about 5 cm depth at each site, and the total of 36 samples obtained are listed in Table 1. To compare the number of fungal propagules in agricultural soil, three soil samples of pineapple, banana and paddy fields were also collected from Iriomote Is. The samples were suspended in physiological salt solution containing 0.85% sodium chloride. Four isolation methods were applied: incubation at 45°C, treatment with 50% ethanol for 15 min., heat treatment at 70°C for 15 min., and the standard dilution plate method. Plates for heat incubation and for other methods were incubated at 45°C and 24°C for three to four days, respectively. Single colonies were picked up from the plates under a dissecting microscope and transferred to malt agar slants. The isolation medium was malt extract-yeast extract-agar in which distilled water

Table 1. List of mangrove mud samples collected in Okinawa, and the number of species isolated from each sample.

| Sample No. | Date sampled | Locality                      | Predominant vegetation   | Number of species isolated |
|------------|--------------|-------------------------------|--|----------------------------|
| 1          | 25/1/'94     | Urauchi river<br>Iriomote Is. | <i>Rhizophora stylosa</i><br><i>Kandelia candel</i>                            | 3                          |
| 2          | „            | „                             | „  | 2                          |
| 3          | „            | „                             | „  | 3                          |
| 4          | „            | „                             | „  | 4                          |
| 5          | „            | „                             | „  | 2                          |
| 6          | „            | „                             | „  | 0                          |
| 7          | „            | „                             | „  | 3                          |
| 8          | „            | „                             | „  | 3                          |
| 9          | 26/1/'94     | Shiira river<br>Iriomote Is.  | <i>Avicennia marina</i><br><i>Rhizophora stylosa</i><br><i>Sonneratia alba</i> | 8                          |
| 10         | „            | „                             | „  | 2                          |
| 11         | „            | „                             | „  | 7                          |
| 12         | „            | „                             | „  | 13                         |
| 13         | „            | „                             | „  | 3                          |
| 14         | 26/1/'94     | Maira river<br>Iriomote Is.   | <i>Avicennia marina</i><br><i>Rhizophora stylosa</i><br><i>Sonneratia alba</i> | 3                          |
| 15         | „            | „                             | „  | 6                          |
| 16         | „            | „                             | „  | 3                          |
| 17         | „            | „                             | „  | 4                          |
| 18         | „            | „                             | „  | 9                          |
| 19         | „            | „                             | „  | 1                          |
| 20         | „            | „                             | „  | 4                          |
| 21         | „            | „                             | „  | 7                          |
| 22         | „            | „                             | „  | 11                         |
| 23         | „            | „                             | „  | 0                          |
| 24         | 27/1/'94     | Nagura river<br>Ishigaki Is.  | <i>Kandelia candel</i><br><i>Rhizophora stylosa</i>                            | 11                         |
| 25         | „            | „                             | „  | 19                         |
| 26         | „            | „                             | „  | 15                         |
| 27         | „            | „                             | „  | 15                         |
| 28         | 28/1/'94     | Ooura river<br>Okinawa Is.    | <i>Kandelia candel</i><br><i>Bruguiera gymnorhiza</i>                          | 11                         |
| 29         | „            | „                             | „  | 4                          |
| 30         | „            | „                             | „  | 7                          |
| 31         | „            | „                             | „  | 2                          |
| 32         | „            | „                             | „  | 5                          |
| 33         | 28/1/'94     | Kesaji river<br>Okinawa Is.   | <i>Rhizophora stylosa</i>  | 0                          |
| 34         | „            | „                             | „  | 0                          |
| 35         | „            | „                             | „  | 5                          |
| 36         | „            | „                             | „  | 2                          |

was replaced by 2.0% artificial sea water (Jamarin S; Jamarin Laboratory, Osaka) containing tetracycline antibiotics ( $50 \mu\text{g/ml}$ ) to inhibit bacterial growth, as previously reported (3, 4, 9). To isolate fungi decomposing chitin, trap method using a crab shell was tried. Tolerance to sodium chloride was tested for some isolates.

### Results and Discussion

Fig. 2 shows the number of fungi in each sample by the dilution plate method.

The average number of fungi for each river site ranged from  $4.5 \times 10^2$  to  $2.3 \times 10^3$  per gram of dry soil, whereas agricultural soil samples of pineapple, banana and paddy fields ranged from  $7.5 \times 10^4$  to  $2.1 \times 10^5$ . Four mud samples collected from the Urauchi, Maira and Kesaji rivers contained no viable fungal propagules (Table 1) even though four isolation methods were tried. This was also confirmed by the direct soil plate method and trap method using the crab shell. It indicates a poor distribution of fungal propagules in the mangrove mud. The reason is considered to be the semi-anaerobic condition and the high content of heavy metals in the mud.

Table 2 lists all the species of fungi isolated from the 36 samples. Forty-three species in 36 genera were identified and classified into 16 species in 11 genera of Ascomycotina, 25 species in 21 genera of Deuteromycotina, 2 species in 2 genera of Zygomycotina, and 2 unidentified species of Basidiomycotina.

Almost all the species identified were common, typical soil fungi which have been recorded worldwide (1, 2, 3, 4, 9). Both the total numbers of fungus species detected (Table 2) and the number of species in each sample were low in all 36 samples.

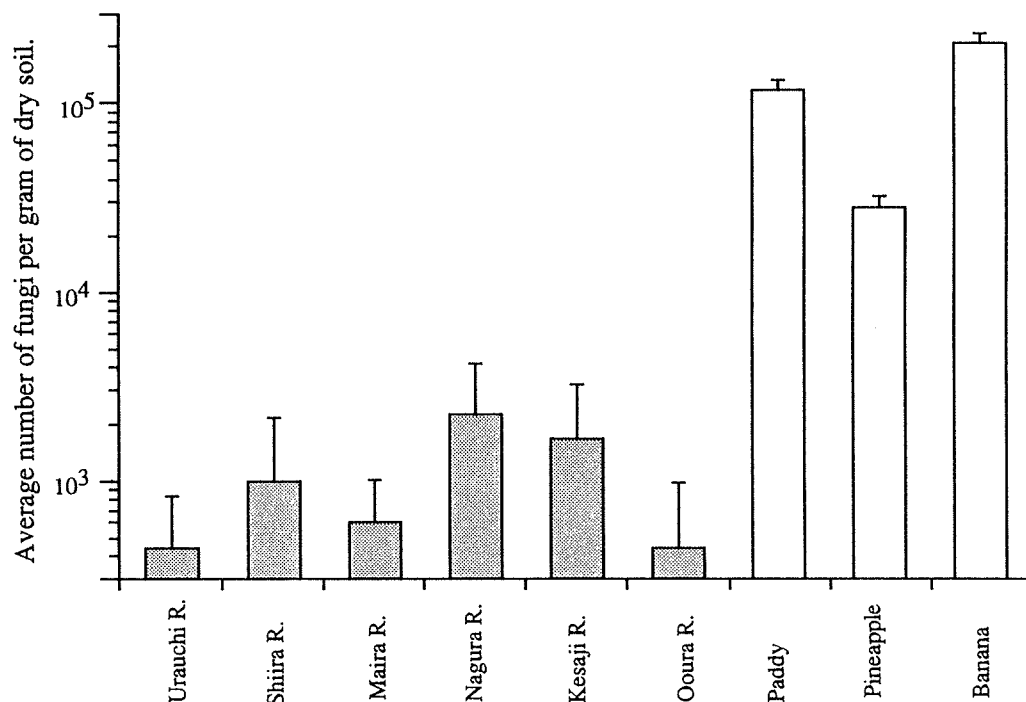


Fig. 2. Number of fungi isolated each site by the dilution plate method.

Eleven genera of Ascomycotina were encountered in this survey. *Talaromyces flavus* (Klocker) Stolk & Samson var. *flavus* (22.2%), *T. stipitatus* C. R. Benjamin apud Stolk & Samson (11.1%) and *T. wortmannii* C. R. Benjamin apud Stolk & Samson (11.1%) occurred frequently. It is considered that these species are isolated in higher frequency by the ethanol and heat treatments of soil samples, although they have often been detected from soil worldwide (1, 2, 3, 4, 9).

Table 2. Fungi isolated from mangrove muds by four isolation methods.

| Species detected  | Sample No.                      | Method <sup>a</sup> | Frequency <sup>b</sup> (%) |
|---|---------------------------------|---------------------|----------------------------|
| <b>ASCOMYCOTINA</b>   |                                 |                     |                            |
| <i>Achaetomium macrosporum</i> Rai et al  | 11                              | D                   | 2.8                        |
| <i>Chaetomium aureum</i> Chivers  | 29                              | D                   | 2.8                        |
| <i>Emericella nidulans</i> (Eidam) Vuillemin var. <i>nidulans</i>                                       | 1                               | H                   | 2.8                        |
| <i>Eupenicillium parvum</i> (Raper & Fennell) Stolk & Scott   | 9, 12                           | T, E                | 5.6                        |
| <i>Eurotium rubrum</i> König et al.   | 27                              | E                   | 2.8                        |
| <i>Microascus cinereus</i> (Emile-Weil & Gaudin) Curzi  | 20                              | D                   | 2.8                        |
| <i>Neosartorya fischeri</i> (Wehmer) Malloch & Cain var. <i>glabra</i> (Fennell & Raper) Malloch & Cain | 24, 25, 27                      | H, T                | 8.3                        |
| <i>N. quadricincta</i> (Yuill) Malloch & Cain   | 13, 27, 28                      | H, T                | 8.3                        |
| <i>Penicillioptosis clavariaefomis</i> Solms-Laubach  | 12                              | E                   | 2.8                        |
| <i>Talaromyces flavus</i> (Klocker) Stolk & Samson var. <i>flavus</i>                                   | 9, 10, 12, 24, 25, 27, 35       | H, T, E, D          | 22.2                       |
| <i>T. ohiensis</i> Pitt   | 25                              | T                   | 2.8                        |
| <i>T. helicus</i> C.R. Benjamin apud Stolk & Samson var. <i>helicus</i>                                 | 24, 25                          | T, E                | 5.6                        |
| <i>T. stipitatus</i> C.R. Benjamin apud Stolk & Samson  | 24, 25, 26, 27                  | T, E, D             | 11.1                       |
| <i>T. wortmannii</i> C.R. Benjamin apud Stolk & Samson  | 11, 18, 21, 24                  | D                   | 11.1                       |
| <i>Thermoascus aurantiacus</i> Miehe  | 27, 30                          | H                   | 5.6                        |
| <i>Thielavia terricola</i> (Gilman & Abott) Emmons  | 24                              | H                   | 2.8                        |
| <i>Westerdykella multispora</i> (Saito & Minoura) Cejp & Milko  | 25                              | D                   | 2.8                        |
| <b>DEUTEROMYCOTINA</b>  |                                 |                     |                            |
| <i>Acremonium albamense</i> Morgan-Jones  | 18, 24, 25, 26, 27, 36          | H                   | 16.7                       |
| <i>A. terricola</i> (Miller et al.) W. Gams   | 27                              | D                   | 2.8                        |
| <i>Acremonium</i> spp.  | (18) <sup>c</sup>               | D                   | 50.0                       |
| <i>Arthriniium phaeospermum</i> (Corda) E.B. Ellis  | 25                              | E                   | 2.8                        |
| <i>Aspergillus clavatus</i> Desmazières   | 12, 17                          | T, D                | 5.6                        |
| <i>A. fumigatus</i> Fresenius   | 30                              | H                   | 2.8                        |
| <i>A. terreus</i> Thom  | 14, 17, 18, 22, 24, 25, 26, 27  | H, D                | 22.2                       |
| <i>Chalara</i> sp.  | 12                              | D                   | 2.8                        |
| <i>Cladosporium cladosporioides</i> (Fresenius) de Vries  | 11, 28                          | D                   | 5.6                        |
| <i>Coniothyrium</i> spp.  | 4, 7, 8, 11, 18, 24, 26, 29, 35 | D                   | 25.0                       |
| <i>Exophiala</i> sp.  | 32                              | D                   | 2.8                        |

Table 2. (Continued).

| Species detected   | Sample No.                                | Method <sup>a</sup> | Frequency <sup>b</sup> (%) |
|--|---|---------------------|----------------------------|
| <i>Fusarium</i> sp.                                      | 35  | D                   | 2.8                        |
| <i>Gliocladium virens</i> Miller et al.                  | 25  | D                   | 2.8                        |
| <i>Gliocladium</i> sp.                                   | 9   | D                   | 2.8                        |
| <i>Metarhizium anisopliae</i> (Metschnikoff) Sorokin     | 12, 26                                    | D                   | 5.6                        |
| <i>Nodulisporium</i> sp.                                 | 3   | E                   | 2.8                        |
| <i>Paecilomyces lilacinus</i> (Thom) Samson              | 12, 22, 25                                | D                   | 8.3                        |
| <i>Paecilomyces</i> spp.                                 | 12, 22, 35                                | D                   | 8.3                        |
| <i>Penicillium citrinum</i> Thom                         | 26  | D                   | 2.8                        |
| <i>P. corylophilum</i> Dierckx                           | 18  | D                   | 2.8                        |
| <i>P. crustosum</i> Thom                                 | 9, 12, 25, 27, 28, 32                     | D                   | 16.7                       |
| <i>P. janthinellum</i> Biourge                           | 21, 25                                    | D                   | 5.6                        |
| <i>P. purpurogenum</i> Stoll                             | 9, 11, 12, 15, 17, 18, 19, 20, 22, 26, 28 | D                   | 30.6                       |
| <i>P. rugulosum</i> Thom                                 | 16  | D                   | 2.8                        |
| <i>Penicillium</i> spp.                                  | 9, 18                                     | D                   | 5.6                        |
| <i>Pestalotiopsis</i> sp.                                | 25  | D                   | 2.8                        |
| <i>Phialophora fastigiata</i> (Lagerberg & Melin) Conant | 1, 9, 21, 27                              | D                   | 11.1                       |
| <i>Phialophora</i> spp.                                  | 3, 4, 11                                  | D                   | 8.3                        |
| <i>Phoma herbarum</i> Westend                            | 13, 24                                    | D                   | 5.6                        |
| <i>Phoma</i> spp.  | (19)                                      | D                   | 52.8                       |
| <i>Phomopsis</i> spp.                                    | 13, 21, 30, 32                            | D                   | 11.1                       |
| <i>Scopulariopsis brumptii</i> Salvanet-Duval            | 26, 29                                    | D                   | 5.6                        |
| <i>Scopulariopsis</i> spp.                               | 16, 22                                    | D                   | 5.6                        |
| <i>Thermophymatospora fibrigera</i> Udagawa et al.       | 27  | H                   | 2.8                        |
| <i>Trichoderma aureoviride</i> Rifai                     | 12, 22, 25                                | D                   | 8.3                        |
| <i>T. harzianum</i> Rifai                                | 17, 18, 20, 25, 26, 27, 30                | D                   | 19.4                       |
| <i>T. koningii</i> Oudemans                              | 36  | D                   | 2.8                        |
| <i>T. pseudokoningii</i> Rifai                           | 10  | D                   | 2.8                        |
| <i>Trichoderma</i> spp.                                  | 24, 25                                    | D                   | 5.6                        |
| <i>Virgaria nigra</i> (Link) Nees ex S.F. Gray           | 20  | D                   | 2.8                        |
| ZYGOMYCOTINA   |   |                     |                            |
| <i>Mucor hiemalis</i> Wehmer f. <i>hiemalis</i>          | 25  | D                   | 2.8                        |
| <i>Rhizomucor pusillus</i> (Lindt) Schipper              | 26  | H                   | 2.8                        |
| BASIDIOMYCOTINA  |   |                     |                            |
| Unidentified species                                     | 1, 22                                     | E, D                | 5.6                        |
| Sterile mycelium   | (26)                                      | E, D                | 69.4                       |

a: H, heat incubation; E, ethanol treatment; T, heat treatment; D, dilution plate. b: Number of positive samples/total number of samples. c: Total number of samples in which the fungi were detected.

Forty-five genera of Deuteromycotina were detected. *Acremonium alabamense* Morgan-Jones, *Aspergillus terreus* Thom, *Penicillium crustosum* Thom, *P. purpurogenum* Stoll, *Trichoderma harzianum* Rifai were frequently isolated from 16.7% to 30.6% of all samples collected. These species are commonly isolated from various soils and materials in many parts of the world (1, 2, 3, 4, 9). Many strains of *Phoma* spp. were isolated. Almost all of these strains are considered to adapt to the conditions of mangrove mud by the formation of enduring tissues such as pycnidia.

Only two species of Zygomycotina were detected, although these species are dominant in cold areas and forest soil (1, 3, 4, 9). The scarce distribution of Zygomycotina is probably due to the deficiency of dissolved oxygen in mangrove mud.

Two unidentified species of Aphyllophorales in Basidiomycotina were isolated. These species had typical clamp connections, but primordia or fruit bodies were not formed during cultivation on sawdust plus rice bran. Some fungi which occur in low frequency in mud were also detected in this investigation. A strain of *Penicillium clavariaeformis* Solms-Laubach belonging to Ascomycotina was isolated from a sample from the Shiira river in Iriomote Is. This species has only been isolated from Iriomote Is. in Japan with its anamorphic state. *Thermophymatospora fibriger* Udagawa et al. and *Virgaria nigra* (Link) Nees ex S. F. Gray belonging to Deuteromycotina were detected from Nagura river in Ishigaki Is. and Maira river in Iriomote Is., respectively. The former species was originally isolated from soil of a date palm plantation in Iraq as a thermotolerant fungus of basidiomycetous Hyphomycetes. The latter is often reported worldwide from various kinds of wood and leaves, but it is rare in soil.

Using the trap method, strains of *Acremonium strictum* W. Gams, *Aspergillus niger* v. Tieghem, *A. terreus* Thom, *Fusarium* sp. *Paecilomyces lilacinus* (Thom) Samson, *Scopulariopsis brumptii* Solvanet-Duval, and *Talaromyces stipitatus* C. R. Benjamin apud Stolk & Samson were isolated. Other than *T. stipitatus*, these fungi are known to decompose chitin and have been isolated from saltmarshes by Domsh et al. (1980).

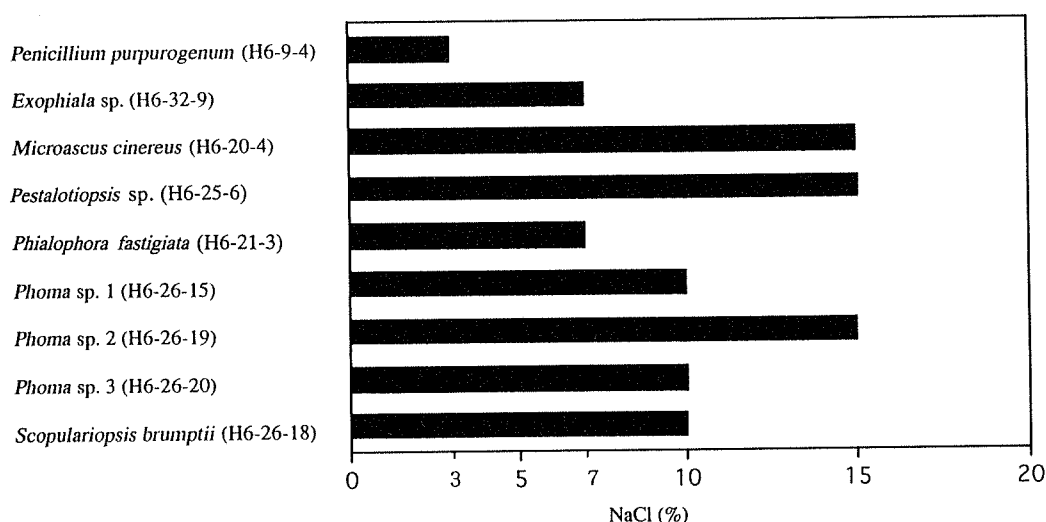


Fig. 3. Salinity range for growth of some isolates. (Growth on malt extract agar containing NaCl after incubation for three weeks at 24 °C.)



Figure 3 shows the results of the test for sodium chloride tolerance. The fungi tested grew up to the concentration of 7.0–15.0% NaCl. These fungi, which are termed as osmophiles, have been isolated and reported most frequently from sugar, salted food products and concentrated fruit juices (1). They are probably able to adapt to the high osmotic pressure that results when mangrove mud dries up at high temperature.

The fungal flora of mangrove mud in Okinawa, which is semi-anaerobic and has a high content of heavy metals, was characterized by fewer species and fewer propagules than those of agricultural soil samples.

A part of this research was supported by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science and Culture, Japan, No. 05760256 to A. Nakagiri.

### References

- 1) Domsch, K.H., W. Gams, and T.H. Anderson. 1980. *Compendium of soil fungi*. Academic Press, London.
- 2) Huang, L.H. and J.A. Schmitt. 1975. Soil microfungi of central and southern Ohio. *Mycotaxon* 3: 55–88.
- 3) Ito, T., M. Ueda, and T. Yokoyama. 1981. Thermophilic and thermotolerant fungi in paddy field soils. *IFO Res. Commun.* 10: 20–32.
- 4) Ito, T. 1993. Changes of fungal flora in soil after a bonfire. *IFO Res. Commun.* 16: 63–85.
- 5) Rai, J.N., J.P. Tewari, and K.G. Mukerji. 1969. Mycoflora of mangrove mud. *Mycopathol. Mycol. Appl.* 38: 17–31.
- 6) Swart, H.T. 1958a. An investigation of mycoflora in the soil of some mangrove swamps. North-Holland Publishing Co. Amsterdam.
- 7) Swart, H.T. 1963a. Further investigation of the mycoflora in the soil of some mangrove swamps. *Acta bot. Neerl.* 12: 98–111.
- 8) Wakushima, S., S. Kuraishi, and N. Sakurai. 1994. Soil salinity and pH in Japanese mangrove forests and growth of cultivated mangrove plants in different soil conditions. *K. Plant. Res.* 107: 39–46.
- 9) Yokoyama, T., T. Ito, and Y.O. Yin. 1989. Filamentous fungi isolated from soils in the Xinjiang Uighur Autonomous region, China. *IFO Res. Commun.* 14: 118–142.

# Mycoflora of the Rhizospheres of Mangrove Trees

Tadayoshi ITO and Akira NAKAGIRI

## Summary

Mycoflora of the rhizospheres of mangrove trees collected from Okinawa Pref. in Japan was investigated. After washing the collected mangrove roots and surrounding soil samples, their root- and soil-inhabiting fungi were isolated by washing and dilution plate methods, respectively. *Pestalotiopsis* sp.-1 and *Trichoderma harzianum* were dominant species on the rhizoplane of mangrove trees, and these two species were considered to be the main components of mangrove rhizoplane mycoflora. The genera *Acremonium*, *Coniothyrium*, *Penicillium*, *Phoma* and *Trichoderma*, known terrestrial fungi, were isolated from the washed root soils. Thus, the mycoflora of the mangrove rhizoplane differed from that of its surrounding soil.

Keywords: Mycoflora, mangrove rhizosphere, terrestrial fungi, root- and soil-inhabiting fungi

The microorganisms in the rhizosphere of higher plants are known to grow by using dead plant debris or secretions from plant root tissues (8). They are also reported to form a kind of micro-community in the rhizosphere (8). The soils of mangrove forests in tropical and subtropical regions are reported to be semi-aerobic, low in nutrients, and to have higher concentrations of heavy metals and higher salinity than terrestrial soils (9). There have been few reports on the mycofloras in such adverse conditions in the mangrove rhizosphere. The present investigation treats mangrove rhizosphere samples collected from estuaries in the South-West Islands of Okinawa prefecture.

## Materials and Methods

*Sampling sites.* Twenty-two healthy root samples of *Avicennia marina* Vierh., *Rhizophora stylosa* Griff., *Sonneratia alba* J.A. Sm., *Bruguiera gymnorrhiza* Lamk., *Kandelia candel* Druce and *Lumnitzera racemosa* Willd. were collected from estuaries of the Maira, Shiira and Urauchi rivers of Iriomote Is. and the Nagura and Fukidou rivers of Ishigaki Is., Okinawa prefecture, Japan in October 1995 (Table 1).

Table 1. List of mangrove rhizosphere samples collected in Okinawa.

| Sample No. | Date sampled | Locality                      | Collected Samples   |
|------------|--------------|-------------------------------|---|
| H7-18-23   | 24/10/'95    | Maira river<br>Iriomote Is.   | <i>Avicennia marina</i><br><i>Rhizophora stylosa</i><br><i>Sonneratia alba</i>    |
| H7-24-31   | 24/10/'95    | Shiira river<br>Iriomote Is.  | <i>Bruguiera gymnorrhiza</i><br><i>Avicennia marina</i><br><i>Sonneratia alba</i> |
| H7-32-35   | 25/10/'95    | Urauchi river<br>Iriomote Is. | <i>Kandelia candel</i>  |
| H7-36-37   | 26/10/'95    | Nagura river<br>Ishigaki Is.  | <i>Lumnitzera racemosa</i>  |
| H7-38-39   | 26/10/'95    | Fukido river<br>Ishigaki Is.  | <i>Rhizophora stylosa</i>   |

**Isolation methods.** Slender root samples were cut into sections of 5 cm in length and washed with physiological saline solution. Five sections of each sample were put on each of two plates containing cornmeal agar and incubated at 15°C for 4 wk.

All fungi appearing during the incubation were isolated at 2, 3 and 4 wk under the dissecting microscope. Soil fungi were isolated by the dilution plate method from the soil suspension obtained in the first washing. Isolates were then transferred to half-concentration malt extract agar slant.

**Isolation medium.** Cornmeal agar containing 50 µg/ml of the tetracycline antibiotics was used for the slender mangrove root samples. For soil samples, malt-yeast extract agar containing the same sort and concentration of antibiotic was used.

**Identification of isolates.** Isolates were inoculated on plates of potato-carrot, malt extract, potato-sucrose and oatmeal agars and incubated at 24°C for appropriate periods. To identify the isolates, one representative strain of each species was used.

## Results and discussion

Table 2 lists the fungi isolated from mangrove root samples and their frequency of isolation. Isolated species mainly belong to the taxon Deuteromycotina.

The sample of *Rhizophora stylosa* yielded the largest number of isolates, 18 strains of 18 species; and *Kandelia candel* yielded the fewest, 6 strains of 2 species.

*Trichoderma harzianum* Rifai was detected in the highest frequency from the 22 samples used (50.0% frequency) and from all six species of mangrove trees. This fungus is well known from plant debris and soil worldwide. It has also been isolated from the rhizospheres of poplar, pine, tobacco, beet and wheat, and is reported to decompose cellulose and starch (1). Therefore, this fungus is considered to have a high affinity for mangrove roots. The second dominant species was *Pestalotiopsis* sp-1. (27.3% frequency). Species of this genus are known as parasites which causes the *Pestalotia* disease of apple, chestnut, persimmon, loquat and coconut palm trees and the leaf spot disease of

Table 2. Fungi isolated from roots of mangrove trees and their frequency of isolation.

| Species                             | Host           |          |          |          |          |          | Frequency (%) <sup>a</sup> |
|-------------------------------------|----------------|----------|----------|----------|----------|----------|----------------------------|
|                                     | So. alba       | Rh. sty. | Br. gym. | Av. mar. | Ka. can. | Lu. rac. |                            |
| <i>Acremonium</i> sp. <sup>b</sup>  | 1 <sup>c</sup> | 1        |          |          |          | 1        | 13.6                       |
| <i>Alternaria alternata</i>         | 1              | 1        |          |          |          |          | 9.1                        |
| <i>Aspergillus aculeatus</i>        |                |          |          | 1        |          |          | 4.5                        |
| <i>Cladosporium cladosporioides</i> |                | 1        |          |          |          |          | 4.5                        |
| <i>Cylindrocarpon destructans</i>   | 1              | 1        |          |          |          | 1        | 13.6                       |
| Coelomycetes                        |                | 3        |          |          |          |          | 13.6                       |
| <i>Engyodontium album</i>           |                |          |          | 1        |          |          | 4.5                        |
| <i>Eupenicillium</i> sp.            |                |          | 1        |          |          |          | 4.5                        |
| <i>Fusarium moniliforme</i>         | 1              |          | 1        |          |          |          | 9.1                        |
| <i>Fusarium</i> sp. 1               |                | 1        |          |          |          |          | 4.5                        |
| <i>Gliocladium virens</i>           |                |          |          |          |          | 1        | 4.5                        |
| <i>Gliomastix murorum</i>           |                |          |          | 1        |          |          | 4.5                        |
| <i>Myrothecium</i> sp.              |                | 1        |          |          |          |          | 4.5                        |
| <i>Pestalotiopsis</i> sp.-1         | 1              | 1        | 1        |          | 2        | 1        | 27.3                       |
| <i>Penicillium citrinum</i>         |                | 1        | 2        |          |          |          | 13.6                       |
| <i>Pencillium</i> sp. 1             | 1              | 1        | 1        |          |          |          | 13.6                       |
| <i>Pencillium</i> sp. 2             |                |          | 1        | 1        |          |          | 9.1                        |
| <i>Pencillium</i> sp. 3             |                |          | 1        | 1        |          |          | 9.1                        |
| <i>Pencillium</i> sp. 4             |                |          | 1        | 1        |          |          | 9.1                        |
| <i>Phoma</i> sp. 1                  |                | 1        |          |          |          |          | 4.5                        |
| <i>Phoma</i> sp. 2                  |                | 1        |          |          |          |          | 4.5                        |
| <i>Scolecobasidium humicola</i>     |                |          |          | 1        |          |          | 4.5                        |
| <i>Trichoderma aureoviride</i>      |                |          | 1        |          |          |          | 4.5                        |
| <i>Trichoderma harzianum</i>        | 3              | 1        | 1        | 1        | 4        | 1        | 50.0                       |
| <i>Trichoderma</i> sp.              | 1              |          |          |          |          |          | 4.5                        |
| <i>Virgaria nigra</i>               |                |          |          | 1        |          |          | 4.5                        |
| <i>Volutina concentrica</i>         |                | 1        |          |          |          |          | 4.5                        |
| <i>Westerdykella dispersa</i>       |                | 1        |          |          |          |          | 4.5                        |
| Unidentified strains                | 2              | 1        | 1        | 1        |          | 1        | 27.3                       |
| Total number of strains             | 12             | 18       | 12       | 10       | 6        | 6        |                            |
| Number of samples                   | 4              | 4        | 4        | 4        | 4        | 2        | 22                         |

a: Total number of positive samples / total number of samples.

b: Bold type shows species detected in the previous investigation (1994).

c: Number of positive samples in each mangrove tree.

maidenhair and Japanese cedar (5, 6). This is the first time it has been detected from mangrove roots.

The species isolated in this investigation agree closely with those of an earlier investigation in October 1994 (unpublished). Therefore, it is considered that the dominant species of the mangrove rhizosphere are *Trichoderma harzianum* and *Pestalotiopsis*

Table 3. Fungi isolated from soil of washed mangrove roots by dilution plate method.

| Species                                      | No. of positive samples | Frequency (%) <sup>a</sup> |
|--|-------------------------|----------------------------|
| <b>DEUTEROMYCOTINA</b>                       |                         |                            |
| <i>Acremonium</i> spp.                       | 11                      | 50.0                       |
| <i>Albophoma yamanashiensis</i>              | 2                       | 9.1                        |
| <i>Arthrimum phaeospermum</i>                | 1                       | 4.5                        |
| <i>Aspergillus clavatus</i>                  | 2                       | 9.1                        |
| <i>Aspergillus niger</i>                     | 1                       | 4.5                        |
| <i>Cladosporium cladosporioides</i>          | 6                       | 27.3                       |
| <i>Coniothyrium</i> spp.                     | 12                      | 54.5                       |
| <i>Exophiala</i> sp.                         | 5                       | 22.7                       |
| <i>Fusarium</i> spp.                         | 8                       | 36.4                       |
| <i>Gliocladium roseum</i>                    | 3                       | 13.6                       |
| <i>Gliocladium virens</i>                    | 2                       | 9.1                        |
| <i>Gliocladium</i> sp.                       | 1                       | 4.5                        |
| <i>Gliomastix murorum</i>                    | 1                       | 4.5                        |
| <i>Metarhizium anisopliae</i>                | 3                       | 13.6                       |
| <i>Myrothecium</i> sp.                       | 1                       | 4.5                        |
| <i>Nodulisporium</i> sp.                     | 3                       | 13.6                       |
| <i>Paecilomyces lilacinus</i>                | 8                       | 36.4                       |
| <i>Paecilomyces</i> spp.                     | 5                       | 22.7                       |
| <i>Penicillium citrinum</i>                  | 6                       | 27.3                       |
| <i>P. corylophilum</i>                       | 2                       | 9.1                        |
| <i>P. crustosum</i>                          | 3                       | 13.6                       |
| <i>P. funiculosum</i>                        | 2                       | 9.1                        |
| <i>P. janthinellum</i>                       | 4                       | 18.2                       |
| <i>P. purpurogenum</i>                       | 8                       | 36.4                       |
| <i>Penicillium</i> spp.                      | 3                       | 13.6                       |
| <i>Pestalotiopsis</i> sp.                    | 1                       | 4.5                        |
| <i>Phialophora fastigiata</i>                | 5                       | 22.7                       |
| <i>Phialophora</i> spp.                      | 6                       | 27.3                       |
| <i>Phoma</i> spp.                            | 13                      | 59.1                       |
| <i>Phomopsis</i> spp.                        | 2                       | 9.1                        |
| <i>Scopulariopsis</i> spp.                   | 5                       | 22.7                       |
| <i>Stilbum</i> sp.                           | 2                       | 9.1                        |
| <i>Trichoderma aureoviride</i>               | 2                       | 9.1                        |
| <i>T. harzianum</i>                          | 10                      | 45.5                       |
| <i>T. koningii</i>                           | 3                       | 13.6                       |
| <i>T. pseudokoningii</i>                     | 2                       | 9.1                        |
| <i>Trichoderma</i> spp.                      | 3                       | 13.6                       |
| <b>ASCOMYCOTINA</b>                          |                         |                            |
| <i>Achaetomium macrosporum</i>               | 1                       | 4.5                        |
| <i>Talaromyces flavus</i> var. <i>flavus</i> | 1                       | 4.5                        |
| <b>ZYGOMYCOTINA</b>                          |                         |                            |
| <i>Cunninghamella</i> sp.                    | 1                       | 4.5                        |
| <i>Mortierella</i> sp.                       | 1                       | 4.5                        |
| <i>Mucor</i> sp.                             | 1                       | 4.5                        |
| Sterile mycelium                             | 16                      | 72.7                       |

a: Number of positive samples / a total number of samples.

sp.-1. To investigate the correlation between fungi and mangrove roots, these two strains were inoculated on the sterilized root of *Rhizophora stylosa*. It is confirmed that the strain of *Trichoderma harzianum* invaded into the intercellular space and sporulated in the air space, and *Pestalotiopsis* sp.-1 strain exfoliated the cortical tissue and formed pycnidia in the mangrove root tissue. *Penicillium citrinum* Thom, a terrestrial fungus, was used as a control. It does not invade the root tissues and formed colonies only on the root surface. It is suggested that the former two species are the main components of the mangrove rhizoplane.

Table 3 shows the species detected and their frequency from 22 samples of soil surrounding roots.

Species of *Acremonium* Link, *Coniothyrium* Corda, *Fusarium* Link, *Paecilomyces* Bainnier, *Penicillium* Link, *Phoma* Sacc. and *Trichoderma* Pers. were the dominant species detected from these samples. These results agreed well with those of mud samples taken from mangrove forest in January 1994 and examined by the dilution plate method (4) and with those of mangrove mud in India (7). No difference was found between the mycoflora of the mangrove root soil and the soils of paddy, pineapple, plant garden and pasture concurrently collected from Iriomote Is. of Okinawa (4) or the soils of paddy and vegetable fields in Osaka prefecture (2, 3). Therefore, the fungi detected in mangrove root soil are considered to have been carried there from land by river.

It was confirmed that mycoflora on the mangrove rhizoplane differs from that of the surrounding root soil. In particular, *Trichoderma harzianum* and *Pestalotiopsis* sp.-1 are suggested an affinity for mangrove roots. Further experiments are needed to confirm the ecological distribution of these fungi.

A part of this study was supported by Fujiwara Natural History Foundation.

### References

- 1) Domsch, K.H., W. Gams, and T.H. Anderson. 1980. Compendium of soil fungi. Academic Press, London.
- 2) Ito, T., M. Ueda, and T. Yokoyama. 1981. Thermophilic and thermotolerant fungi in paddy field soils. IFO Res. Commun. 10: 20-32.
- 3) Ito, T. 1993. Changes of fungal flora in soil after a bonfire. IFO Res. Commun. 16: 63-85.
- 4) Ito, T. and A. Nakagiri. 1997. A mycofloral study on mangrove mud in Okinawa, Japan. IFO Res. Commun. 18: 32-39.
- 5) Japan Plant Protection Association (ed.). 1975-1984. Common names of economic plant diseases in Japan. Vol. 1-4. (In Japanese).
- 6) Japan Plant Protection Association (ed.). 1984. Common names of economic plant diseases in Japan. Vol. 5. (In Japanese).
- 7) Swart, H.T. 1963a. Further investigation of mycoflora in the soil of some mangrove swamps. Acta bot. Neerl. 12: 98-111.
- 8) Tsuru, S. 1981. Koutoushokubutu to biseibutu 1. In Dojyoubiseibutukenkyuukai (ed.) Tsuchinobiseibutu p. 173-200. Hakuyusha, Tokyo. (In Japanese).
- 9) Wakushima, S., S. Kuraishi, and N. Sakurai. 1994. Soil salinity and pH in Japanese mangrove forests and growth of cultivated mangrove plant in different soil conditions. J. Plant Res. 107: 39-46.

## Preliminary Study of Endophytic Fungi in Evergreen Plants from Ishigaki and Iriomote Islands

Izumi OKANE, Akira NAKAGIRI and Tadayoshi ITO

### Summary

Endophytic fungi were isolated from 21 evergreen plants from Ishigaki and Iriomote islands in Okinawa Pref. Some endophytic fungi were found in all plants examined. Xylariaceous fungus and *Phyllosticta* spp. were isolated from about half of the plants tested. *Pestalotiopsis* spp. were isolated from 7 plants, *Phomopsis* spp. and *Colletotrichum gloeosporioides* were found in 6 plants each. *Alternaria alternata*, *Nigrospora oryzae*, *Cladosporium cladosporioides*, *Acremonium* spp., *Gliocladium roseum*, *Phoma* sp., *Curvularia* sp. and *Coccomyces* sp. were also isolated from several plants.

Key words: endophytic fungi, evergreen plants, subtropical region in Japan.

Endophytic fungi within aerial parts of vascular plants have been extensively studied over the past 15 years. Most of these investigations involved plants from temperate regions (16), although plants from subtropical and tropical regions have also been investigated by several researchers (1, 3, 8, 17, 20). Endophytic fungi have been found in a wide range of flowering, mainly dicotyledonous plants and conifers (16). Most reports of endophytes occurring in monocotyledonous plants concern grasses, in which the role of some of these endophytic fungi on the development of their host is beginning to be understood (7). In Japan, though several investigations of endophytic fungi have been carried out on conifers, grasses and ericaceous plants (9, 10, 11, 12, 15), further investigations are necessary to clarify ecological and species diversity of endophytic fungi on various plants. Because of its hot, moist climate during the growing season and diverse vascular plant flora, Japan is predicted to have a very rich flora of endophytic fungi (4).

In this study we report the incidence of endophytic fungi within leaves of evergreen plants from Ishigaki and Iriomote islands, Okinawa Pref. This is a preliminary study for clarification of ecological and species diversity of endophytic fungi.

### Materials and methods

Samples were collected in October 1995 from Ishigaki and Iriomote islands in Okinawa Pref., which are located at 24°N lat., 124°E long. approximately. Plants examined are shown in Table 1. They are evergreens, mostly distributed in the subtropi-

Table 1. Plants investigated from Ishigaki and Iriomote islands.

| Plant (Japanese common name)                                      | Family (Japanese)            | Distribution in Japan  |
|---|------------------------------|--|
| <i>Pinus luchuensis</i><br>(Ryukyu-matsu)                         | Pinaceae<br>(Matsu)          | South of Tokara Isl.   |
| <i>Quercus miyagii</i><br>(Okinawa-urajirogashi)                  | Fagaceae<br>(Buna)           | Ryukyu Isl.  |
| <i>Artocarpus integrifolius</i><br>(Paramitsu)                    | Moraceae<br>(Kuwa)           | cultivated   |
| <i>Ficus</i> sp.<br>(Ougon-gajyumaru)                             | Moraceae<br>(Kuwa)           | Yakushima Isl., Ryukyu Isl.                                  |
| <i>Ficus septica</i><br>(Ooba-inubiwa)                            | Moraceae<br>(Kuwa)           | Ryukyu Isl.  |
| <i>Schima wallichii</i><br>(Ijyu)                                 | Theaceae<br>(Tsubaki)        | Bonin Isl., Ryukyu Isl.                                      |
| <i>Garcinia subelliptica</i><br>(Fukugi)                          | Clusiaceae<br>(Terihaboku)   | Ryukyu Isl.  |
| <i>Calophyllum inophyllum</i><br>(Terihaboku)                     | Clusiaceae<br>(Terihaboku)   | Bonin Isl., Ryukyu Isl.                                      |
| <i>Distylium racemosum</i><br>(Isunoki)                           | Hamamelidaceae<br>(Mansaku)  | Honshu, Shikoku, Kyushu, Ryukyu Isl.                         |
| <i>Cassia glauca</i><br>(Kobanosenna)                             | Legminosae<br>(Mame)         | cultivated   |
| <i>Leucaena leucocephala</i><br>(Gin-nemu)                        | Legminosae<br>(Mame)         | Ryukyu Isl.  |
| <i>Poinciana regia</i><br>(Hououboku)                             | Legminosae<br>(Mame)         | South of Kyusyu  |
| <i>Mangifera indica</i><br>(Mango)                                | Anacardiaceae<br>(Urushi)    | cultivated   |
| <i>Heritiera littoralis</i><br>(Sakishima-suounoki)               | Sterculiaceae<br>(Aogiri)    | Ryukyu Isl.  |
| <i>Rhizophora stylosa</i><br>(Yaeyama-hirugi)                     | Rhizophoraceae<br>(Hirugi)   | South of Okinawa Isl.  |
| <i>Vaccinium wrightii</i><br>(Giima)                              | Ericaceae<br>(Tsutsuji)      | Ryukyu Isl.  |
| <i>Diospyros ferrea</i> var. <i>buxifolia</i><br>(Ryukyu-kokutan) | Ebenaceae<br>(Kakinoki)      | Ryukyu Isl.  |
| <i>Psychotria rubra</i><br>(Bochoji)                              | Rubiaceae<br>(Akane)         | Yakushima Isl., Bonin Isl. to Ryukyu Isl.                    |
| <i>Messerschmidia argentea</i><br>(Monpanoki)                     | Boraginaceae<br>(Murasaki)   | Tokara Isl. to Ryukyu Isl.                                   |
| <i>Scaevola sericea</i><br>(Kusatobera)                           | Goodeniaceae<br>(Kusatobera) | Yakushima Isl., Tanegashima Isl.,<br>Ryukyu Isl., Bonin Isl. |
| <i>Crossostephium chinense</i><br>(Mokubyakkou)                   | Compositae<br>(Kiku)         | Tokara Isl. to Ryukyu Isl., Bonin Isl.                       |



cal regions of Japan, the southern part of the Tokara Islands, the Ryukyu Islands and the Bonin Islands. Twenty-one plant species were investigated in this study. Healthy leaves were collected and put into paper bags, which were covered with polyethylene bags to keep a moderate humidity. These were held between two boards made of braided pieces of bamboo and carried to the laboratory of IFO (Institute for Fermentation, Osaka) in Osaka.

In our experiments, the leaves were rigorously surface-sterilized before culturing the endophytes. Surface-sterilization was effected by immersion in 70% ethanol solution for 1 min and sodium hypochlorite solution (1% available chlorine) for 2 min. The leaves were rinsed in sterile distilled water and put into sterile paper towels for 3 h to remove water from the surface of the leaves. The number of leaves tested varied with the plant. After surface sterilization and drying, the leaves were divided into several segments. They were serially placed on half-strength malt extract agar medium in 90-mm plates and incubated at room temperature for 3 months. The fungi growing out of the segments during the incubation period were isolated and identified.

### Results and Discussion

Table 2 shows the number of hosts colonized by endophytic fungi isolated. Table 3 shows the frequency on each plant of the five major endophytic fungi.

Some endophytic fungi were isolated from all plants. Endophytic fungi are thought to occur ubiquitously in a wide range of evergreen plants in the subtropical region of Japan, though the frequency of the fungi isolated may not be reliable because an insufficient number of leaves was examined in the case of certain plants.

Xylariaceous fungus and *Phyllosticta* spp. were isolated from about a half of the plants examined, that is, 11 and 10 plants, respectively. Eight plants of them were common hosts of these fungi. Xylariaceous fungus and *Phyllosticta* spp. are expected as

Table 2. Number of hosts colonized by fungi isolated.

| Fungus                                | Number of hosts | %  |
|---------------------------------------|-----------------|----|
| Xylariaceous fungus                   | 11              | 52 |
| <i>Phyllosticta</i> spp.              | 10              | 48 |
| <i>Pestalotiopsis</i> sp. 1           | 3               | 14 |
| <i>Pestalotiopsis</i> sp. 2           | 4               | 19 |
| <i>Phomopsis</i> spp.                 | 6               | 29 |
| <i>Colletotrichum gloeosporioides</i> | 6               | 29 |
| <i>Alternaria alternata</i>           | 1               | 5  |
| <i>Nigrospora oryzae</i>              | 2               | 10 |
| <i>Cladosporium cladosporioides</i>   | 1               | 5  |
| <i>Acremonium</i> sp. 1               | 1               | 5  |
| <i>Acremonium</i> sp. 2               | 4               | 19 |
| <i>Gliocladium roseum</i>             | 3               | 14 |
| <i>Phoma</i> sp.                      | 1               | 5  |
| <i>Curvularia</i> sp.                 | 2               | 10 |
| <i>Coccomyces</i> sp.                 | 2               | 10 |
| Unidentified coelomycete              | 1               | 5  |
| Sterile dark mycelia                  | 5               | 24 |
| Sterile light mycelia                 | 7               | 33 |

Table 3. Colonization frequencies of major endophytic fungi isolated from evergreen plants from Ishigaki and Iriomote islands.

| Plant   | Xylariaceous<br>fungus | <i>Phyllosticta</i><br>spp. | <i>Pestalotiopsis</i><br>spp. | <i>Phomopsis</i><br>spp. | <i>Colletotrichum</i><br><i>gloeosporioides</i> | Total number of fungi<br>isolated in this study |
|---|------------------------|-----------------------------|-------------------------------|--------------------------|---|---|
| <i>Pinus luchuensis</i>                       | 4/9 (44)               |                             | 1/9 (11)                      |                          |   | 3   |
| <i>Quercus miyagii</i>                        | 2/3 (66)               | 1/3 (33)                    |                               |                          |   | 3   |
| <i>Artocarpus integrifolius</i>               | 3/3 (100)              |                             |                               | 1/3 (33)                 |   | 3   |
| <i>Ficus</i> sp.                              | 2/6 (33)               | 3/6 (50)                    |                               |                          |   | 2   |
| <i>Ficus septica</i>                          |                        |                             |                               |                          | 1/3 (33)  | 3   |
| <i>Schima wallichii</i>                       | 1/4 (25)               | 3/4 (75)                    |                               |                          |   | 3   |
| <i>Garcinia subelliptica</i>                  | 5/6 (83)               | 1/6 (16)                    |                               |                          |   | 3   |
| <i>Calophyllum inophyllum</i>                 |                        | 1/6 (16)                    |                               |                          |   | 5   |
| <i>Distylium racemosum</i>                    | 2/10 (20)              | 1/10 (10)                   |                               |                          |   | 4   |
| <i>Cassia glauca</i>                          | 2/6 (33)               | 4/6 (66)                    |                               |                          |   | 3   |
| <i>Leucaena leucocephala</i>                  |                        |                             |                               |                          | 2/19 (10)                                       | 4   |
| <i>Poinciana regia</i>                        |                        |                             |                               | 2/18 (11)                | 1/18 (5)  | 3   |
| <i>Mangifera indica</i>                       | 1/3 (33)               | 1/3 (33)                    |                               |                          |   | 2   |
| <i>Heritiera littoralis</i>                   |                        |                             | 1/5 (20)                      | 1/5 (20)                 |   | 3   |
| <i>Rhizophora stylosa</i>                     |                        |                             |                               |                          | 1/3 (33)  | 3   |
| <i>Vaccinium wrightii</i>                     | 4/10 (40)              | 7/10 (70)                   | 1/10 (10)                     |                          | 3/10 (30)                                       | 4   |
| <i>Diospyros ferrea</i> var. <i>buxifolia</i> |                        | 4/6 (66)                    | 1/6 (16)                      |                          | 1/6 (16)  | 4   |
| <i>Psychotria rubra</i>                       | 2/3 (66)               |                             | 1/3 (33)                      |                          |   | 4   |
| <i>Messerschmidia argentea</i>                |                        |                             | 2/3 (66)                      | 3/3 (100)                |   | 3   |
| <i>Scaevola sericea</i>                       |                        |                             | 1/6 (16)                      | 5/6 (83)                 |   | 4   |
| <i>Crossosiphium chinense</i>                 |                        |                             |                               | 1/12 (8)                 |   | 3   |
| Number of hosts<br>colonized by the fungus    | 11                     | 10                          | 7                             | 6                        | 6   |   |

\* Number of leaves colonized by the fungus/Total number of leaves examined (%)

to be major endophytic fungi in this study.

Hyphomycetous imperfect states of xylariaceous fungi have been isolated from some plant families including subtropical and tropical species and are considered to appear commonly among census list of endophytes (17, 18). *Noduliosporium gregarium* (Berk. & Curt.) Meyer and *Noduliosporium*-anamorph of *Hypoxylon fragiforme* (Pers. ex Fr.) Kickx were found in tropical plant species belong to Araceae, Bromeliaceae and Orchidaceae (17). Cultural and isozymic investigations on endophytic species of *Xylaria* were carried out by Rodrigues et al. (1993)(21). Though xylariaceous fungi are known as saprobes or weak parasites on a wide range of plants, they are considered to relate in harmony with some hosts.

*Phyllosticta* is considered the quintessential endophyte genus (4). This taxon has been widely noted as an endophyte (5, 6, 19) and is expected among the endophyte flora whenever a new host plant is investigated. Okane (unpublished) has found that *Guignardia* sp. with a *Phyllosticta* anamorph which is similar to *Guignardia vaccinii* Shear (anamorph: *Phyllosticta vaccinii* Earle) in morphology is apparently ubiquitous on leaves of several ericaceous plants. *Guignardia vaccinii* ( $\equiv$  *Botryosphaeria vaccinii* (Shear) Barr), which has not been reported in Japan, is known as a latent infective weak-pathogen on cranberry (*Vaccinium macrocarpon*) (24). *Phyllosticta* spp. have been isolated from healthy leaves of 63 plant species among 93 plants investigated in Kyoto (frequency of approximately 70%) (Okane, unpublished). It can be expected that many more species will be identified on Japanese plants and that their ecological diversity will be clarified.

*Phomopsis* spp. and *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. were isolated from 6 plants each. *Poinciana regia* was a common host of them though these two fungi were not isolated from the same leaf. *Phomopsis* is particularly widespread, having been found in a wide range of angiospermous trees (2). Webber and Gibbs (1984)(23) have reported the widespread occurrence of *Phomopsis oblonga* (Desmaz.) Traverso as an endophytic fungus in the outer bark of *Ulmus* species in northern and eastern parts of England and in northern Wales. *Colletotrichum gloeosporioides* has been shown to form subcuticular hyphae or appressoria which remain dormant on the young leaves, flowers and fruit of *Citrus* and which resume growth only as the leaves age or the fruit ripen (22). Such behavior has been also observed in *Guignardia citricarpa* Kiely on *Citrus* (13). *Phomopsis* and *Colletotrichum* have been found as common endophytic fungi on ericaceous plants (Okane, unpublished). These two genera are considered to be represented in the endophytic floras of a wide variety of plants in Japan.

*Pestalotiopsis* sp. 1 was isolated from 3 plants, *Heritiera littoralis*, *Vaccinium wrightii* and *Psychotria rubra*, and *Pestalotiopsis* sp. 2 was isolated from 4 plants, *Pinus luchuensis*, *Dispyros ferrea* var. *buxifolia*, *Messerschmidia argentea* and *Scaevola sericea*. Common hosts of these two species were not found. *Pestalotiopsis* is known as a facultative and latent infective parasite, which has been isolated from old leaves, frequently with species of *Phoma*, *Cladosporium*, *Alternaria* etc. Though *Pestalotiopsis* was not isolated from *Rhizophora stylosa*, one of the common mangrove trees in Ryukyu Isl., Nakagiri et al. (1989)(14) have reported that *Pestalotiopsis* sp., *Acremonium* spp. and *Cladosporium cladosporioides* were isolated from the leaves of some of mangrove trees as the first group

in the fungal succession on these plants from the site, and *Pestalotiopsis* was found to be parasitized by *Halophytophthora* which invaded the leaves just after leaf fall. It is expected to be observed similar ecological phenomenon between *Pestalotiopsis* which occur the leaves of other various evergreen plants in this site and other fungi which invade the leaves later.

*Acremonium* spp. were isolated from *Artocarpus integrifolius*, *Calophyllum inophyllum*, *Leucaena leucocephala*, *Rhizophora stylosa* and *Scaevola sericea* in low frequencies. *Acremonium* and related fungi have been shown to enter into a mutualistic symbiosis with several grass species (7). This fungal group, as a member of the endophytic flora, can be expected to have beneficial effects not only on grasses but also on various dicotyledonous plants. *Alternaria alternata*, *Cladosporium cladosporioides* and *Phoma* sp. were isolated from *Messerschmidia argentea*, *Leucaena leucocephala* and *Psychotria rubra*, respectively. *Nigrospora oryzae* was isolated from *Pinus luchuensis* and *Scaevola sericea*. *Gliocladium roseum* was isolated from three plants, *Ficus septica*, *Rhizophora stylosa* and *Cassia glauca*. *Curvularia* sp. was isolated from *Calophyllum inophyllum* and *Crossostephium chinense*. *Coccomyces* sp. was isolated from *Schima wallichii* (Thaceae) and *Psychotria rubra* (Rubiaceae). *Coccomyces martiniae* Hansf. have been isolated from *Eucalyptus viminalis* in high frequency with *Coniothyrium* sp. (1, 3). In this study, *Leptostroma* and *Phialocephala*, which have been found in several pines in Japan (11), were not isolated from *Pinus luchuensis*.

Larger samples of leaves from various stages are required for more reliable investigation of the colonization frequency and the host preference of endophytic fungi, although it is considered that some endophytic fungi exist ubiquitously within the plants examined.

The possible role of most of endophytic fungi as mutualistic symbionts has not been proved for plant/insect systems, but several recent reports suggest that the possibility should be investigated further (4). Most plant-parasitic fungi, except for those which have been proved to have a symbiotic relationship with their hosts, like mycorrhizal fungi and grass endophytes, may be easily suspected to only absorb nutrition from their host or to commit wrongs one of their days. Further investigations on ecological and species diversity are necessary to clarify their essence.

### References

- 1) Bertoni, M. D. and D. Cabral. 1988. Phyllosphere of *Eucalyptus viminalis* II: Distribution of endophytes. *Nova Hedwigia* 46: 491-502.
- 2) Boddy, L. and G. S. Griffith. 1989. Role of endophytes and latent invasion in the development of decay communities in sapwood of angiospermous trees. *Sydowia* 41: 41-73.
- 3) Cabral, D. 1985. Phyllosphere of *Eucalyptus viminalis*: Dynamics of fungal populations. *Trans. Br. Mycol. Soc.* 85: 501-511.
- 4) Carroll, G. C. 1990. Fungal endophytes in vascular plants: Mycological research opportunities in Japan. *Trans. Mycol. Soc. Japan* 31: 103-116.
- 5) Carroll, G. C. and F. E. Carroll. 1978. Studies on the incidence of coniferous needle endophytes in the Pacific Northwest. *Can. J. Bot.* 56: 3034-3043.
- 6) Carroll, F. E., E. Müller and B. C. Sutton. 1977. Preliminary studies on the incidence of

- needle endophytes in some European conifers. *Sydowia* 29: 87–103.
- 7) Clay, K. 1989. Clavicipitaceous endophytes of grasses: their potencial as biological agents. *Mycol. Res.* 92: 1–12.
  - 8) Dreyfuss, M. and O. Petrini. 1984. Further investigations on the occurrence and distribution of endophytic fungi in tropical plants. *Botanica Helvetica* 94: 33–40.
  - 9) Hata, K. and K. Futai. 1993. Effect of needle aging on the total colonization rates of endophytic fungi on *Pinus thunbergii* and *Pinus densiflora* needles. *J. Jpn. For. Soc.* 75: 338–341.
  - 10) Hata, K. and K. Futai. 1995. Endophytic fungi associated with healthy pine needles and needles infested by the pine needle gall midge, *Thecodiplosis japonensis*. *Can. J. Bot.* 73: 384–390.
  - 11) Hata, K. and K. Futai. 1996. Variation in fungal endophyte populations in needles of the genus *Pinus*. *Can. J. Bot.* 74: 103–114.
  - 12) Koga, H., T. Kimigakuhuro, T. Tsukiboshi and T. Uematsu. 1993. Incidence of endophytic fungi in perennial ryegrass in Japan. *Ann. Phytopathol. Soc. Japan* 59: 180–184.
  - 13) McOnie, K. C. 1967. Germination and infection of *Citrus* by ascospores of *Guignardia citricarpa* in relation to control of black spot. *Phytopathol.* 57: 743–746.
  - 14) Nakagiri, A., S. Tokumasu, H. Araki, S. Koreeda and T. Tubaki. 1989. Succession of fungi in decomposing mangrove leaves in Japan. *Proceedings of the 5th International Symposium on Microbial Ecology*, p. 297–301.
  - 15) Okane, I., A. Nakagiri and T. Ito. 1996. *Discostroma tricellulare*, a new endophytic ascomycete with a *Seimatosporium* anamorph isolated from *Rhododendron*. *Can. J. Bot.* 74: 1334–1344.
  - 16) Petrini, O. 1986. Taxonomy of endophytic fungi of aerial plant tissues. In: N. J. Fokkema and J. van den Heuvel (ed) *Microbiology of the Phyllosphere*, p. 175–187. Cambridge University Press.
  - 17) Petrini, O. and M. Dreyfuss. 1981. Endophytische Pilze in epiphytischen Areceae, Bromeliaceae und Orchidaceae. *Sydowia* 34: 135–148.
  - 18) Petrini, L. and O. Petrini. 1985. Xylariaceous fungi as endophytes. *Sydowia* 38: 216–234.
  - 19) Petrini, O., J. Stone and F. E. Carroll. 1982. Endophytic fungi in evergreen shrubs of Western Oregon: a preliminary study. *Can. J. Bot.* 60: 789–796.
  - 20) Rodrigues, K. F. and G. J. Samuels. 1990. Preliminary study of endophytic fungi in a tropical palm. *Mycol. Res.* 94: 827–830.
  - 21) Rodrigues, K. F., A. Leuchtmann and O. Petrini. 1993. Endophytic species of *Xylaria*: cultural and isozymic studies. *Sydowia* 45: 116–138.
  - 22) Tokunaga, Y. and I. Ohira. 1973. Latent infection of anthracnose on *Citrus* in Japan. *Rept. Tottori Mycol. Inst. (Japan)* 10: 693–702.
  - 23) Webber, J. and J. N. Gibbs. 1984. Colonization of elm bark by *Phomopsis oblonga*. *Trans. Br. Mycol. Soc.* 82: 348–352.
  - 24) Weidemann, G. J. and D. M. Boone. 1984. Development of latent infections on cranberry leaves inoculated with *Botryosphaeria vaccinii*. *Phytopathol.* 74: 1041–1043.

## Amplification and Sequencing of Mitochondrial Cytochrome C Oxidase Subunit II Gene for Phylogenetic Analysis of Yeast

Yasuyoshi NAKAGAWA and Kozaburo MIKATA

### Summary

For phylogenetic analysis of yeasts, amplification and sequencing of mitochondrial cytochrome c oxidase subunit II (COX II) gene were performed. PCR primers for the amplification of COX II gene were designed from the conserved regions of the COX II gene sequences. With these primers, the DNA fragments amplified from *Arthroascus javanensis* IFO 1848<sup>T</sup> (<sup>T</sup>= type strain), *Debaryomyces hansenii* var. *hansenii* IFO 0083<sup>T</sup>, *Rhodospiridium toruloides* IFO 0559<sup>T</sup> and *Saccharomyces cerevisiae* IFO 10217<sup>T</sup> had the predicted fragment size. A phylogenetic tree based on the COX II sequences does not conflict with that of small subunit rRNA (18S rRNA). The frequency of base substitutions in COX II gene was higher (at least 3.3 times) than that of the 18S rRNA gene. These results suggest that COX II sequence comparison is a suitable tool for the phylogenetic analysis of closely related yeasts and fungi.

Keywords: Cytochrome c oxidase subunit II, phylogeny, mitochondria, yeast

Phylogenetic analysis of yeasts and fungi has been performed mainly based on small subunit rRNA (18S rRNA) sequencing (1, 2, 13, 17, 20). However, the base differences in 18S rRNA sequence are too small to analyze phylogenetic relationships among closely related yeasts and fungi. The analysis of more rapidly evolving genes is suitable for the phylogenetic analysis of closely related organisms. It is known that mitochondrial DNA evolved faster than genomic DNA (3). In this study, we evaluated the amplification and sequencing of mitochondrial cytochrome c oxidase subunit II gene for phylogenetic analysis of yeasts.

### Materials and Methods

*Strains and cultivation.* The strains examined were *Arthroascus javanensis* IFO 1848<sup>T</sup> (<sup>T</sup>= type strain), *Debaryomyces hansenii* var. *hansenii* IFO 0083<sup>T</sup>, *Rhodospiridium toruloides* IFO 0559<sup>T</sup> and *Saccharomyces cerevisiae* IFO 10217<sup>T</sup>. They were cultivated

at 28°C in medium containing (per liter) 10.0 g of glucose, 5.0 g of peptone (Difco Laboratories, Detroit, USA), 3.0 g of yeast extract (Difco) and 3.0 g of malt extract (Difco) (pH 5.6).

*Preparation of mitochondrial DNA.* We prepared mitochondrial DNA from ascomycetous yeasts in isopycnic cesium chloride gradients, using the fluorescent DNA-binding dye bisbenzimidazole (Sigma, St. Louis, USA) (8, 11).

*Amplification and sequencing of cytochrome c oxidase subunit II gene.* COX II gene was amplified by a PCR (18) using TaKaRa *Taq* (Takara Shuzo, Kyoto, Japan) and primers, CYT2S120 (5'-GAATTACATGATAATATWATGT) and CYT2A620 (5'-GATACTTGATTAAWCKICCAGG). The conditions used for thermal cycling were as follows: denaturation of the target DNA at 94°C for 2 min, followed by 30 cycles consisting of denaturation at 94°C for 1 min, primer annealing at 40°C for 1 min, and primer extension at 72°C for 1 min. After the last cycle, the reaction mixture was kept at 72°C for 5 min, then cooled to 4°C. The 0.5 kb amplified fragment was separated by agarose gel electrophoresis and purified by SpinBind DNA recovery system for agarose gels (FMC BioProducts, Rockland, USA). The methods used for cloning of the purified fragments have been described previously in detail (16). The single-stranded DNA and cloned plasmid material were sequenced by using BcaBEST Dideoxy Sequencing Kit (Takara Shuzo) in combination with [ $\alpha$ -<sup>35</sup>S] dATP. The primers used for sequencing were BcaBEST Primer M13-47 and RV-P (Takara Shuzo).

*Phylogenetic analysis.* COX II sequences of the strains examined and sequences of reference organisms obtained from the literature (5, 6, 9, 14, 15) were aligned with the *Saccharomyces cerevisiae* sequence. The CLUSTAL V software package (10) was used to generate evolutionary distances ( $K_{nuc}$  values (12)) and similarity values and to construct a phylogenetic tree by using the neighbor-joining method (19) and  $K_{nuc}$  values. The topology of the phylogenetic tree was evaluated by the bootstrap resampling method (7) with 1,000 replicates.

*Nucleotide sequence accession numbers.* The sequence data have been deposited with DDBJ and will appear in the DDBJ, EMBL, GSDB and NCBI Nucleotide Sequence Databases with the accession numbers from D55725 to D55728.

## Results and Discussion

PCR primers for amplification of the COX II gene were designed from two conserved regions (position 112 to 133 and position 607 to 629, *Saccharomyces cerevisiae* numbering system) of the COX II gene sequences of three species of ascomycetous yeast, *Hansenula saturnus* (14), *Kluyveromyces lactis* (9) and *Saccharomyces cerevisiae* (4), and three species of filamentous ascomycetes, *Aspergillus nidulans* (6), *Neurospora crassa* (15) and *Podospora anserina* (5). PCR amplification of the COX II gene was attempted for the following strains: *Arthroascus javanensis* IFO 1848<sup>T</sup>, *Debaryomyces hansenii* var. *hansenii* IFO 0083<sup>T</sup>, *Rhodospiridium toruloides* IFO 0559<sup>T</sup> and *Saccharomyces cerevisiae* IFO 10217<sup>T</sup>. The major PCR products had predicted fragment size (c.a. 0.5 kb). The determined sequences of *Arthroascus javanensis* IFO 1848<sup>T</sup>, *Debaryomyces hansenii* var.

*hansenii*, IFO 0083<sup>T</sup>, and *Saccharomyces cerevisiae* IFO 10217<sup>T</sup> were 467 bp long, and that of *Rhodospiridium toruloides* IFO 0559<sup>T</sup> was 473 bp long. The obtained sequence of *Saccharomyces cerevisiae* IFO 10217<sup>T</sup> is 100% identical to the published COX II gene sequence of *Saccharomyces cerevisiae* (4).

The levels of COX II gene sequence similarity among Hemiascomycetes (ascomycetous yeasts) range from 73.1% to 89.0%, and those among Euascomycetes (filamentous ascomycetes) range from 75.9% to 89.2% (Table 1). Against these relatively high similarity values, COX II gene sequence similarity between Hemiascomycetes and Euascomycetes is less than 66.8%, that between Hemiascomycetes and *Rhodospiridium toruloides* (Basidiomycota) is less than 63.2%, and that between Euascomycetes and *Rhodospiridium toruloides* is less than 62.5%.

A phylogenetic tree constructed by the neighbor-joining method and the  $K_{\text{nuc}}$  values

Table 1. Similarity values for sequences of cytochrome c oxidase subunit II gene and 18S rRNA<sup>1)</sup>.

| Organism  | % Similarity |      |      |      |      |      |      |      |      |
|---|--------------|------|------|------|------|------|------|------|------|
|   | 1            | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    |
| 1 <i>Saccharomyces cerevisiae</i>                   |              | 97.0 | -2)  | -    | 95.9 | 89.3 | -    | 88.9 | 86.2 |
| 2 <i>Kluyveromyces lactis</i>                       | 89.0         |      | -    | -    | 95.3 | 89.8 | -    | 89.1 | 85.5 |
| 3 <i>Hansenula saturnus</i>                         | 80.0         | 82.1 |      | -    | -    | -    | -    | -    | -    |
| 4 <i>Arthroascus javanensis</i>                     | 78.9         | 80.4 | 73.1 |      | -    | -    | -    | -    | -    |
| 5 <i>Debaryomyces hansenii</i> var. <i>hansenii</i> | 78.5         | 76.5 | 74.8 | 76.5 |      | 90.1 | -    | 89.8 | 86.6 |
| 6 <i>Podospora anserina</i>                         | 66.8         | 66.6 | 65.5 | 65.7 | 65.1 |      | -    | 97.1 | 85.7 |
| 7 <i>Aspergillus nidulans</i>                       | 64.7         | 64.7 | 60.8 | 66.0 | 64.0 | 78.0 |      | -    | -    |
| 8 <i>Neurospora crassa</i>                          | 64.2         | 65.1 | 64.2 | 62.5 | 64.4 | 89.2 | 75.9 |      | 85.8 |
| 9 <i>Rhodospiridium toruloides</i>                  | 63.2         | 61.6 | 58.8 | 60.6 | 61.4 | 62.5 | 59.7 | 59.5 |      |

1) The similarity values at upper right are for 18S rRNA sequences and those at lower left are for cytochrome c oxidase subunit II gene sequences.

2) -, not determined.

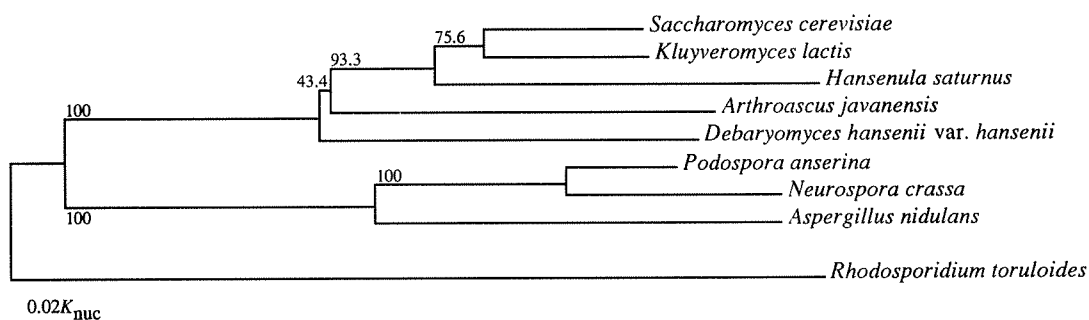


Fig. 1. Phylogenetic tree derived from cytochrome c oxidase subunit II gene sequences. Scale bar = 0.02  $K_{\text{nuc}}$  in nucleotide sequences. The lengths of the vertical lines are not significant. The numbers on the branches refer to the confidence limit (expressed as a percentage) estimated by the bootstrap analysis with 1,000 replicates.



Table 2.  $K_{\text{nuc}}$  values for sequences of cytochrome c oxidase subunit II gene and 18S rRNA<sup>1)</sup>.

| Organism  | $K_{\text{nuc}}$ values |       |                 |       |       |       |       |       |       |
|---|-------------------------|-------|-----------------|-------|-------|-------|-------|-------|-------|
|   | 1                       | 2     | 3               | 4     | 5     | 6     | 7     | 8     | 9     |
| 1 <i>Saccharomyces cerevisiae</i>                   |                         | 0.031 | - <sup>2)</sup> | -     | 0.042 | 0.117 | -     | 0.122 | 0.154 |
| 2 <i>Kluyveromyces lactis</i>                       | 0.119                   |       | -               | -     | 0.049 | 0.111 | -     | 0.118 | 0.162 |
| 3 <i>Hansenula saturnus</i>                         | 0.234                   | 0.205 |                 | -     | -     | -     | -     | -     | -     |
| 4 <i>Arthroascus javanensis</i>                     | 0.249                   | 0.229 | 0.335           |       | -     | -     | -     | -     | -     |
| 5 <i>Debaryomyces hansenii</i> var. <i>hansenii</i> | 0.254                   | 0.282 | 0.308           | 0.282 |       | 0.107 | -     | 0.110 | 0.148 |
| 6 <i>Podospora anserina</i>                         | 0.440                   | 0.444 | 0.463           | 0.461 | 0.471 |       | -     | 0.030 | 0.160 |
| 7 <i>Aspergillus nidulans</i>                       | 0.479                   | 0.479 | 0.556           | 0.454 | 0.491 | 0.260 |       | -     | -     |
| 8 <i>Neurospora crassa</i>                          | 0.486                   | 0.471 | 0.486           | 0.521 | 0.482 | 0.117 | 0.291 |       | 0.159 |
| 9 <i>Rhodospidium toruloides</i>                    | 0.508                   | 0.537 | 0.597           | 0.560 | 0.542 | 0.520 | 0.578 | 0.583 |       |

<sup>1)</sup> The  $K_{\text{nuc}}$  values at upper right are for 18S rRNA sequences and those at lower left are for cytochrome c oxidase subunit II gene sequences.

<sup>2)</sup> -, not determined.

shows that the organisms used in this study divided into three groups: Hemiascomycetes, Euascomycetes and *Rhodospidium toruloides* (Fig. 1). This topology was supported by a confidence value of 100% in bootstrap analysis. The phylogenetic tree based on the COX II gene sequences does not conflict with that of 18S rRNA sequences. (1, 2, 17).

Two deletions, each consisting of three bases were found after positions 193 and 208 in all Hemiascomycetes. These deletions were not found in Euascomycetes and *Rhodospidium toruloides*. These facts suggest that these deletions occurred after Hemiascomycetes branched off from the others.

We compared the  $K_{\text{nuc}}$  values and the similarity values of COX II gene sequences with those of 18S rRNA sequences. As shown in Table 1 and 2, the frequency of base substitutions in the COX II gene was higher than that of 18S rRNA. Among Hemiascomycetes, 18S rRNA sequence similarity and  $K_{\text{nuc}}$  values range from 95.3% to 97.0% and from 0.031 to 0.049, while COX II gene sequence similarity and  $K_{\text{nuc}}$  values range from 73.1% to 89.0% and from 0.119 to 0.335, respectively.  $K_{\text{nuc}}$  values derived from COX II gene are at least 3.3 times higher than those for 18S rRNA. Though more sequence data of COX II gene are needed, these results suggest that COX II sequence comparison may be a suitable tool for phylogenetic analysis of closely related organisms.

We thank Dr. Yuzo Yamada, professor emeritus of Shizuoka University, for reviewing the manuscript and giving us valuable suggestions.

### References

- 1) Berbee, M.L. and J.W. Taylor. 1992. Detecting morphological convergence in true fungi, using 18S rRNA gene sequence data. *Biosystems* 28: 117-125.
- 2) Berbee, M.L. and J.W. Taylor. 1993. Dating the evolutionary radiations of the true fungi. *Can. J. Bot.* 71: 1114-1127.
- 3) Bruns, T.D. and T.M. Szaro. 1992. Rate and mode differences between nuclear and

- mitochondrial small-subunit rRNA genes in mushrooms. *Mol. Biol. Evol.* 9: 836-855
- 4) Coruzzi, G. and A. Tzagoloff. 1979. Assembly of the mitochondrial membrane system. DNA sequence of subunit 2 of yeast cytochrome oxidase. *J. Biol. Chem.* 254: 9324-9330.
  - 5) Cummings, D.J., F. Michel, J.M. Domenico, and K.L. McNally. 1990. Mitochondrial DNA sequence analysis of the cytochrome oxidase subunit II gene from *Podospora anserina*. A group IA intron with a putative alternative splice site. *J. Mol. Biol.* 212: 287-294.
  - 6) Dyson, N.J., T.A. Brown, J.A. Ray, R.B. Waring, C. Scazzocchio, and R.W. Davis. 1989. Processing of mitochondrial RNA in *Aspergillus nidulans*. *J. Mol. Biol.* 208: 587-599.
  - 7) Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783-791.
  - 8) Garber, R.C. and O.C. Yoder. 1983. Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal, and plasmid components. *Anal. Biochem.* 135: 416-422.
  - 9) Hardy, C.M. and G.D. Clark-Walker. 1990. Nucleotide sequence of the cytochrome oxidase subunit 2 and val-tRNA genes and surrounding sequence from *Kluyveromyces lactis* K8 mitochondrial DNA. *Yeast* 6: 403-410.
  - 10) Higgins, D. G., A. J. Bleasby, and R. Fuchs. 1992. CLUSTAL V: improved software for multiple sequence alignment. *Comput. Applic. Biosci.* 8: 189-191.
  - 11) Hudspeth, M.E.S., D.S. Shumard, K.M. Tatti, and L.I. Grossman. 1980. Rapid purification of yeast mitochondrial DNA in high yield. *Biochim. Biophys. Acta* 610: 221-228.
  - 12) Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111-120.
  - 13) Kurtzman, C.P. 1992. rRNA sequence comparisons for assessing phylogenetic relationships among yeasts. *Int. J. Syst. Bacteriol.* 42: 1-6.
  - 14) Lawson, J.E. and D.W. Deters. 1985. Nucleotide sequence of the mitochondrial cytochrome c oxidase subunit II gene in the yeast *Hansenula saturnus*. *Curr. Genet.* 9: 351-360.
  - 15) Macino, G. and G. Morelli. 1983. Cytochrome oxidase subunit 2 gene in *Neurospora crassa* mitochondria. *J. Biol. Chem.* 258: 13230-13235.
  - 16) Nakagawa, Y., T. Sakane, and A. Yokota. 1996. Transfer of "*Pseudomonas riboflavina*" (Foster 1944), a gram-negative, motile rod with long-chain 3-hydroxy fatty acids, to *Devosia riboflavina* gen. nov., sp. nov., nom. rev. *Int. J. Syst. Bacteriol.* 46: 16-22.
  - 17) Nishida, H. and J. Sugiyama. 1994. Archiascomycetes: detection of a major new lineage within the Ascomycota. *Mycoscience* 35: 361-366.
  - 18) Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.
  - 19) Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425.
  - 20) Yamada, Y. and H. Kawasaki. 1989. The molecular phylogeny of the Q9-equipped basidiomycetous yeast genera *Mrakia* Yamada et Komagata and *Cystofilobasidium* Oberwinkler et Bandoni based on the partial sequences of 18S and 26S ribosomal ribonucleic acids. *J. Gen. Appl. Microbiol.* 35: 173-183.

## Conidium Development of An Aero-aquatic Hyphomycete, *Peyronelina glomerulata*

Akira NAKAGIRI and Tadayoshi ITO

### Summary

Conidium morphology and its developmental process in *Peyronelina glomerulata* are clarified by observing natural and cultured materials under a scanning electron microscope. Morphological characteristics of conidia and conidiophores show the adaptation of this fungus in the production and dispersal of conidia in aquatic habitats.

Keywords: aero-aquatic fungus, conidial development, *Peyronelina glomerulata*.

In the course of studies of aquatic fungi in the Bousou Peninsula, Chiba Pref., an aero-aquatic hyphomycete, *Peyronelina glomerulata* Arnoud ex Fisher, Webster & Kane, was found on a submerged decomposing culm of *Cyperus* sp. collected from the margin of a freshwater pond. *Peyronelina* is a monotypic genus and *P. glomerulata* has been reported only from France, U. K. and Canada (1,2,3). The fungus forms crown-shaped conidia comprised of curved arms surrounding a central pile of subglobose cells. Conidia at various stages of development were obtained by incubating the fungus on natural substrates in a moist chamber and by culturing the isolates on agar media. Morphology and the developmental process of the conidium was observed in detail under a scanning electron microscope (SEM).

### Materials and Methods

**Collection.** Decomposing twigs and leaves submerged in water were collected from the Kamega-Jo pond, Misaki-cho, Isumi-gun, Chiba Pref., on 11 Dec. 1995. They were incubated in shallow water in a Petri dish at room temperature (20-25 °C). After several weeks, aquatic and aero-aquatic fungi, such as *Diplocladiella* sp., *Spirosphaera* sp. and *Canderabrum brocciatum* Tubaki, appeared on aerated parts of twigs and culms. After three months of incubation, conidia of *P. glomerulata* were found on the surface of a wet culm of *Cyperus* sp. Continuing incubation by adding water enabled us to observe many

conidia at various developmental stages.

*Isolation.* Single conidia were isolated with a fine needle on Cornmeal agar (CMA, Nissui, Tokyo) plates containing 0.01% of penicillin and streptomycin.

*Observation under SEM.* Small pieces of the natural substrate and agar blocks with conidia were fixed with 1% osmium tetroxide at 4 °C for 12 h. Because the conidia were easily detached from the substrate and floated on the surface of the fixative fluid, they were fixed by putting the materials in a small chamber filled with the vapor of the fixative. The fixed material was dehydrated in ethanol and isoamyl acetate, then critical point dried before coating with platinum. Observation was carried out with a JSM-5400 (JEOL Ltd.) operated at 15 kv.

## Results

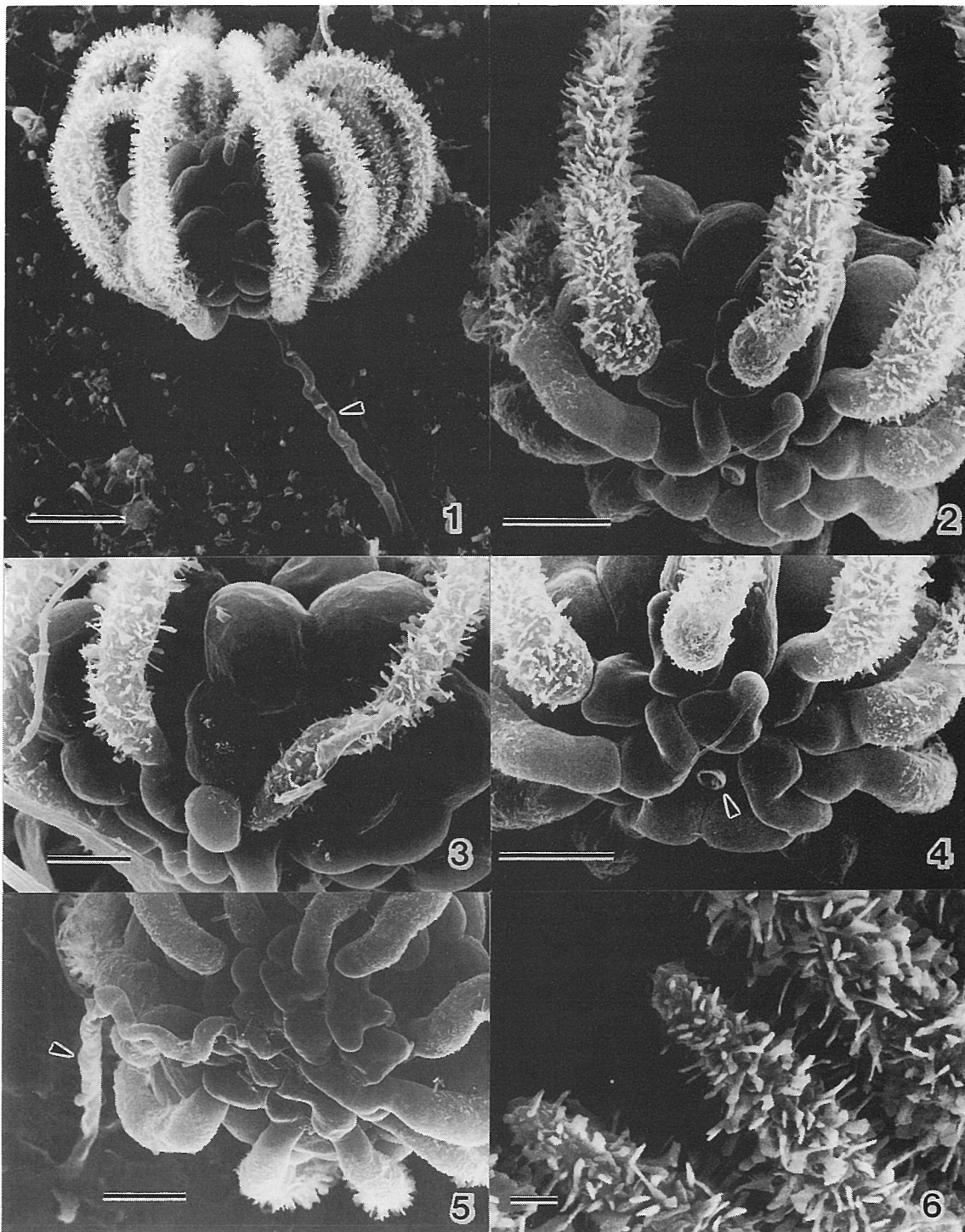
### *Morphology of conidia*

Mature conidia are crown-shaped and composed of central subglobose cells and surrounding arms (Fig. 1). The subglobose cells, 4–7  $\mu\text{m}$  in diam, originate from basal cells of the arms, and 20–30 cells are successively formed by budding (Fig. 3). From 7 to 17 arms arise from the base of the conidium. Each arm is composed of arm cells and a basal cell (Fig. 2). The latter cells are formed by repetitive branching of the primary cell of the conidium attached to the conidiophore (Figs. 4,5). The branched basal cells intricate together and form a disc at the base of conidium (Fig. 5). The arms, 30–60 X 2–3  $\mu\text{m}$ , are attenuate and curved at the apex to gather at the center of the top of the conidium. The arm cells are covered with flat, flake-like spicules, 1–2 X 0.2–0.4  $\mu\text{m}$ , though the basal part of the arm cells is poorly covered (Fig. 4) and the apical part is covered with short spicules (Fig. 6). Conidiophores, 30–45  $\mu\text{m}$  long, arise from a creeping hypha in the substrate. The conidiophore twists or spirals and connects to the center of the basal disc of the conidium (Figs. 1,4,5).

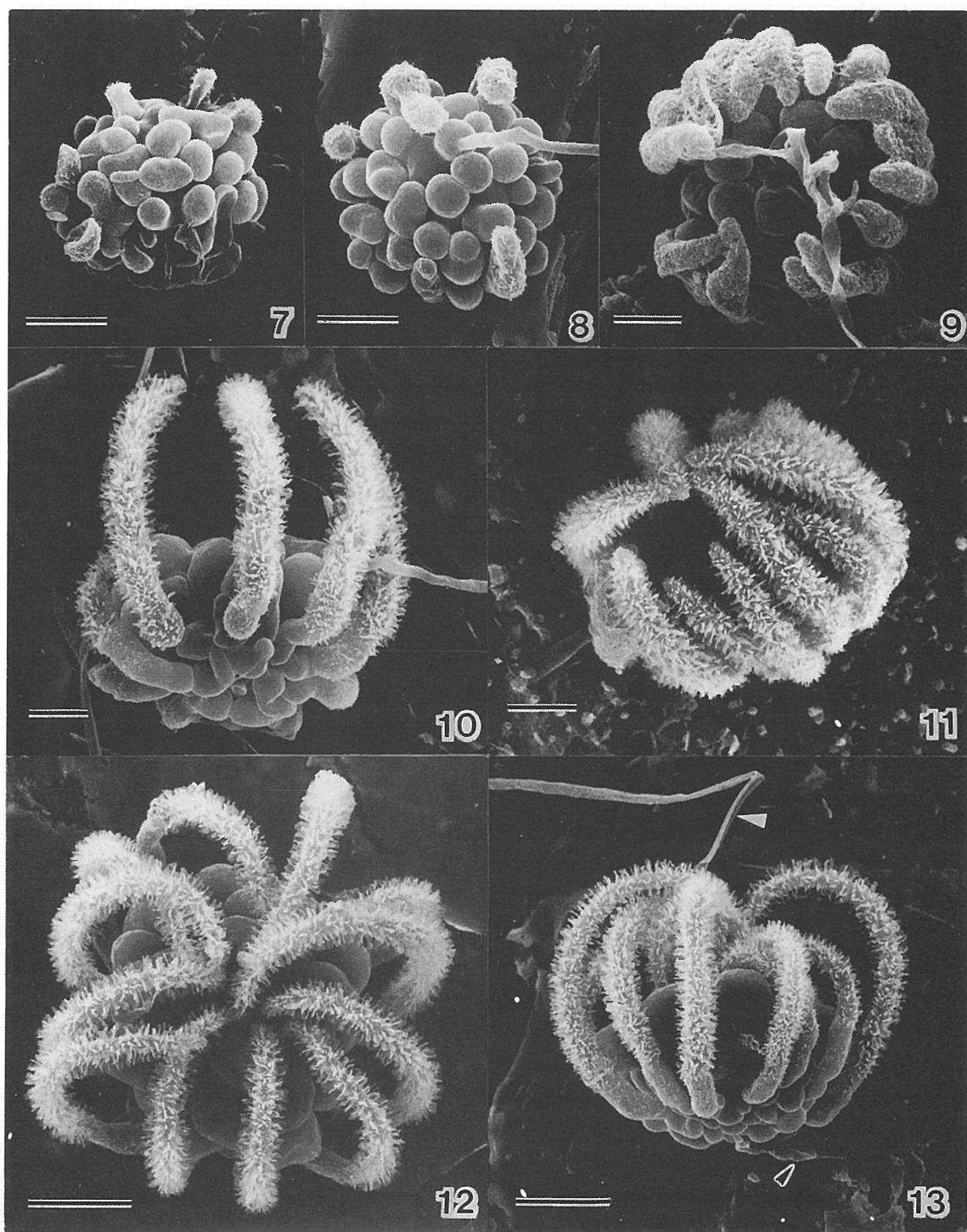
### *Developmental process of conidia*

A conidium in the earliest developmental stage observed on the natural substrate is shown in Fig. 7. The conidium has subglobose cells on a basal disc which is composed of bulbous intricate cells. From the bulbous cells (= the arm basal cells), the subglobose cells emerge by budding, and several arm initials arise from the marginal part of the basal disc. The subglobose cells multiply by budding and the arms grow upward (Fig. 8). Many arms emerge from the basal disc (Fig. 9). At this stage, ornamentation on the arm cell has not yet developed. Then, the arms grow further upward and spicules begin to develop on the surface (Fig. 10). Elongated arms curve at the apices to enclose the pile of subglobose cells (Fig. 11). The tips of the arms gather at the center of the conidium (Fig. 12). The enclosed subglobose cells increased in number to 20–30 cells and in size to 4–7  $\mu\text{m}$  in diam. Finally, the conidium becomes crown-shaped. The arms continue to grow to make a space inside the crown, which serves to entrap air (Fig. 13).

Conidia were observed to germinate from the central subglobose cells, never from the arm cells. Often, a hypha germinates from a subglobose cell, and elongates to more than

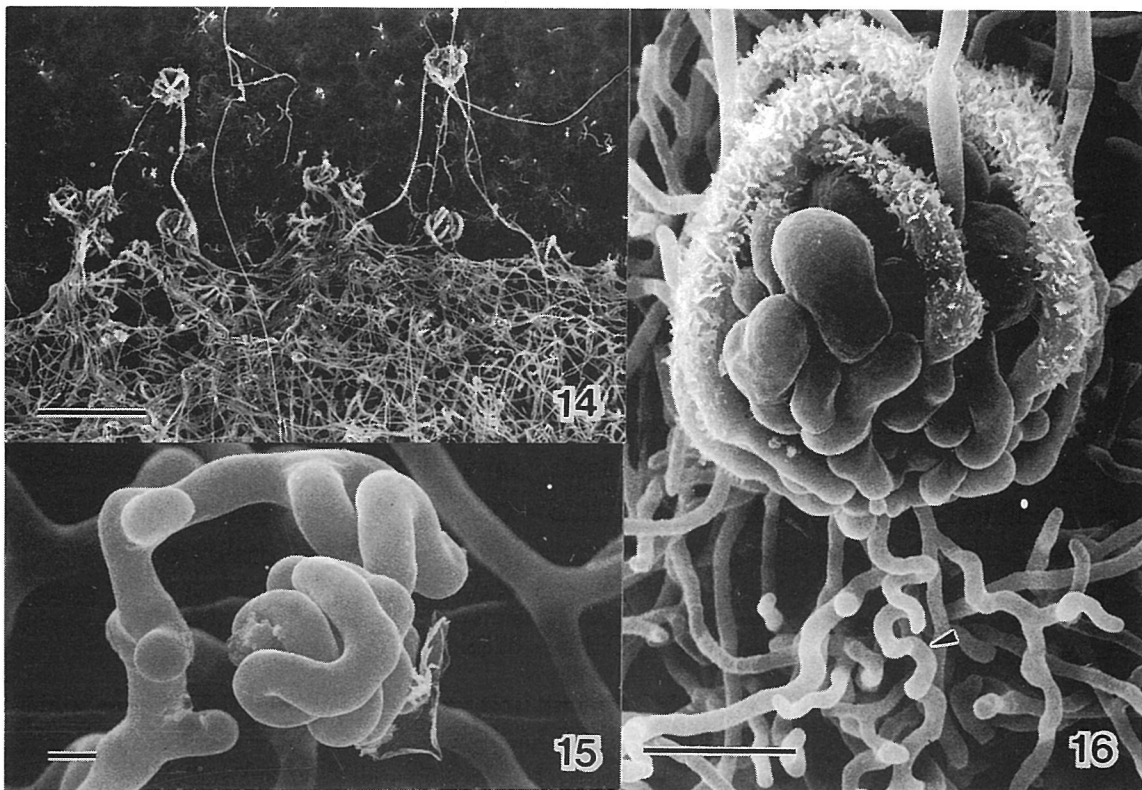


Figs. 1-6. *Peyronelina glomerulata*. 1. A crown-shaped conidium produced on a conidiophore (arrow). 2. Arms arising from arm basal cells. 3. Subglobose cells inside the conidium multiplied by budding. 4 & 5. A disc-shaped base of conidium, composed of intricately branching arm basal cells (arrow in Fig. 4, a detachment scar of conidiophore; arrow in Fig. 5, a twisting conidiophore attaching to the base of conidium). 6. Apices of arms covered with flake-like spicules. (Bars: 1 = 10  $\mu\text{m}$ ; 2-5 = 5  $\mu\text{m}$ ; 6 = 1  $\mu\text{m}$ )



Figs. 7-13. Conidium development of *Peyronelina glomerulata*. 7-13. The developmental process is explained in the text (black arrow in Fig. 13, a conidiophore; white arrow in Fig. 13, hypha germinated from the subglobose cell of conidium). (Bars: 7-11=5  $\mu$ m; 12,13=10  $\mu$ m)





Figs. 14-17. *Peyronelina glomerulata* (IFO 32867) cultured on CMA plate. **14.** Colony edge forming conidia on the surface of the medium. **15.** An intricate hyphal ball formed on the surface of mycelium. **16.** Conidium produced in culture (arrow: twisting conidiophore). (Bars: 14 = 100  $\mu$ m; 15 = 1  $\mu$ m; 16 = 10  $\mu$ m)

100  $\mu$ m and becomes erect (Fig. 13).

The single-conidium isolates IFO 32867 (AN-1505), AN-1506 and AN-1507 readily produced conidia on CMA plates (Fig. 14). In culture, an intricate hyphal ball (Fig. 15), which may be the initial structure of a conidium, was observed on the surface of mycelium. Conidia formed on the agar medium were similar to those on the natural substrate, but often the arms failed to enclose completely the central subglobose cells (Fig. 16). Conidiophores are twisted (Fig. 16), as seen in the specimen on the natural substrate.

### Discussion

This is the first report describing the developmental process of the peculiarly shaped conidium of *P. glomerulata* and the first published report of this species from Japan, though two strains were previously deposited in Japan Collection of Microorganisms (JCM) as JCM 9266 and 9267 by Dr. Y. Tsurumi. The two strains were isolated from dead leaves submerged in ponds (pers. commun. from Dr. Tsurumi). *Peyronelina glomerulata* was originally isolated by Arnaud (1) from the surface of perithecia of *Lasiosphaeria* sp. on vegetative debris in France and was not suggested to be a member of aero-aquatic fungi. However, Fisher et al. (2) found this fungus from submerged plant

materials (wood blocks of Scots pine, *Pinus sylvestris*; pinnules of bracken, *Pteridium aquilinum*; leaf petiole of an unidentified tree) collected in U.K. and redescribed the species with Latin diagnosis to make the taxon name validly published. They considered it to be an aero-aquatic fungus. Our study supports their view, because this fungus produced conidia on the aerated parts of a dead culm of *Cyperus* sp. submerged in a shallow water, and the conidia float on the surface of water by entrapping an air bubble inside the arms of the conidium. Morphological characteristics of the fungus observed in this study mostly accord with those described by Fisher et al. (2). However, while they showed that branches (= arms) were straight under very moist conditions, our material did not show this property. They also mentioned that this fungus failed to sporulate on several agar media including corn meal agar, and had to be colonized on bracken leaf in aerated water and incubated on moist filter paper for sporulation. In contrast, our isolates readily produced conidia on the surface of CMA plates. In spite of these differences, the overall similarity in morphology and size of conidia warrants our identification.

*Peyronelina glomerulata* is an aero-aquatic fungus adapting well to aquatic habitats by forming a floatable propagule entrapping an air bubble inside its arms (3). The spiculate ornamentation on the arms was observed to be hydrophobic, as suggested by Fisher et al. (2), and may work effectively for keeping an air bubble inside the arms and floating on the surface of water. We observed a twisting conidiophore elongated up to 45  $\mu\text{m}$  in accord with the conidium development. The flexible conidiophore may serve to keep the developing conidium on the surface of water if water level fluctuates, for example, due to rainfall. A single germinating hypha erecting into the air from a subglobose cell was often observed when the conidia were kept in a moist chamber. This phenomenon was also observed by Dr. Tsurumi on his materials (pers. commun. from Dr. Tsurumi). It is not clear whether this is just the germination of a conidium under moist conditions or the erect hypha works for dispersal or entrapment of a liberated conidium in the aquatic habitat.

We thank Dr. Y. Tsurumi (Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd.) for valuable information about his isolates. This study was supported by the grant from the Chiba Historical Materials Research Foundation to A. Nakagiri.

#### References

- 1) Arnaud, G. 1952. Mycologie concrete. Genera. Bulletin trimestriel de la Societe Mycologique de France 68: 181-223.
- 2) Fisher, P.J., J. Webster and D.F. Kane. 1976. *Peyronelina glomerulata* from submerged substrata in Britain. Trans. Br. Mycol. Soc. 67: 351-354.
- 3) Michaelides, J. and B. Kendrick. 1982. The bubble-trap propagules of *Beverwykella*, *Helicoon* and other aero-aquatic fungi. Mycotaxon 14: 247-260.



## Descriptive Catalogue of IFO Fungus Collection XV.

In the routine identification work on fungi isolated in Japan, and in checks of the list of the fungal taxa preserved in the IFO culture collection for published records of their occurrence in Japan, many taxa have been found to be either new to Japan or obscurely or insufficiently described. In some cases, the first record of a fungus in Japan gives only the name of its taxon, without an adequate description of the species concerned. The object of this series is to provide descriptions of the fungi preserved or newly deposited in the IFO fungus collection and/or in the IFO herbarium and to contribute to our knowledge of the fungal flora of Japan.

The authors of the descriptions of these fungal taxa are shown in parentheses.

- 100. *Chaetomium cupreum* Ames** (Figs. 1-3) Sordariales  
Mycologia 41: 642 (1949); von Arx et al., Nova Hedwigia, Beih. 84: 22 (1986); Ootani,  
Micol. Flora of Japan Vol. 3, No. 3, p. 37 (1995), Yokendo LTD, Tokyo.  
Syn: *Chaetomium rufum* Ramakrishnan, Proc. Ind. Acad. Sci., B, 38: 118 (1953).  
*Chaetomium trilaterale* Chivers var. *cupreum* (Ames) J.C. Cooke, Mycologia 65:  
1218 (1973).

Colonies on potato carrot agar with a daily growth rate of 5.6-5.8 mm at 24°C, creeping on agar surface, pale pink, velvety to floccose, thin at the margin with a sector; reverse uncolored to pale pink. On oatmeal agar with a daily growth rate of 5.4-6 mm, yellowish red, floccose with a sector; reverse purplish red. On potato sucrose agar with a daily growth rate of 6-6.2 mm, purplish red, floccose; reverse purplish red. On malt extract agar with a daily growth rate of 5.9-6.1 mm, brownish red at the central area, pale brownish red at the margin, floccose to velvety at the margin; reverse white to pale gray. Aerial hyphae white. Ascomata borne on the agar surface, at first globose, becoming ovoid, ostiolate, 100-120  $\mu\text{m}$ , with a brown wall of angular, mostly 5-9  $\mu\text{m}$  cells. Ascomatal hairs arcuate, incurved, apically 1 to 3 coiled, roughened, bright red or orange-red in reflected light, 4-5  $\mu\text{m}$  wide near the base, tapering to the apex, septate. Lateral hairs slender, straight or slightly arcuate, sometimes loosely 1 to 3 coiled at the apex, minutely roughened, septate, 3-3.5  $\mu\text{m}$  wide at the base. Asci clavate, evanescent, 8-spored, 32-36  $\times$  13-15  $\mu\text{m}$ . Ascospores reniform or lunate, hyaline and dextrinoid when young, later becoming pale brown to pale yellowish brown, 10-13  $\times$  5-6  $\mu\text{m}$ , with a single apical germ pore.

At 37 °C, growth is moderate, with immature ascomata.

Hab.: manure soil, Iriomote Is., Taketomi-cho, Yaeyama-gun, Okinawa Pref, Japan, 24

Oct. 1995 (IFO 32696=T. Ito H6E-98-4, IFO H-12200).

This species is confirmed by von Arx et al. (1986) and Ootani (1995) as a distinct species. It differs from *C. trilaterale* Chivers by the red coloured ascomatal hairs, reniform and uniporate ascospores.

The fungus often isolated from soil, dung, vegetable rubbish and wilted pineapple plants in Canada, France, India, Japan, Madagascar, Panama, Spain and other countries.

(T. Ito and A. Nakagiri)

**101. *Engyodontium album*** (Limber) de Hoog (Figs. 4-6) Hyphomycetes  
Persoonia 10: 33 (1978); de Hoog, Persoonia 12: 135 (1984).

Syn: *Tritirachium album* Limber, Mycologia 32: 27 (1940).

*Beauveria alba* (Limber) Saccas, Rev. Mycol. 13: 64 (1948).

*Tritirachium fungicola* Shvartsman et al., Flora sporoyykh rastenii  
Kazakhstana VIII-I: Moniliales, Alma Ata. (1973).

*Sporotrichum gorlenkoanum* Kuritzina & Sizova, Mikol. Fitopatol. 1:  
342 (1967).

Colonies on potato carrot agar with a daily growth rate of 3.3-3.6 mm at 24°C, consisting of a basal felt of vegetative mycelium and bearing abundant conidia, white, floccose, thin at the margin; reverse uncolored. On oatmeal agar with a daily growth rate of 3.1-3.3 mm, white, floccose; reverse uncolored. On potato sucrose agar with a daily growth rate of 3.6-3.9 mm, white, floccose, rising on the central area; reverse uncolored to pale yellow. On malt extract agar with a daily growth rate of 3.3-3.6 mm, white, rising on the central area, velvety at the margin; reverse uncolored to pale yellow. Mycelium matted or creeping on agar surface, hyaline, smooth-walled, 2-2.5  $\mu$ m wide. Conidiophores hyaline, thin- and smooth-walled, branching strictly verticillate, 2-2.5  $\mu$ m wide. Conidiogenous cells arising singly or in whorls from the conidiophores, straight, tapering towards the tip, polyblastic, forming conidia on elongated rhachides, up to 50  $\mu$ m long, 1.5-2  $\mu$ m wide at the base. Conidia ellipsoid when young, later becoming to obovoid, with acuminate base, hyaline, smooth-walled, one-celled. 2-2.5  $\times$  1.5-2  $\mu$ m.

At 37°C, growth is nil.

Hab.: roots of *Avicennia marina* Vierh., Shiira river, Iriomote Is., Taketomi-cho, Yaeyama-gun, Okinawa Pref, Japan, 24 Oct. 1995 (IFO 32828=T. Ito H7-22-4, IFO H-12215).

The species was originally described by Limber (1940) as *Tritirachium album* and it was transferred to the genus *Beauveria* Vuill. (*B. alba*) by Saccas (1948). de Hoog (1978) established the new genus *Engyodontium* de Hoog and separated it from *Beauveria* by its strictly verticillate branch and denticles structures.

The fungus is isolated from human skin, fresco, air, *Pisum sativum* L. and human brain abscess in New Zealand, Netherlands, Rumania, Japan and Israel.

(T. Ito and A. Nakagiri)

**102. *Chaetomella raphigera* Swift**

(Figs. 7–14) Coelomycetes

Mycologia 22: 165 (1930)

Syn: *Chaetomella terricola* Rama Rao, Mycopath. Mycol. appl. 19: 255 (1963)*Volutellospora raphigera* (Swift) Thirum. & Mathur in Mathur & Thirum., Sydowia 18: 38 (1964).*Volutellospora cinnamomea* Thirum. & Mathur in Mathur & Thirum., Sydowia 18: 39 (1964)*Volutellospora terricola* (Rama Rao) Mathur & Thirum., Sydowia 18: 39 (1964)*Chaetomella cinnamomea* (Thirum. & Mathur) Petrak, Sydowia 18: 378 (1964)*Chaetomella terricola* Rama Rao var. *mysorensis* Nag Raj & Govindu, Sydowia 23: 112 (1969)

Colonies on five-times-diluted V-8 Juice agar (1/5V-8A) white without aerial hyphae, extending up to 49–50 mm in diam at 25 C in 7 days, forming conidiomata on the surface of the medium. On potato sucrose agar, colony reverse red-brown, forming abundant conidiomata. Hyphae hyaline. Conidiomata subglobose to ovoid, fan- or shell-shaped when young, 127–212  $\mu\text{m}$  long, 66–97  $\mu\text{m}$  wide, 104–140  $\mu\text{m}$  high, dark brown to black, with 12–28 setae over the surface. Setae filiform, inflated or hooked at the apex, 1–4-septate, 42–80  $\mu\text{m}$  long, 3–4  $\mu\text{m}$  wide at the base, 5–7  $\mu\text{m}$  wide at the apex, brown at the base, pale brown at the apex. Conidiophores arising from the bottom of the conidioma, filiform, 1.5–2  $\mu\text{m}$  in diam, hyaline, asymmetrically branching to form phialides. Conidiogenous cells phialidic, filiform, attenuate, hyaline, (5–)50–90  $\times$  1–1.5  $\mu\text{m}$ . Conidia fusiform to elliptical, one-celled, hyaline, 5–12(–14)  $\times$  1.5–3  $\mu\text{m}$  ( $\bar{x}$  = 7.0  $\times$  2.3  $\mu\text{m}$ ), with or without mucilaginous material deposited at both ends. At maturation, a conidial mass is pushed out from the top of conidioma. Conidia are also produced in a naked conidioma which is composed of conidiophores and marginal setae. The naked conidiomata resembling those of *Volutella* species are often formed in culture.

Hab.: on submerged fallen green leaf of *Bruguiera gymnorrhiza* Lamk., Ouke pond, Minami-Daito Island, Okinawa Pref., Japan, 26 Sep. 1996 (IFO 32878 = AN-1512, IFO H-12212; AN-1513).

From Japan, this fungus has been isolated by Matsushima (1975) from soils in Hachijo Is. and Iriomote Is. Worldwide, it has been reported from tropical and subtropical regions (Sutton & Sarbhay, 1976). However, the literature contains contradictory descriptions of the conidia. Conidia having no appendages were originally described in *Chaetomella raphigera* by Swift (1930), and later by Sutton & Sarbhay (1976) and Sutton (1980). The same conidia were described in *C. terricola* by Rama Rao (1963) and *Volutellospora cinnamomea* by Matsushima (1971). However, conidia equipped with mucilage material at both ends were illustrated in *Volutellospora cinnamomea* by Mathu & Thirumalachar (1964) and *C. terricola* var. *mysorensis* by Nag Raj & Govindu (1969). All the above species were treated as synonymous by Sutton & Sarbhay (1976) and Sutton (1980). This contradiction may indicate confusion in the species taxonomy. However, our specimen

shows that some conidia, especially smaller ones, are equipped with mucilaginous material at both ends, while others, mostly larger ones, are not. This observation may support Sutton & Sarbhay's treatment, but further critical studies on the specimens of each synonymous species are necessary to clarify this problem.

(A. Nakagiri and T. Ito)

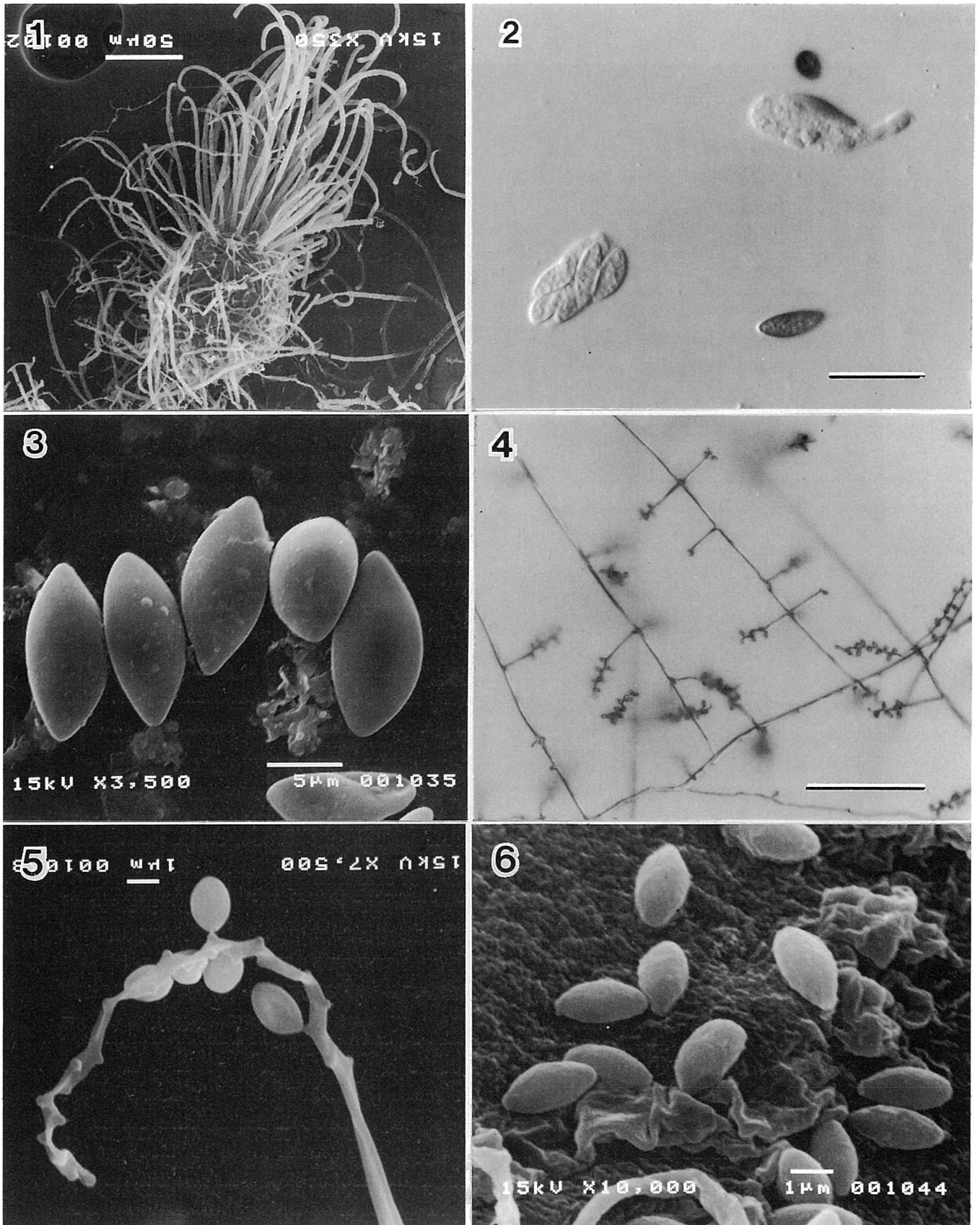
**103. *Pleurothecium recurvatum*** (Morgan) von Hohnel. (Fig. 15-19) Hyphomycetes Ber. Deutsch. Bot. Ges. 37: 154 (1919); Goos, Mycologia 61: 1048 (1969)

Colonies on corn meal agar white to pale brown, extending slowly, hyphae septate, hyaline to pale brown, 2-3  $\mu\text{m}$  in diam. Best sporulation occurred on corn meal agar. Conidiophores arising from the mycelium, singly or in clusters, septate, dark, narrowed and paler at the apex, sometimes branching, frequently swollen and rhizoidal at the base, 252-440  $\mu\text{m}$  ( $\bar{x}$ =290  $\mu\text{m}$ ) in length and 4-8  $\mu\text{m}$  in diam. Conidia arising as blown-out portions of the conidiophore wall from successively produced growing points, the conidiogenous cell elongating with production of successive conidia and recurving to produce a "helicoid cyme". Conidia borne upon blunt teeth, and collecting in moist heads, hyaline, 3-septate, naviculate to subcylindrical, the apical cell rounded, and the basal cell truncate, 15-27  $\times$  5-9  $\mu\text{m}$  ( $\bar{x}$ =22  $\times$  7  $\mu\text{m}$ ).

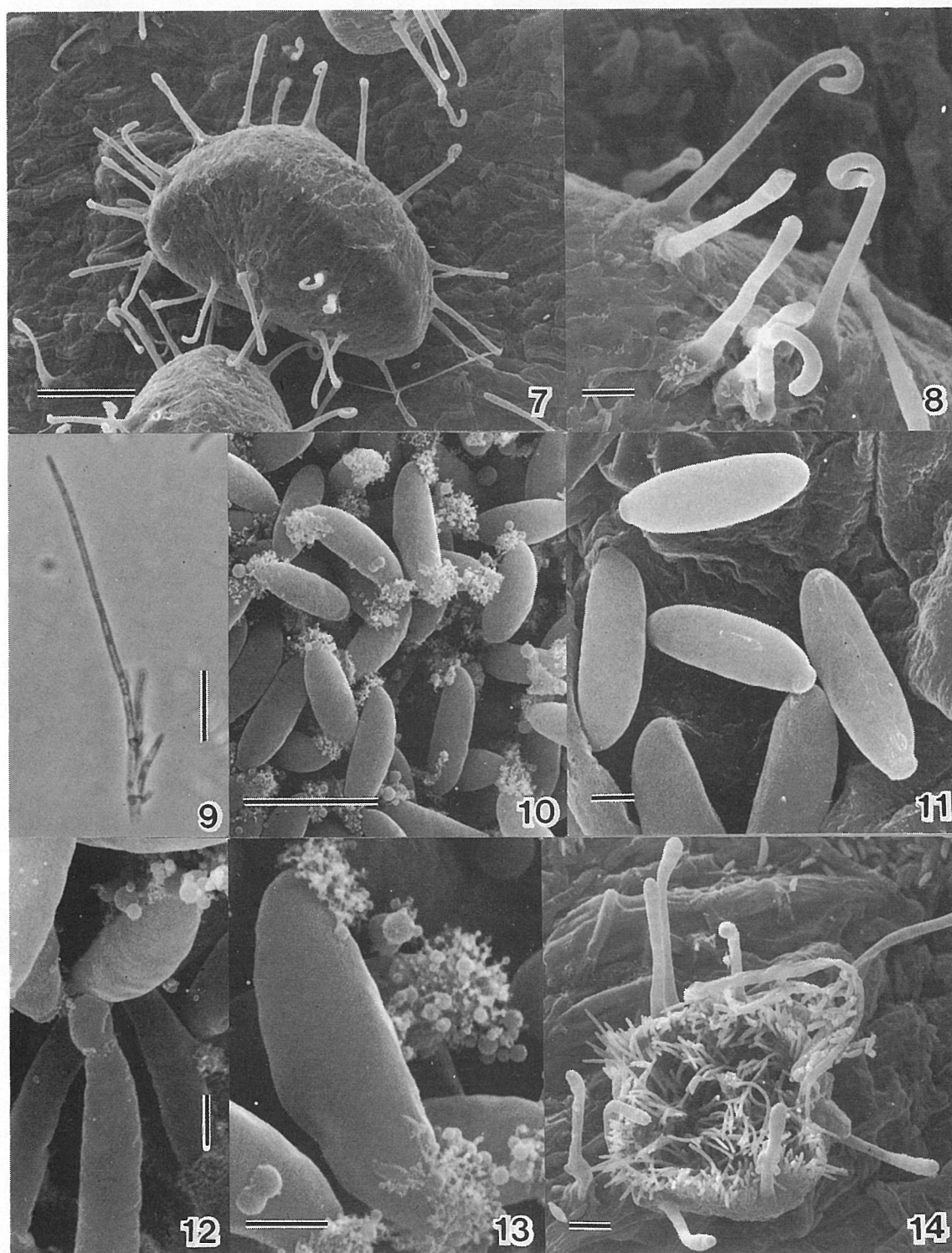
Hab.: on rotten wood, Saijyo-cyo, Hiba-gun, Hiroshima pref., 26 Sept. 1996 (IFO 32879 = I. Okane IOC 1236)

This fungus was isolated from chips of rotten wood. During a culturing period on the substrata, which were incubated in a moist chamber, this fungus grew vigorously on a cup fungus, *Scutellinia* sp. which had occurred on the substrata. The term "helicoid cyme" for the conidiogenous apparatus of this fungus was used by Morgan (1895) and is consistent with the definition given by Lawrence (1951), "Taxonomy of vascular plants".

(I. Okane, A. Nakagiri and T. Ito)



Figs. 1-3. *Chaetomium cupreum* (IFO 32696). 1. Ascoma. 2. Immature asci. 3. Ascospores. 4-6. *Engyodontium album* (IFO 32828). 4. Conidiophores with conidia. 5. Sympodial conidiogenous cell with conidia. 6. Conidia. (Bars: 1 & 4 = 50  $\mu$ m; 2 = 20  $\mu$ m; 3 = 5  $\mu$ m; 5 & 6 = 1  $\mu$ m).



Figs. 7-14. *Chaetomella raphigera* (IFO 32878). 7. Conidioma with setae formed on 1/5V-8A. 8. Setae. 9. Conidiophore with phialides. 10. & 13. Conidia with mucilaginous materials at both ends. 11. Conidia without the mucilaginous material. 12. Phialide forming a conidium. 14. Naked conidioma exposing phialides and surrounded by setae. (Bars: 7 = 50  $\mu\text{m}$ ; 8, 9, 14 = 10  $\mu\text{m}$ ; 10 = 5  $\mu\text{m}$ ; 11, 12, 13 = 1  $\mu\text{m}$ )



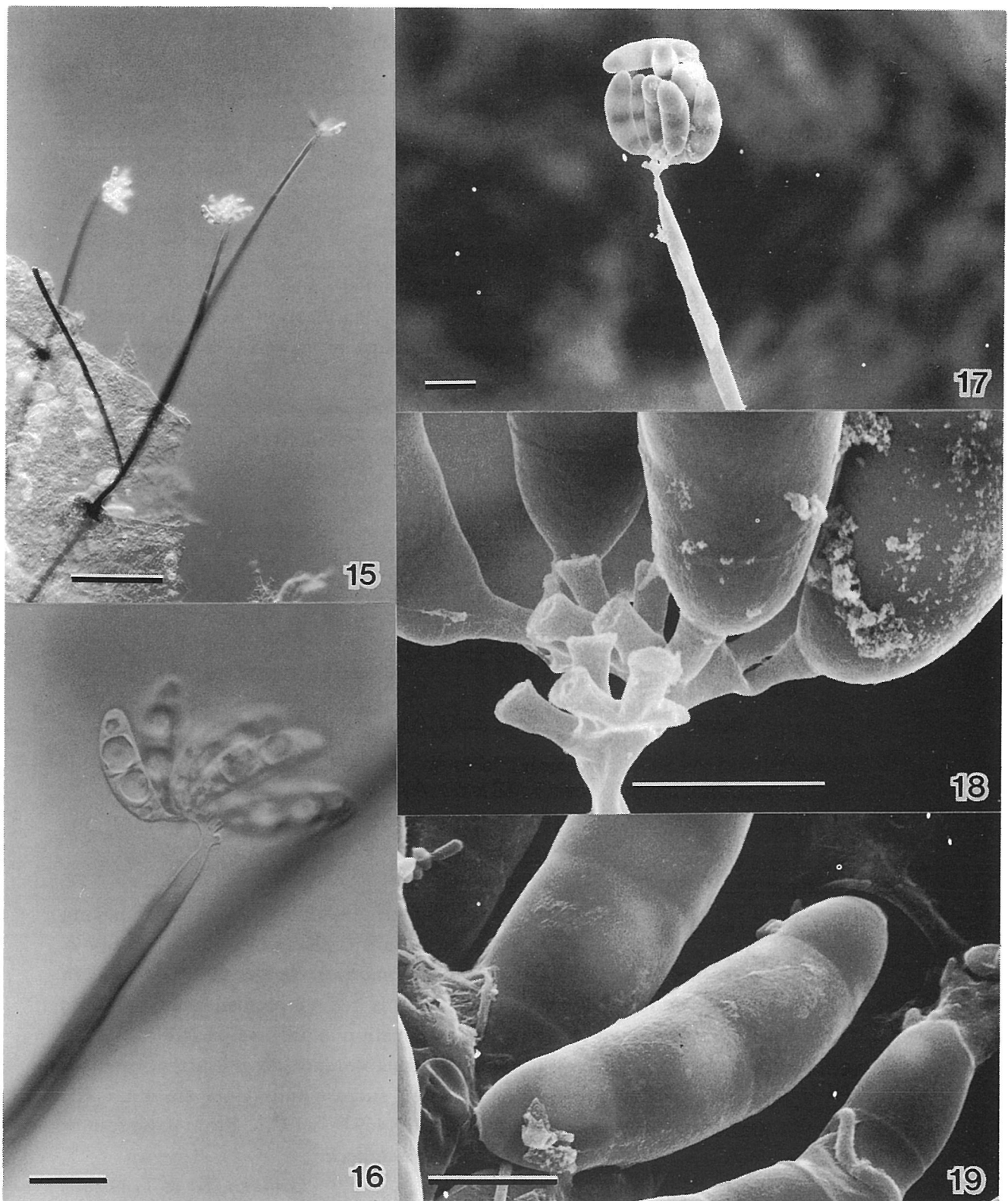


Fig. 15-19. *Pleurothecium recurvatum* (IFO 32879). 15. Erect conidiophore forming conidia. 16. Conidia formed upon blunt teeth. 17. Conidia collecting in moist head. 18. Conidiogenous cells. 19. Conidia. (Bars: 15=100  $\mu$ m, 16 and 17=10  $\mu$ m, 18 and 19=5  $\mu$ m)

## Descriptive Catalogue of IFO Actinomycetes Collection III

The purpose of this catalogue is to describe the taxonomic properties of strains which have been reidentified as different species in routine identification work on the IFO actinomycetes collection. The authors of the descriptions are shown in parentheses.

7. *Streptomyces baldaccii* (Farina & Rocci) Witt & Stackebrandt  
Witt, D. and E. Stackebrandt, Int. J. Syst. Bacteriol., 41: 456 (1991)

### IFO 3491

Strain IFO 3491 was deposited under the name of "*Streptomyces* sp.," having originally been designated as *Streptomyces rubrireticuli* Waksman & Henrici. The phenotypic characters of this strain closely resemble those of *Streptomyces baldaccii* IFO 14693<sup>T</sup> (T=type strain) as follows: aerial mass color is white, pink to red, substrate mycelium color is red to dark red, melanin formation is positive on International *Streptomyces* Project (ISP) medium 6 but negative on ISP medium 7. Glucose and inositol are utilized for growth, but arabinose, fructose, mannitol, raffinose, rhamnose, sucrose and xylose are not. The level of DNA relatedness between this strain and *Streptomyces baldaccii* IFO 14693<sup>T</sup> is 88%. From these results, IFO 3491 is identified as a strain of *Streptomyces baldaccii*.

(Hatano, K. & T. Nishii)

### IFO 13862

Strain IFO 13862 was deposited under the name of "*Streptomyces* sp.," having originally been designated as *Streptomyces fervens* subsp. *phenomyceticus* Hamada & Umezawa. However, this strain closely resembles *Streptomyces baldaccii* IFO 14693<sup>T</sup> in phenotypic characters, as follows: aerial mass color is red to reddish gray, substrate mycelium color is reddish brown to red, melanin formation is positive on ISP medium 6 and weakly positive on ISP medium 7. Glucose, fructose and inositol are utilized for growth, but arabinose, mannitol, raffinose, rhamnose, sucrose and xylose are not. The level of DNA relatedness between this strain and IFO 14693<sup>T</sup> is 79%. From these results, IFO 13862 is identified as a strain of *Streptomyces baldaccii*.

(Hatano, K. & T. Nishii)

8. *Streptomyces ehimensis* (Shibata et al.) Witt & Stackebrandt  
Witt, D. and E. Stackebrandt, Int. J. Syst. Bacteriol., 41: 456 (1991)



**IFO 3417**

This strain was deposited under the name of "*Streptomyces* sp.," having originally been designated as *Streptomyces rubrireticuli* Waksman & Henrici. In phenotypic characters, however, it closely resembles those of *Streptomyces ehimensis* IFO 12858<sup>T</sup>, as follows: aerial mass color is white, red and/or gray, substrate mycelium color is yellowish brown to brown, melanin formation is positive on ISP media 6 and 7. Glucose, fructose and inositol are utilized for growth, but arabinose, mannitol, raffinose, rhamnose, sucrose and xylose are not. The level of DNA relatedness between this strain and IFO 12858<sup>T</sup> is 88%. From these results, IFO 3417 is identified as a strain of *Streptomyces ehimensis*.

(Hatano, K. and T. Nishii)

**IFO 13802**

This strain was deposited under the name of "*Streptomyces* sp.," having originally been designated as *Streptoverticillium rimofaciens* Niida. The phenotypic characters of this strain, however, closely resemble those of *Streptomyces ehimensis* IFO 12858<sup>T</sup>, as follows: the aerial mass color is yellow, gray and/or red, substrate mycelium color is brown to dark brown, melanin formation is positive on ISP medium 6 but negative on ISP medium 7. Glucose, fructose, inositol and mannitol are utilized for growth, but arabinose, raffinose, rhamnose, sucrose and xylose are not. The level of DNA relatedness between IFO 13802 and IFO 12858<sup>T</sup> is 92%. From these findings, IFO 13802 is identified as a strain of *Streptomyces ehimensis*.

(Hatano, K. and T. Nishii)

9. *Streptomyces hachijoensis* (Hosoya et al.) Witt & Stackebrandt  
Witt, D. and E. Stackebrandt, Int. J. Syst. Bacteriol., 41: 456 (1991)

**IFO 13808**

Strain IFO 13808 was deposited under the name of "*Streptomyces* sp.," having originally been designated as *Streptoverticillium taitoensis* Oh-iwa et al. However, its phenotypic characters resemble those of *Streptomyces hachijoensis* IFO 12782<sup>T</sup>, except that it bears aerial mycelia sparsely. The phenotypic characters of IFO 13808 are as follows: aerial mass color is white to yellow, substrate mycelium color is yellowish brown to brown, melanin formation is negative on ISP media 1, 6 and 7. Glucose and inositol are utilized for growth, but arabinose, fructose, mannitol, raffinose, rhamnose sucrose and xylose are not. The DNA relatedness to *Streptomyces hachijoensis* IFO 12782<sup>T</sup> is 85%. Thus, IFO 13808 is identified as a strain of *Streptomyces hachijoensis*. This identification is supported by the report of Labeda (Int. J. Syst. Bacteriol., 46: 699 (1996)).

(Hatano, K. and T. Nishii)

10. *Streptomyces cochleatus* Nakagaito et al.

Nakagaito, Y., A. Yokota and T. Hasegawa, J. Gen. Appl. Microbiol., 38: 105 (1992)

Having reexamined utilization of carbon sources and nitrate reduction of *Stre-*

Table 1. Utilization of carbon sources in *Streptomyces cochleatus* IFO 14767 and IFO 14768<sup>T</sup>.

|                   | IFO 14767 |     | IFO 14768 <sup>T</sup> |     |
|-------------------|-----------|-----|------------------------|-----|
|                   | A*        | B** | A*                     | B** |
| L-Arabinose       | +         | +   | +                      | +   |
| D-Fructose        | —         | +   | —                      | —   |
| i-Inositol        | —         | —   | —                      | —   |
| D-Mannitol        | —         | +   | —                      | —   |
| Raffinose         | +         | —   | —                      | —   |
| L-Rhamnose        | —         | —   | —                      | —   |
| Sucrose           | —         | —   | —                      | —   |
| D-Xylose          | +         | —   | —                      | —   |
| D-Glucose         | +         | +   | +                      | +   |
| Nitrate reduction | —         | +   | —                      | +   |

A\* : Nakagaito, Y., et al., J. Gen. Appl. Microbiol., 38: 105 (1992)

B\*\* : This study

*ptomyces cochleatus* IFO 14767 and IFO 14768<sup>T</sup>, we correct some of the reported physiological properties of these strains as shown in Table 1.

Levels of DNA relatedness between IFO 14767 and IFO 14768<sup>T</sup>, and between IFO 14767 and *Streptomyces paracochleatus* IFO 14769<sup>T</sup> are 11–22% and 2–13%, respectively. These results indicate that strain IFO 14767 belongs to neither *Streptomyces cohleatus* nor *Streptomyces paracochleatus*. Strain IFO 14767 is not available for distribution because its taxonomic status is ambiguous.

(Hatano, K., T. Nishii and T. Tamura)

#### 11. *Streptomyces paracochleatus* Nakagaito et al.

Nakagaito, Y., A. Yokota and T. Hasegawa, J. Gen. Appl. Microbiol., 38: 105 (1992)

Nakagaito et al. reported that *Streptomyces paracochleatus* IFO 14769<sup>T</sup> utilized glucose and fructose for growth, but not arabinose, inositol, mannitol, raffinose, rhamnose, sucrose and xylose. However, we correct this report as follows: *Streptomyces paracochleatus* IFO 14769<sup>T</sup> utilizes arabinose, glucose and xylose for growth, but not fructose, inositol, mannitol, raffinose, rhamnose and sucrose.

(Hatano, K., T. Nishii and T. Tamura)

CATALOGUE OF NEWLY ACCEPTED STRAINS  
FEBRUARY - NOVEMBER 1996

The cultures involved in the following catalogue can be distributed under the  
same condition as strains listed IFO LIST OF CULTURES 10th Edition

| IFO             | Name   | Temp | Med |
|-----------------|--|------|-----|
| (T=Type strain) |  |      |     |
| 10853           | T <i>Candida akabanensis</i><br>JCM 9115 -- T. Nakase, NK-4, frass of a vine.  | 28   | 108 |
| 10854           | T <i>Candida fukuyamanensis</i><br>JCM 9396 -- T. Nakase -- T. Hatano, KO-15, water of pond Ao.  | 25   | 108 |
| 10855           | T <i>Candida pseudoglaebosa</i><br>JCM 2168 -- CBS 6715 -- T. Furukawa, soil.  | 25   | 108 |
| 10856           | T <i>Candida sojae</i><br>JCM 1644 -- AJ 4787 -- T. Nakase, extraction process of water-soluble<br>substance of defatted soybean flakes. | 25   | 108 |
| 10857           | <i>Arxula adenivorans</i><br>CBS 7370 -- J.P. van der Walt, soil.  | 24   | 112 |
| 10858           | T <i>Arxula adenivorans</i><br>CBS 8244 -- W.J. Middelhoven, soil.   | 24   | 112 |
| 10859           | <i>Arxula terrestris</i><br>CBS 7376 -- J.P. van der Walt, soil.   | 24   | 112 |
| 10860           | T <i>Metschnikowia agaves</i><br>CBS 7744 -- M.-A. Lachance, blue agave.   | 24   | 112 |
| 10861           | <i>Metschnikowia agaves</i><br>CBS 7745 -- M.-A. Lachance, blue agave.   | 24   | 112 |
| 10862           | <i>Saccharomyces castellii</i><br>CBS 2913 -- J.L. Etchells, fermenting cucumber.  | 24   | 112 |
| 10863           | <i>Saccharomyces castellii</i><br>CBS 4310 -- A. Capriotti, soil.  | 24   | 112 |
| 10864           | <i>Saccharomyces castellii</i><br>CBS 3006 -- J.L. Etchells, fermenting cucumber.  | 24   | 112 |

|       |   |  |    |     |
|-------|---|--|----|-----|
| 10865 |   | <i>Saccharomyces castellii</i>   | 24 | 112 |
|       |   | CBS 3007 -- J.L. Etchells, Fermenting cucumber.                                    |    |     |
| 10866 |   | <i>Saccharomyces sp.</i>   | 24 | 112 |
|       |   | CBS 6463 -- VKM Y-1659, fermenting grapes.   |    |     |
| 10867 |   | <i>Saccharomyces sp.</i>   | 24 | 112 |
|       |   | CBS 6904 -- J.P. van der Walt, soil.   |    |     |
| 10868 |   | <i>Saccharomyces castellii</i>   | 24 | 112 |
|       |   | CBS 7188 -- W.J. Middelhoven, ensiled maize.                                       |    |     |
| 10869 |   | <i>Saccharomyces servazzii</i>   | 24 | 112 |
|       |   | CBS 6865 -- V.K. Hazu.   |    |     |
| 10870 |   | <i>Saccharomyces servazzii</i>   | 24 | 112 |
|       |   | CBS 7721 -- R. Kappe, faeces.  |    |     |
| 10871 | T | <i>Candida boidinii</i>  | 25 | 108 |
|       |   | JCM 9604 -- NRRL Y-2332 -- C. Ramírez, tanning fluid.                              |    |     |
| 10872 | T | <i>Candida stellimalicola</i>  | 25 | 108 |
|       |   | JCM 3546 -- M. Suzuki, T-53 -- W. Daengsubha, star apple.                          |    |     |
| 10873 | T | <i>Stephanoascus farinosus</i>   | 17 | 108 |
|       |   | CBS 140.71 -- W. Gams, carpophore Hirneola auricula-judae.                         |    |     |
| 10874 |   | <i>Stephanoascus farinosus</i>   | 17 | 108 |
|       |   | CBS 563.74 -- W. Gams, carpophore Hirneola auricula-judae.                         |    |     |
| 10875 | T | <i>Pseudozyma flocculosa</i>   | 24 | 108 |
|       |   | CBS 167.88 -- L.A. Shaw, LAS-012, Erysiphe polygoni on leaf of Trifolium pratense. |    |     |
| 10877 | T | <i>Pseudozyma rugulosa</i>   | 24 | 108 |
|       |   | CBS 170.88 -- L.A. Shaw, LAS-016, moldyleaves of Zea mays.                         |    |     |
| 10879 | T | <i>Stephanoascus smithiae</i>  | 24 | 108 |
|       |   | CBS 7522.1 -- IGC 4646, soil.  |    |     |
| 10880 |   | <i>Stephanoascus smithiae</i>  | 24 | 108 |
|       |   | CBS 7522.2 -- IGC 4647, soil.  |    |     |
| 10881 |   | <i>Filobasidium elegans</i>  | 24 | 108 |
|       |   | R.J. Bandoni, 75-8197-A2 MS, inflorescens scape of Yucca brevifolia.               |    |     |
| 10882 |   | <i>Filobasidium elegans</i>  | 24 | 108 |
|       |   | R.J. Bandoni, 75-8197-A4 MS, inflorescens scape of Yucca brevifolia.               |    |     |
| 10883 |   | <i>Filobasidium floriforme</i>   | 17 | 108 |
|       |   | R.J. Bandoni, 75-8702-1 MS, weathered scape of Yucca cf. rupicola.                 |    |     |
| 10884 |   | <i>Filobasidium floriforme</i>   | 17 | 108 |

|       |   |    |     |
|-------|---|----|-----|
|       | R.J. Bandoni, 75-8702-2 MS, weathered scape of <i>Yucca</i> cf. <i>rupicola</i> .                           |    |     |
| 10885 | <i>Filobasidium floriforme</i>  | 17 | 108 |
|       | R.J. Bandoni, 75-8702-1A, weathered scape of <i>Yucca</i> cf. <i>rupicola</i> .                             |    |     |
| 10886 | <i>Filobasidium floriforme</i>  | 17 | 108 |
|       | R.J. Bandoni, 75-8702-1B, weathered scape of <i>Yucca</i> cf. <i>rupicola</i> .                             |    |     |
| 10887 | <i>Filobasidium globisporum</i>   | 24 | 108 |
|       | R.J. Bandoni, 75-8197-B3 MS, weathered leaves of <i>Yucca breuifolia</i> .                                  |    |     |
| 10888 | <i>Filobasidium globisporum</i>   | 24 | 108 |
|       | R.J. Bandoni, 75-8197-B4 MS, weathered leaves of <i>Yucca breuifolia</i> .                                  |    |     |
| 10889 | <i>Pichia membranaefaciens</i>  | 24 | 108 |
|       | HUT 7295 -- K. Kodama, Y 124.   |    |     |
| 10890 | <i>Pichia membranaefaciens</i>  | 24 | 108 |
|       | HUT 7297 -- K. Kodama, Y 233.   |    |     |
| 10891 | <i>Pichia membranaefaciens</i>  | 24 | 108 |
|       | HUT 7303 -- K. Kodama, Y 460.   |    |     |
| 10892 | <i>Pichia membranaefaciens</i>  | 24 | 108 |
|       | HUT 7304 -- K. Kodama, Y 311.   |    |     |
| 10893 | <i>Pichia membranaefaciens</i>  | 24 | 108 |
|       | HUT 7302 -- K. Kodama, Y 463.   |    |     |
| 13183 | <i>Streptomyces aureofaciens</i>  | 28 | 228 |
|       | IFO (K. Nakazawa) -- Lederle Labs, UV-8.  |    |     |
| 15911 | <i>Methylobacterium extorquens</i>  | 30 | 330 |
|       | IFO (T. Sakane) -- Welding Res. Inst., Osaka Univ. (Y. Kikuchi; SUS-E; corrosion of stainless steel welds). |    |     |
| 15912 | <i>Methylobacterium fujisawaense</i>  | 30 | 330 |
|       | IFO (T. Sakane) -- Welding Res. Inst., Osaka Univ. (Y. Kikuchi; Cu-F; corrosion of copper pipe).            |    |     |
| 15913 | <i>Staphylococcus</i> sp.   | 30 | 203 |
|       | IFO (T. Sakane) -- Welding Res. Inst., Osaka Univ. (Y. Kikuchi; Cu-A; corrosion of copper pipe).            |    |     |
| 15914 | <i>Sphingomonas</i> sp.   | 30 | 203 |
|       | IFO (T. Sakane) -- Welding Res. Inst., Osaka Univ. (Y. Kikuchi; SUS-A; corrosion of stainless steel welds). |    |     |
| 15915 | <i>Sphingomonas</i> sp.   | 30 | 203 |
|       | IFO (T. Sakane) -- Welding Res. Inst., Osaka Univ. (Y. Kikuchi; Cu-B; subterranean water).                  |    |     |

|       |   |   |    |     |
|-------|---|---|----|-----|
| 15916 |   | <i>Sphingomonas sp.</i>   | 30 | 203 |
|       |   | IFO (T. Sakane) -- Welding Res. Inst., Osaka Univ. (Y. Kikuchi; Cu-C; subterranean water).      |    |     |
| 15917 |   | <i>Sphingomonas sp.</i>   | 30 | 203 |
|       |   | IFO (T. Sakane) -- Welding Res. Inst., Osaka Univ. (Y. Kikuchi; Cu-D; subterranean water).      |    |     |
| 15918 | T | <i>Actinomadura nitritigenes</i>  | 28 | 266 |
|       |   | DSM 44137 -- A. Lipski, stamm L46, experimental biofilters.                                     |    |     |
| 15919 |   | <i>Saccharomonospora viridis</i>  | 37 | 266 |
|       |   | DSM 43671 -- N. Agre, VKM A-802, soil.  |    |     |
| 15920 | T | <i>Streptomyces fervens</i> subsp. <i>melrosporus</i>   | 28 | 279 |
|       |   | DSM 40905 -- T.G. Pridham -- NRRL 3117 -- UC 2459, soil.  |    |     |
| 15921 | T | <i>Nocardia transvalensis</i>   | 28 | 227 |
|       |   | DSM 43405 -- R.E. Gordon -- IMRU 3426 -- NCTC 2392 -- A. Pijper & B. Pullinger, mycetoma pedis. |    |     |
| 15926 | T | <i>Streptomyces cinnamoneus</i> subsp. <i>sparsus</i>   | 28 | 227 |
|       |   | ATCC 25185 -- M.J. Thirumalachar.   |    |     |
| 15927 |   | <i>Streptomyces cinnamoneus</i> subsp. <i>albosporus</i>  | 28 | 227 |
|       |   | ATCC 25186 -- M.J. Thirumalachar, soil.   |    |     |
| 15928 | T | <i>Streptomyces cinnamoneus</i> subsp. <i>lanosus</i>   | 28 | 227 |
|       |   | ATCC 25187 -- M.J. Thirumalachar, soil.   |    |     |
| 15929 |   | <i>Streptomyces olivoreticuli</i> subsp. <i>cellulophilus</i>                                   | 28 | 227 |
|       |   | ATCC 21632.   |    |     |
| 15930 |   | <i>Vibrio alginolyticus</i>   | 30 | 325 |
|       |   | IFO (A. Nakagiri; Lb-3; decomposing thallus of <i>Cystoceira prolifera</i> ).                   |    |     |
| 15933 | T | <i>Thermomonospora curvata</i>  | 50 | 304 |
|       |   | JCM 3096 -- KCC A-0096 -- A. Henssen, B9.   |    |     |
| 15936 | T | <i>Cytophaga fermentans</i>   | 30 | 331 |
|       |   | IAM 14302 -- NCIMB 2218 -- ATCC 19072 -- H. Veldkamp, marine mud.                               |    |     |
| 15938 | T | <i>Cytophaga latercula</i>  | 25 | 333 |
|       |   | IAM 14305 -- ATCC 23177 -- R.A. Lewin, SIO-1, seawater aquarium outflow.                        |    |     |
| 15940 | T | <i>Persicobacter diffluens</i>  | 25 | 333 |
|       |   | IAM 14117 -- NCIMB 1402 -- R.A. Lewin -- R. Freitas, B1, black sandy mud.                       |    |     |
| 15941 | T | <i>Flammeovirga aprica</i>  | 25 | 333 |
|       |   | IAM 14298 -- ATCC 23126 -- R.A. Lewin, JL-4, rocky sand.  |    |     |

- |       |   |   |    |     |
|-------|---|---|----|-----|
| 15942 | T | <i>Flavobacterium psychrophilum</i>   | 17 | 332 |
|       |   | IAM 14308 -- NCIMB 1947 -- G.L. Bullock-- E.J. Ordal, 3068, coho salmon.                      |    |     |
| 15943 | T | <i>Flavobacterium columnare</i>   | 20 | 334 |
|       |   | IAM 14301 -- NCIMB 2248 -- E.J. Ordal, I-S-2c1, diseased salmonid fish.                       |    |     |
| 15944 | T | <i>Flavobacterium saccharophilum</i>  | 25 | 802 |
|       |   | IAM 14309 -- NCIMB 2072 -- M.O. Moss, 024, silt water interface, River Wey.                   |    |     |
| 15945 | T | <i>Flavobacterium pectinovorum</i>  | 25 | 802 |
|       |   | IAM 14307 -- NCIMB 9059 -- B.C.J.G. Knight -- M.J. Dorey, 81, soil.                           |    |     |
| 15946 | T | <i>Flexibacter maritimus</i>  | 30 | 333 |
|       |   | IAM 14317 -- NCIMB 2154 -- H. Wakabayashi, R-2, diseased red sea bream.                       |    |     |
| 15947 | T | <i>Flexibacter ovolyticus</i>   | 15 | 333 |
|       |   | IAM 14318 -- NCIMB 13127 -- G.H. Hansen, EKD 002, adherent epiflora of Atlantic halibut eggs. |    |     |
| 15948 | T | <i>Marinilabilia salmonicolor</i>   | 30 | 331 |
|       |   | IAM 14310 -- NCIMB 2216 -- ATCC 19041 -- H. Veldkamp -- J. Lascelles, marine mud.             |    |     |
| 15951 |   | <i>Kocuria varians</i>  | 30 | 203 |
|       |   | CCM 2132 -- K. Komagata, 3-1, oil brine.  |    |     |
| 15952 |   | <i>Kocuria varians</i>  | 30 | 203 |
|       |   | CCM 2133 -- K. Komagata, 6-1, oil brine.  |    |     |
| 15953 |   | <i>Kocuria varians</i>  | 30 | 203 |
|       |   | CCM 2189 -- OUT 8092.   |    |     |
| 15954 |   | <i>Kocuria varians</i>  | 30 | 203 |
|       |   | CCM 2430 -- NCTC 7565 -- T. Gibson, G40, milk.  |    |     |
| 15955 |   | <i>Kocuria varians</i>  | 30 | 203 |
|       |   | CCM 2431 -- NCTC 7566 -- T. Gibson, G92, milk.  |    |     |
| 15956 | T | <i>Streptomyces althioticus</i>   | 28 | 231 |
|       |   | ATCC 19724 -- E.B. Shirling -- H. Yamaguchi.  |    |     |
| 15957 | T | <i>Paenibacillus amylolyticus</i>   | 37 | 802 |
|       |   | Res. Lab., Higeta Shoyu Co., Ltd. (O. Shida; HSCC 434) -- NRRL NRS-290.                       |    |     |
| 15958 | T | <i>Paenibacillus chibaensis</i>   | 37 | 802 |
|       |   | Res. Lab., Higeta Shoyu Co., Ltd. (O. Shida; HSCC 442) -- NRRL B-142 -- FDA, PCI221.          |    |     |
| 15959 | T | <i>Paenibacillus illinoisensis</i>  | 37 | 802 |
|       |   | Res. Lab., Higeta Shoyu Co., Ltd. (O. Shida; HSCC 309) -- NRRL NRS-1356, soil.                |    |     |

|       |   |   |    |     |
|-------|---|---|----|-----|
| 15960 | T | <i>Actinomadura glomerata</i><br>JCM 9376.  | 28 | 304 |
| 15961 | T | <i>Actinomadura longicatena</i><br>JCM 9377.  | 28 | 304 |
| 15962 | T | <i>Actinoplanes minutisporangius</i><br>JCM 9458 -- ATCC 49415 -- M.P. Lechevalier, IMRU LL-A-60 --<br>J.S. Ruan, A-60, soil.                               | 28 | 266 |
| 15963 | T | <i>Micromonospora chersina</i><br>JCM 9459 -- ATCC 53710 -- Bristol-MyersSquibb Res. Inst. M956-1, soil.  | 28 | 227 |
| 15964 | T | <i>Pilimelia terevasa</i><br>JCM 3091 -- KCC A-0091 -- W.D. Kane, soil.   | 28 | 227 |
| 15968 | T | <i>Chitinophaga pinensis</i><br>DSMZ 2588 -- UQM 2034 -- V. Sangkhobol & V.B.D. Skerman, pine litter.   | 22 | 272 |
| 15969 |   | <i>Chitinophaga pinensis</i><br>DSMZ 2589 -- UQM 2036 -- V. Sangkhobol & V.B.D. Skerman, fresh water.   | 22 | 272 |
| 15970 | T | <i>Flexibacter aurantiacus</i><br>DSMZ 6792 -- H. Reichenbach, strain Fx a2 -- ATCC 23107 -- R.A. Lewin,<br>DWO -- M. Dworkin, garden soil.                 | 30 | 272 |
| 15971 |   | <i>Persicobacter diffluens</i><br>NCIMB 1430 -- R.A. Lewin, LIM-1, beach mud.   | 25 | 335 |
| 15972 |   | <i>Persicobacter diffluens</i><br>NCIMB 1465 -- R.A. Lewin, NN-3 -- E. Kwei & J. Teya, coarse<br>greenish-brown sand.                                       | 25 | 335 |
| 15973 |   | <i>Flexibacter aggregans</i><br>NCIMB 1389 -- R.A. Lewin, QQ-1, brown sand.   | 25 | 335 |
| 15974 |   | <i>Flexibacter aggregans</i><br>NCIMB 1391 -- R.A. Lewin, QQ-11, brown sand.  | 25 | 335 |
| 15975 |   | <i>Flexibacter aggregans</i><br>NCIMB 1432 -- R.A. Lewin, Q-3 -- Oceanographic Inst. in Split,<br>Yugoslavia, fine light brown sand.                        | 25 | 335 |
| 15976 | T | <i>Flexibacter aggregans</i><br>NCIMB 1443 -- R.A. Lewin, NN-13 -- E. Kwei & J. Teya, green-brown sand.   | 25 | 335 |
| 15977 | T | <i>Flexibacter aggregans</i> subsp. <i>catalaticus</i><br>NCIMB 1418 -- R.A. Lewin, HI-3 -- J. Quast & R. Haight, under<br>rozen sand, upper littoral zone. | 25 | 335 |
| 15978 | T | <i>Flexibacter aurantiacus</i> subsp. <i>copepodarum</i>  | 25 | 335 |



|       |   |   |    |     |
|-------|---|---|----|-----|
|       |   | NCIMB 1394 -- R.A. Lewin, COP -- B.T. Lang, offshore copepod.   |    |     |
| 15979 |   | <i>Flexibacter tractuosus</i>   | 25 | 335 |
|       |   | NCIMB 1393 -- R.A. Lewin, T13, brown mud.   |    |     |
| 15980 |   | <i>Flexibacter tractuosus</i>   | 25 | 335 |
|       |   | NCIMB 1416 -- R.A. Lewin, HI15 -- J. Quast & R. Haight, underneath frozen sand in upper littoral zone.                      |    |     |
| 15981 |   | <i>Flexibacter tractuosus</i>   | 25 | 335 |
|       |   | NCIMB 1429 -- R.A. Lewin, JK11 -- W. Stephenson, brown sand.  |    |     |
| 15982 | T | <i>Microscilla arenaria</i>   | 25 | 335 |
|       |   | NCIMB 1413 -- R.A. Lewin, HJ1, brown sand.  |    |     |
| 15983 | T | <i>Microscilla sericea</i>  | 25 | 335 |
|       |   | NCIMB 1403 -- R.A. Lewin, SI07, marine aquarium outflow.  |    |     |
| 15985 |   | <i>Cytophaga lytica</i>   | 25 | 336 |
|       |   | DSM 2039 -- H. Reichenbach, strain Cy l2, sandy mud.  |    |     |
| 15986 |   | <i>Cytophaga lytica</i>   | 25 | 336 |
|       |   | DSM 2040 -- H. Reichenbach, strain Cy l1 -- ATCC 23169 -- R.A. Lewin, WFB-21, seawater aquarium outflow.                    |    |     |
| 15987 | T | <i>Flexithrix dorotheae</i>   | 30 | 336 |
|       |   | DSMZ 6795 -- H. Reichenbach, strain Ft d1 -- ATCC 23163 -- R.A. Lewin, QQ3, marine silt.                                    |    |     |
| 15988 | T | <i>Flexibacter litoralis</i>  | 25 | 336 |
|       |   | DSM 6794 -- H. Reichenbach, strain Fx l1 -- ATCC 23117 -- R.A. Lewin, SIO-4, seawater aquarium.                             |    |     |
| 15989 | T | <i>Flexibacter tractuosus</i>   | 25 | 336 |
|       |   | DSM 4126 -- NCMB 1408 -- R.A. Lewin, strain H-43, sand.   |    |     |
| 15990 |   | <i>Flexibacter aggregans</i>  | 25 | 335 |
|       |   | NCIMB 1464 -- R.A. Lewin, JL 13 -- R.W.Crigg, coarse white sand, upper littoral zone.                                       |    |     |
| 15991 |   | <i>Flexibacter maritimus</i>  | 20 | 333 |
|       |   | NCIMB 2158 -- J.A. Buswell, Dover sole with black spot necrosis.  |    |     |
| 15992 |   | <i>Flexibacter ovolyticus</i>   | 15 | 333 |
|       |   | NCIMB 13128 -- G.H. Hansen, EKC001, adherent epiflora of halibut eggs ( <i>Hippoglossus hippoglossus</i> ).                 |    |     |
| 15993 |   | <i>Flexibacter ovolyticus</i>   | 15 | 333 |
|       |   | NCIMB 13129 -- G.H. Hansen, VKB004, water in egg incubator containing Atlantic halibut ( <i>Hippoglossus hippoglossus</i> ) |    |     |

|       |   |  |    |     |
|-------|---|--|----|-----|
| 15994 | T | <i>Microscilla furvescens</i>  | 25 | 335 |
|       |   | NCIMB 1419 -- R.A. Lewin, TV2 -- M. Wilcox, brown sand.                    |    |     |
| 15995 |   | <i>Microscilla sericea</i>   | 25 | 335 |
|       |   | NCIMB 1392 -- R.A. Lewin, SIO-9, seawater aquarium outflow.                |    |     |
| 16000 | T | <i>Caulobacter subvibrioides</i>   | 30 | 338 |
|       |   | ATCC 15264 -- J. Stove, CB 81, pond water.                                 |    |     |
| 16002 | T | <i>Pseudonocardia spinosa</i>  | 28 | 266 |
|       |   | JCM 3136 -- KCC A-0136 -- A. Henssen, MB SH-1, soil.                       |    |     |
| 16006 |   | <i>Cytophaga hutchinsonii</i>  | 25 | 276 |
|       |   | NCIMB 10782 -- N. Palleroni.   |    |     |
| 16007 |   | <i>Sporocytophaga myxococcoides</i>  | 25 | 276 |
|       |   | NCIMB 8639 -- H. Bortels.  |    |     |
| 16008 |   | <i>Sporocytophaga myxococcoides</i>  | 25 | 276 |
|       |   | NCIMB 10507 -- L.B. Perry -- NCTC -- P.H.H. Gray -- H.B. Hutchinson, soil. |    |     |
| 16009 | T | <i>Cathayosporangium alboflavum</i>  | 28 | 266 |
|       |   | ATCC 51497 -- H. Runmao, SIIA 945112, soil.                                |    |     |
| 16010 |   | <i>Planotetraspora sp.</i>   | 28 | 266 |
|       |   | ATCC 51498 -- H. Runmao, SIIA 942023, soil.                                |    |     |
| 16014 | T | <i>Bergeyella zoohelcum</i>  | 30 | 342 |
|       |   | IAM 14550 -- NCTC 11660 -- CDC, D658, sputum.                              |    |     |
| 16015 |   | <i>Flexibacter maritimus</i>   | 25 | 340 |
|       |   | IAM 14118 -- NCIMB 2153 -- H. Wakabayashi, B-2, Black sea breem.           |    |     |
| 16016 | T | <i>Weeksella virosa</i>  | 30 | 203 |
|       |   | IAM 14551 -- NCTC 11634 -- CDC, 9751, urine.                               |    |     |
| 16020 |   | <i>Cytophaga lytica</i>  | 30 | 335 |
|       |   | ATCC 23157 -- R.A. Lewin, ENS, beach silt.                                 |    |     |
| 16021 |   | <i>Cytophaga lytica</i>  | 30 | 335 |
|       |   | ATCC 23174 -- R.A. Lewin, B-9, seawater.                                   |    |     |
| 16022 |   | <i>Cytophaga lytica</i>  | 30 | 335 |
|       |   | ATCC 23176 -- R.A. Lewin, BON, seawater aquarium outflow.                  |    |     |
| 16024 | T | <i>Flexibacter aurantiacus</i> subsp. <i>excathedrus</i>                   | 30 | 277 |
|       |   | ATCC 23086 -- R.A. Lewin, CR-134, cathederal pool.                         |    |     |
| 16025 |   | <i>Flexibacter flexilis</i>  | 30 | 277 |
|       |   | ATCC 23080 -- R.A. Lewin, CR-81, rivulet.                                  |    |     |
| 16026 |   | <i>Flexibacter flexilis</i>  | 30 | 341 |

|       |   |   |    |     |
|-------|---|---|----|-----|
|       |   | ATCC 23089 -- R.A. Lewin, A-52 -- Soriano.  |    |     |
| 16027 |   | <i>Flexibacter flexilis</i>   | 30 | 341 |
|       |   | ATCC 23096 -- R.A. Lewin, WAR-5, hot spring.  |    |     |
| 16028 | T | <i>Flexibacter flexilis</i> subsp. <i>pelliculosus</i>  | 30 | 277 |
|       |   | ATCC 23098 -- R.A. Lewin, FLE -- J. Holt, water.  |    |     |
| 16030 |   | <i>Flexibacter roseolus</i>   | 30 | 341 |
|       |   | ATCC 23087 -- R.A. Lewin, CR-141, hot spring.   |    |     |
| 16031 | T | <i>Flexibacter roseolus</i>   | 30 | 341 |
|       |   | ATCC 23088 -- R.A. Lewin, CR-155, hot spring.   |    |     |
| 16033 |   | <i>Flexibacter sancti</i>   | 30 | 277 |
|       |   | ATCC 23090 -- R.A. Lewin, BA-23 -- A. Cataldi.  |    |     |
| 16034 |   | <i>Flexibacter sancti</i>   | 30 | 277 |
|       |   | ATCC 23097 -- R.A. Lewin, MIC -- J. Holt, soil from feed lot.                                     |    |     |
| 16035 |   | <i>Flexibacter tractuosus</i>   | 30 | 335 |
|       |   | ATCC 23151 -- R.A. Lewin, EG-13, brown fine mud.  |    |     |
| 16036 |   | <i>Flexibacter tractuosus</i>   | 30 | 335 |
|       |   | ATCC 23152 -- R.A. Lewin, EE-13, red-brown mud.   |    |     |
| 16037 |   | <i>Flexibacter tractuosus</i>   | 30 | 335 |
|       |   | ATCC 23145 -- R.A. Lewin, GH-1, brown, silty sand.  |    |     |
| 16038 |   | <i>Flexibacter tractuosus</i>   | 30 | 335 |
|       |   | ATCC 23191 -- R.A. Lewin, GH-2, brown, silty sand.  |    |     |
| 16041 | T | <i>Flexibacter japonensis</i>   | 30 | 203 |
|       |   | JCM 9735 -- Fujisawa Pharm. Co., Ltd. (T. Fujita; 758; soil).                                     |    |     |
| 16042 | T | <i>Microscilla marina</i>   | 24 | 340 |
|       |   | DSMZ, DSM 4236 -- NCMB 1400 -- ATCC 23134 -- R.A. Lewin, Si0-8, marine aquarium outflow.          |    |     |
| 16043 |   | <i>Cytophaga aurantiaca</i>   | 25 | 276 |
|       |   | IAM 14300 -- NCIMB 8628 -- H. Bortels, pond soil.   |    |     |
| 16046 | T | <i>Gordona aichiensis</i>   | 30 | 227 |
|       |   | JCM 6046 -- M. Tsukamura, E9028 (=62001), human sputa.  |    |     |
| 16047 | T | <i>Gordona bronchialis</i>  | 30 | 271 |
|       |   | JCM 3198 -- KCC A-0198 -- M. Tsukamura, 3410, Sputa of patients with pulmonary disease.           |    |     |
| 16048 | T | <i>Gordona rubropertinctus</i>  | 30 | 271 |
|       |   | JCM 3204 -- KCC A-0204 -- M. Goodfellow, N4 -- NCIB 9664 -- ATCC 14352 -- R.E. Gordon, 154, soil. |    |     |

|       |   |  |    |     |
|-------|---|--|----|-----|
| 16049 | T | <i>Gordona sputi</i>   | 30 | 227 |
|       |   | JCM 3228 -- KCC A-0228 -- M. Tsukamura,3884 (strain Nagura 8539),<br>Sputa of patients with pulmonary disease.                               |    |     |
| 16050 | T | <i>Gordona terrae</i>  | 30 | 271 |
|       |   | JCM 3206 -- KCC A-0206 -- M. Goodfellow, N659 -- M. Mordarska,<br>T5 -- M. Tsukamura,3612, soil.   |    |     |
| 32771 |   | <i>Phytophthora humicola</i>   | 28 | 8   |
|       |   | CBS 200.81 -- P.J. Ann & W.H. Ko, soil of a citrus orchard. PP 7-K-1680  |    |     |
| 32772 |   | <i>Amanita pseudoporphyria</i>   | 24 | 7   |
|       |   | Shiga Forest Res. Cent. (A. Ohta; KY-A1) -- Forest Exp. Stn. Kyoto<br>Pref. (T. Fujita).   |    |     |
| 32773 |   | <i>Boletus reticulatus</i>   | 24 | 20  |
|       |   | Shiga Forest Res. Cent. (A. Ohta; NBR-1) -- Nara Pref. Forest Exp. Stn.<br>(M. Kawai; NBR-1; Quercus myrsinaefolia forest).                  |    |     |
| 32774 |   | <i>Boletus reticulatus</i>   | 24 | 20  |
|       |   | Shiga Forest Res. Cent. (A. Ohta; NBR-2) -- Nara Pref. Forest Exp. Stn.<br>(M. Kawai; NBR-2).  |    |     |
| 32775 |   | <i>Lactarius chrysorrheus</i>  | 24 | 20  |
|       |   | Shiga Forest Res. Cent. (A. Ohta; KY-Lc1) -- Forest Exp. Stn. Kyoto Pref.<br>(T. Fujita).  |    |     |
| 32776 |   | <i>Lyophyllum fumosum</i>  | 24 | 7   |
|       |   | Shiga Forest Res. Cent. (A. Ohta; OK-F1) -- Okayama Pref. Forest Exp. Stn.<br>(T. Takeuchi; Kayou 94).                                       |    |     |
| 32777 |   | <i>Lactarius hatsudake</i>   | 24 | 7   |
|       |   | Shiga Forest Res. Cent. (A. Ohta; KY-H1) -- Forest Exp. Stn. Kyoto Pref.<br>(T. Fujita).   |    |     |
| 32778 |   | <i>Lactarius hatsudake</i>   | 24 | 7   |
|       |   | Shiga Forest Res. Cent. (A. Ohta; LH-1)-- Ishikawa-ken Forest Exp. Stn.<br>(I. Nose; LH-1; Pinus thunbergii forest).                         |    |     |
| 32779 |   | <i>Lyophyllum shimeji</i>  | 24 | 7   |
|       |   | Shiga Forest Res. Cent. (A. Ohta; NG2L)-- Niigata Pref. Forest Exp. Stn.<br>(N. Matsumoto; LS-2; Pinus densiflora - Quercus serrata forest). |    |     |
| 32780 |   | <i>Lyophyllum shimeji</i>  | 24 | 7   |
|       |   | Shiga Forest Res. Cent. (A. Ohta; EH2L)-- Ehime Pref. Forest Exp. Stn.<br>(N. Mori; 150302-3; Quercus serrata - Pinus densiflora forest).    |    |     |
| 32781 |   | <i>Lyophyllum shimeji</i>  | 24 | 7   |

- Shiga Forest Res. Cent. (A. Ohta; LS-1)-- Ishikawa-ken Forest Exp. Stn.  
(I. Nose; LS-1; *Pinus densiflora* forest).
- 32782 *Lyophyllum shimeji* 24 7  
Shiga Forest Res. Cent. (A. Ohta; OK-L2) -- Okayama Pref. Forest Exp.  
Stn. (T. Takeuchi; Chuou 94).
- 32783 *Lyophyllum sykosporum* 24 7  
Shiga Forest Res. Cent. (A. Ohta; OK-SY1) -- Okayama Pref. Forest Exp.  
Stn. (T. Takeuchi; Tottori 91).
- 32784 *Russula subnigricans* 24 7  
Shiga Forest Res. Cent. (A. Ohta; MG-Rs1) -- Miyagi Pref. Forest Exp.  
Stn. (R. Kasuya; 102-1; *Pinus densiflora* forest).
- 32785 *Suillus bovinus* 24 7  
Shiga Forest Res. Cent. (A. Ohta; KY-S1) -- Forest Exp. Stn. Kyoto Pref.  
(T. Fujita).
- 32786 *Suillus grevillei* 24 7  
Shiga Forest Res. Cent. (A. Ohta; OK-S2) -- Okayama Pref. Forest Exp.  
Stn. (T. Takeuchi; Hade 94).
- 32787 *Tricholoma auratum* 24 7  
Shiga Forest Res. Cent. (A. Ohta; IS-K1) -- Ishikawa-ken Forest Exp.  
Stn. (I. Nose; TA-1; *Pinus thunbergii* forest).
- 32788 *Amanita pantherina* 24 7  
Shiga Forest Res. Cent. (A. Ohta; AP2; *Picea abies* forest).
- 32789 *Boletopsis leucomelas* 24 7  
Shiga Forest Res. Cent. (A. Ohta; B11; *Pinus densiflora* forest).
- 32790 *Calvatia craniiformis* 24 7  
Shiga Forest Res. Cent. (A. Ohta; Cc3).
- 32791 *Lactarius chrysorrheus* 24 7  
Shiga Forest Res. Cent. (A. Ohta; Lc5).
- 32792 *Lyophyllum fumosum* 24 7  
Shiga Forest Res. Cent. (A. Ohta; Lf2).
- 32793 *Lactarius hatsudake* 24 7  
Shiga Forest Res. Cent. (A. Ohta; Lh2; *Pinus densiflora* forest).
- 32794 *Lactarius hatsudake* 24 7  
Shiga Forest Res. Cent. (A. Ohta; Lh4; *Pinus densiflora* forest).
- 32795 *Pleurotus calyptratus* 24 1  
Agric. Univ. Athens (G. Zervakis) -- MUCL 28909 -- Cult. Col. Wood

- Destroying Fungi, Czechoslovakia -- A. Czerny, dead tree of *Populus alba*.
- 32796 *Pleurotus cornucopiae* var. *citrinopileatus* 24 1  
Agric. Univ. Athens (G. Zervakis) -- MUCL 28684 -- K.M. Graham,  
forest (Malaysia).
- 32797 *Pleurotus dryinus* 24 1  
Agric. Univ. Athens (G. Zervakis) -- CBS 804.85, *Malus sylvestris*.
- 32798 *Pleurotus eryngii* 24 1  
Agric. Univ. Athens (G. Zervakis; LGAM P63; *Eryngium campestre*).
- 32799 *Penicillium dendriticum* 24 1  
Fac. Integ. Arts and Sci., Hiroshima Univ. (K. Kasai; KK-1; decaying  
cone of *Pinus densiflora*).
- 32800 *Penicillium dendriticum* 24 1  
Fac. Integ. Arts and Sci., Hiroshima Univ. (K. Kasai; KK-2; decaying  
cone of *Pinus densiflora*).
- 32801 *Penicillium dendriticum* 24 1  
Fac. Integ. Arts and Sci., Hiroshima Univ. (K. Kasai; KK-3; decaying  
cone of *Pinus densiflora*).
- 32802 *Gliocephalotrichum bulbilium* 24 5  
Fac. Integ. Arts and Sci., Hiroshima Univ. (K. Kasai; KK-95-1; decaying  
cone of *Pinus densiflora*).
- 32803 *Thysanophora penicillioides* 24 1  
Fac. Integ. Arts and Sci., Hiroshima Univ. (K. Kasai; KK-95-2; decaying  
cone of *Pinus densiflora*).
- 32804 *Rhizopogon rubescens* 24 7  
Shiga Forest Res. Cent. (A. Ohta; R-1) -- Miyagi Pref. Forest. Exp. Stn.  
(T. Hirano; R-1; *Pinus thunbergii* forest).
- 32805 *Tricholoma flavovirens* 24 20  
Shiga Forest Res. Cent. (A. Ohta; KY-K1) -- Forest Exp. Stn. Kyoto Pref.  
(T. Fujita; *Pinus densiflora* forest).
- 32806 *Tricholoma matsutake* 24 7  
Shiga Forest Res. Cent. (A. Ohta; 11-43) -- Miyagi Pref. Forest. Exp. Stn.  
(R. Kasuya; 11-43).
- 32807 *Tricholoma matsutake* 24 7  
Shiga Forest Res. Cent. (A. Ohta; TMT4) -- Tokushima Pref. Forestry  
Technol. Res. Cent. (M. Abe; TMT4; *Pinus densiflora* forest).
- 32808 *Tricholoma ustale* 24 7

|       |   |    |    |
|-------|---|----|----|
|       | Shiga Forest Res. Cent. (A. Ohta; KY-Tu1) -- Forest Exp. Stn. Kyoto Pref. (T. Fujita).                                  |    |    |
| 32809 | <i>Lyophyllum shimeji</i>   | 24 | 7  |
|       | Shiga Forest Res. Cent. (A. Ohta; Ls7; Pinus densiflora forest).  |    |    |
| 32810 | <i>Lyophyllum shimeji</i>   | 24 | 7  |
|       | Shiga Forest Res. Cent. (A. Ohta; Ls13; Pinus densiflora forest).   |    |    |
| 32811 | <i>Pisolithus tinctorius</i>  | 24 | 20 |
|       | Shiga Forest Res. Cent. (A. Ohta; Pt1).   |    |    |
| 32812 | <i>Rhizopogon rubescens</i>   | 24 | 7  |
|       | Shiga Forest Res. Cent. (A. Ohta; Rr4) -- Forest Exp. Stn. Kyoto Pref. (H. Fujita; Pinus thunbergii forest).            |    |    |
| 32813 | <i>Rhizopogon rubescens</i>   | 24 | 7  |
|       | Shiga Forest Res. Cent. (A. Ohta; Rr5) -- Yamaguchi Pref. Forestry Guidance Cent. (Y. Yamada; Pinus thunbergii forest). |    |    |
| 32814 | <i>Sarcodon aspratus</i>  | 24 | 7  |
|       | Shiga Forest Res. Cent. (A. Ohta; Sa2).   |    |    |
| 32815 | <i>Sarcodon aspratus</i>  | 24 | 7  |
|       | Shiga Forest Res. Cent. (A. Ohta; Sa3).   |    |    |
| 32816 | <i>Suillus bovinus</i>  | 24 | 7  |
|       | Shiga Forest Res. Cent. (A. Ohta; Sb12; Pinus densiflora forest).   |    |    |
| 32817 | <i>Suillus luteus</i>   | 24 | 7  |
|       | Shiga Forest Res. Cent. (A. Ohta; Sl2; Pinus densiflora forest).  |    |    |
| 32818 | <i>Suillus luteus</i>   | 24 | 7  |
|       | Shiga Forest Res. Cent. (A. Ohta; Sl9; Pinus densiflora forest).  |    |    |
| 32819 | <i>Tricholoma japonicum</i>   | 24 | 7  |
|       | Shiga Forest Res. Cent. (A. Ohta; Tj1; Pinus densiflora forest).  |    |    |
| 32820 | <i>Tricholoma japonicum</i>   | 24 | 7  |
|       | Shiga Forest Res. Cent. (A. Ohta; Tj3; Pinus densiflora forest).  |    |    |
| 32821 | <i>Tricholoma auratum</i>   | 24 | 20 |
|       | Shiga Forest Res. Cent. (A. Ohta; Tk1; Pinus thunbergii forest).  |    |    |
| 32822 | <i>Tricholoma flavovirens</i>   | 24 | 20 |
|       | Shiga Forest Res. Cent. (A. Ohta; Tk6; Pinus densiflora forest).  |    |    |
| 32823 | <i>Tricholoma matsutake</i>   | 24 | 7  |
|       | Shiga Forest Res. Cent. (A. Ohta; Tm88; Pinus densiflora forest).   |    |    |
| 32824 | <i>Tricholoma robustum</i>  | 24 | 20 |
|       | Shiga Forest Res. Cent. (A. Ohta; Tr5; Pinus densiflora forest).  |    |    |

|       |   |    |   |
|-------|---|----|---|
| 32825 | <i>Tricholoma ustale</i>  | 24 | 7 |
|       | Shiga Forest Res. Cent. (A. Ohta; Tu1).   |    |   |
| 32826 | <i>Albophoma yamanashiensis</i>   | 24 | 5 |
|       | IFO (T. Ito; T. Ito H770-40-7; soil).   |    |   |
| 32827 | <i>Albophoma yamanashiensis</i>   | 24 | 5 |
|       | IFO (T. Ito; T. Ito H7-27S-3; soil of Bruguiera gymnorhiza root).   |    |   |
| 32828 | <i>Engyodontium album</i>   | 24 | 2 |
|       | IFO (T. Ito; T. Ito H7-22-4; root of Avicennia marina).   |    |   |
| 32829 | <i>Leiothecium ellipsoideum</i>   | 24 | 5 |
|       | IFO (T. Ito; T. Ito H770-48-9; soil).   |    |   |
| 32830 | <i>Peziza ostracoderma</i>  | 24 | 1 |
|       | IFO (T. Ito; T. Ito H770-44-2; soil).   |    |   |
| 32831 | <i>Mortierella umbellata</i>  | 24 | 1 |
|       | Sugadaira Montane Res. Cent., Univ. of Tsukuba (Y. Degawa; DM 271 (A1); soil under Quercus mirsinaefolia forest). |    |   |
| 32832 | <i>Mortierella umbellata</i>  | 24 | 1 |
|       | Sugadaira Montane Res. Cent., Univ. of Tsukuba (Y. Degawa; DM 273 (A2); soil under Castanopsis sieboldii forest). |    |   |
| 32833 | <i>Mortierella umbellata</i>  | 24 | 1 |
|       | Sugadaira Montane Res. Cent., Univ. of Tsukuba (Y. Degawa; DM 274 (A3); soil of Miscanthus sinensis field).       |    |   |
| 32834 | <i>Mortierella umbellata</i>  | 24 | 1 |
|       | Sugadaira Montane Res. Cent., Univ. of Tsukuba (Y. Degawa; DM 272 (B1); soil under Quercus mirsinaefolia forest). |    |   |
| 32835 | <i>Mortierella umbellata</i>  | 24 | 1 |
|       | Sugadaira Montane Res. Cent., Univ. of Tsukuba (Y. Degawa; DM 275 (B2); soil under Machilus thunbergii forest).   |    |   |
| 32836 | <i>Mortierella umbellata</i>  | 24 | 1 |
|       | Sugadaira Montane Res. Cent., Univ. of Tsukuba (Y. Degawa; DM 276 (B3); soil under Machilus thunbergii forest).   |    |   |
| 32837 | <i>Gelasinospora reticulata</i>   | 24 | 8 |
|       | FMR 5490 (J. Guarro; forest soil).  |    |   |
| 32838 | <i>Ascodesmis nigricans</i>   | 24 | 8 |
|       | FMR 5503 (J. Guarro; forest soil).  |    |   |
| 32839 | <i>Microascus longirostris</i>  | 24 | 8 |
|       | FMR 5506 (J. Guarro; garden soil).  |    |   |



|       |   |    |   |
|-------|---|----|---|
| 32840 | <i>Gelasinospora calospora</i>  | 24 | 8 |
|       | FMR 5508 (J. Guarro; forest soil).  |    |   |
| 32841 | <i>Gelasinospora santi-florii</i>   | 24 | 8 |
|       | FMR 5509 (J. Guarro; forest soil).  |    |   |
| 32842 | <i>Sporormiella minima</i>  | 24 | 8 |
|       | FMR 5510 (J. Guarro; garden soil).  |    |   |
| 32843 | <i>Chaetomium jabalpurens</i>   | 24 | 8 |
|       | FMR 5512 (J. Guarro; garden soil).  |    |   |
| 32844 | <i>Gelasinospora retispora</i>  | 24 | 8 |
|       | FMR 5513 (J. Guarro; garden soil).  |    |   |
| 32845 | <i>Coniochaeta saccardoi</i>  | 24 | 8 |
|       | FMR 5514 -- S.K. Abdullah, unidentified plant debris submerged in water.  |    |   |
| 32846 | <i>Zopfiella longicaudata</i>   | 24 | 8 |
|       | FMR 5542 -- S.K. Abdullah, sheep dung.  |    |   |
| 32847 | <i>Preussia typharum</i>  | 24 | 8 |
|       | FMR 5543 -- S.K. Abdullah, unidentified plant debris in water.  |    |   |
| 32848 | <i>Zopfiella leucotricha</i>  | 24 | 8 |
|       | FMR 5544 -- S.K. Abdullah, Phragmites sp.   |    |   |
| 32849 | <i>Colletotrichum acutatum</i>  | 24 | 1 |
|       | Shikoku Nat. Agric. Exp. Stn., T. Sato -- Nagano Pref. Fruit Tree Exp. Stn. (A. Iijima; GCP 11; Prunus domestica).        |    |   |
| 32850 | <i>Colletotrichum acutatum</i>  | 24 | 1 |
|       | Shikoku Nat. Agric. Exp. Stn., T. Sato -- Nagano Pref. Fruit Tree Exp. Stn. (A. Iijima; GCP 26; Prunus domestica).        |    |   |
| 32851 | <i>Colletotrichum acutatum</i>  | 24 | 1 |
|       | Shikoku Nat. Agric. Exp. Stn. (T. Sato; PSS 3; Prunus domestica).   |    |   |
| 32852 | <i>Colletotrichum acutatum</i>  | 24 | 1 |
|       | Shikoku Nat. Agric. Exp. Stn. (T. Sato; TSS1) -- Nat. Res. Inst. of Veg. Orn. Pl. and Tea (N. Tezuka; Anemone coronaria). |    |   |
| 32853 | <i>Colletotrichum acutatum</i>  | 24 | 1 |
|       | Shikoku Nat. Agric. Exp. Stn. (T. Sato; AC-1; Anemone coronaria).   |    |   |
| 32854 | <i>Arthrimum phaeospermum</i>   | 24 | 1 |
|       | Life Web, Grad. Schl. of Human and Environ. Stud., Kyoto Univ. (M. Okada; 94-45-6; dead leaf of Phragmites australis).    |    |   |
| 32855 | <i>Apiospora montagnei</i>  | 24 | 1 |
|       | Life Web, Grad. Schl. of Human and Environ. Stud., Kyoto Univ.  |    |   |

- (M. Okada; 95-73-1-4; living leaf of *Phragmites karka*).
- 32856 *Aspergillus japonicus* 24 1  
Life Web, Grad. Schl. of Human and Environ. Stud., Kyoto Univ.
- (M. Okada; 95-45-8-3; living leaf of *Phragmites karka*).
- 32857 *Conioscypha lignicola* 24 1  
Life Web, Grad. Schl. of Human and Environ. Stud., Kyoto Univ.
- (M. Okada; 95-48-2-1; living leaf of *Phragmites karka*).
- 32858 *Epicoccum purpurascens* 24 1  
Life Web, Grad. Schl. of Human and Environ. Stud., Kyoto Univ.
- (M. Okada; 95-57-5-3; living leaf of *Phragmites karka*).
- 32859 *Nigrospora sphaerica* 24 1  
Life Web, Grad. Schl. of Human and Environ. Stud., Kyoto Univ.
- (M. Okada; 95-73-4-1; living leaf of *Phragmites karka*).
- 32860 *Nigrospora oryzae* 24 1  
Life Web, Grad. Schl. of Human and Environ. Stud., Kyoto Univ.
- (M. Okada; 95-41-7-1; living leaf of *Phragmites australis*).
- 32861 *Paecilomyces lilacinus* 24 1  
Life Web, Grad. Schl. of Human and Environ. Stud., Kyoto Univ.
- (M. Okada; 95-56-3-4; living leaf of *Phragmites karka*).
- 32862 *Pithomyces chartarum* 24 1  
Life Web, Grad. Schl. of Human and Environ. Stud., Kyoto Univ.
- (M. Okada; 95-47-3-4; living leaf of *Phragmites karka*).
- 32863 *Pithomyces graminicola* 24 1  
Life Web, Grad. Schl. of Human and Environ. Stud., Kyoto Univ.
- (M. Okada; 95-49-1-1; living leaf of *Phragmites australis*).
- 32864 *Diplocladiella scalaroides* 24 1  
CBS 222.59 -- K. Tubaki, decayed leaf.
- 32865 *Halophytophthora operculata* 24 8  
Mar. Inst., Univ. Georgia (S.Y. Newell; SAP 121; submerged yellow  
leaf of *Avicennia* sp.).
- 32866 *Trimorphomyces papilionaceus* 24 5  
Dept. Botany, Univ. British Columbia (R. Bandoni; 8887-A; stem of *Sasa* sp.).
- 32867 *Peyronelina glomerulata* 24 1  
IFO (A. Nakagiri; AN-1505; submerged decomposing culm of *Cyperus* sp.).
- 32868 *Aegerita candida* 24 1  
FMR 5586 -- S.K. Abdullah, submerged rotten twig. PP 8-K-908

|       |  |    |    |
|-------|--|----|----|
| 32869 | <i>Helicodendron articulatum</i>   | 24 | 1  |
|       | FMR 5499 -- S.K. Abdullah, submerged Quercus spp. leaves.                  |    |    |
| 32870 | <i>Helicodendron conglomeratum</i>   | 24 | 1  |
|       | FMR 5497 -- S.K. Abdullah, submerged oak leaves.                           |    |    |
| 32871 | <i>Helicodendron fractum</i>   | 24 | 1  |
|       | FMR 5500 -- S.K. Abdullah, submerged pine needle.                          |    |    |
| 32872 | <i>Helicodendron paradoxum</i>   | 24 | 1  |
|       | FMR 5498 -- S.K. Abdullah, submerged Quercus spp. leaves.                  |    |    |
| 32873 | <i>Helicodendron tubulosum</i>   | 24 | 1  |
|       | FMR 5502 -- S.K. Abdullah, submerged Betula spp. leaves.                   |    |    |
| 32874 | <i>Helicoon fuscosporum</i>  | 24 | 1  |
|       | FMR 5494 -- S.K. Abdullah, submerged Quercus spp. leaves.                  |    |    |
| 32875 | <i>Helicoon fuscosporum</i>  | 24 | 1  |
|       | FMR 5495 -- S.K. Abdullah, submerged Salix spp. leaves.                    |    |    |
| 32876 | <i>Spirosphaera floriformis</i>  | 24 | 1  |
|       | FMR 5583 -- S.K. Abdullah, submerged Coreolus leaves.                      |    |    |
| 32878 | <i>Chaetomella raphigera</i>   | 24 | 1  |
|       | IFO (A. Nakagiri; AN-1512; submerged fallen leaf of Bruguiera gymnorhiza). |    |    |
| 32879 | <i>Pleurothecium recurvatum</i>  | 24 | 17 |
|       | IFO (I. Okane; IOC 1236; rotten wood).                                     |    |    |
| 32880 | <i>Chaetomium gelasinosporum</i>   | 24 | 2  |
|       | FMR 5735 -- S.K. Abdullah, soil.   |    |    |
| 32881 | <i>Chaetomium jabalpurense</i>   | 24 | 2  |
|       | FMR 5590 -- J. Guarro, soil.   |    |    |
| 32882 | <i>Chaetomium jabalpurense</i>   | 24 | 2  |
|       | FMR 5736 -- J. Guarro, soil.   |    |    |
| 32883 | <i>Chaetomium nigricolor</i>   | 24 | 2  |
|       | FMR 5737 -- J. Guarro, soil.   |    |    |
| 32884 | <i>Chaetomium quadrangulatum</i>   | 24 | 2  |
|       | FMR 5549 -- J. Guarro, soil.   |    |    |
| 32885 | <i>Corynascus sepedonium</i>   | 24 | 2  |
|       | FMR 5593 -- J. Guarro, soil.   |    |    |
| 32886 | <i>Eleutherascus peruvianus</i>  | 24 | 2  |
|       | FMR 5553 -- J. Guarro, soil.   |    |    |
| 32887 | <i>Emericella rugulosa</i>   | 24 | 1  |
|       | FMR 5738 -- S.K. Abdullah, soil.   |    |    |

|       |   |    |   |
|-------|---|----|---|
| 32888 | <i>Emericella rugulosa</i>  | 24 | 1 |
|       | FMR 5754 -- J. Guarro, soil.  |    |   |
| 32889 | <i>Eupenicillium javanicum</i> var. <i>javanicum</i>                        | 24 | 5 |
|       | FMR 5772 -- A. Stchigel, soil.  |    |   |
| 32890 | <i>Gelasinospora santi-florii</i>   | 24 | 8 |
|       | FMR 5556 -- J. Guarro, soil.  |    |   |
| 32891 | <i>Gelasinospora seminuda</i>   | 24 | 8 |
|       | FMR 5653 -- J. Guarro, soil.  |    |   |
| 32892 | <i>Lasiobolus microsporus</i>   | 24 | 1 |
|       | FMR 5589 -- J. Guarro, soil.  |    |   |
| 32893 | <i>Lophotrichus bartlettii</i>  | 24 | 2 |
|       | FMR 5572 -- S.K. Abdullah, sheep dung.                                      |    |   |
| 32894 | <i>Lophotrichus macrosporus</i>   | 24 | 2 |
|       | FMR 5571 -- S.K. Abdullah, sheep dung.                                      |    |   |
| 32895 | <i>Narasimhella hyalinosporea</i>   | 24 | 1 |
|       | FMR 5570 -- J. Guarro, garden soil.   |    |   |
| 32896 | <i>Neurospora africana</i>  | 24 | 2 |
|       | FMR 5547 -- J. Guarro, soil.  |    |   |
| 32897 | <i>Petriella setifera</i>   | 24 | 8 |
|       | FMR 5550 -- J. Guarro, soil.  |    |   |
| 32898 | <i>Clathrosphaerina zalewskii</i>   | 24 | 1 |
|       | FMR 5579 -- S.K. Abdullah, Quercus sp. leaves submerged in a stream.        |    |   |
| 32899 | <i>Pseudaegerita corticalis</i>   | 24 | 1 |
|       | FMR 5581 -- S.K. Abdullah, decaying Salix sp. twig.                         |    |   |
| 50041 | <i>MBT2</i>   |    |   |
|       | RTCI -- T. Niijima(MTU) -- Mark. S. Soloway (Univ. Tennessee)               |    |   |
| 50090 | <i>4G12 hybridoma</i>   |    |   |
|       | Yoshitomi Pharmaceutical Industries, Ltd. (Y. Matsushiro).                  |    |   |
| 50161 | <i>KT-5</i>   |    |   |
|       | IFO (M. Takeuchi).  |    |   |
| 50466 | <i>RC1</i>  |    |   |
|       | Inst. Basic Med. Sci., Univ. of Tsukuba(M. Yamamoto; 8C7; mouse hybridoma). |    |   |

## LIST OF MEDIA

**20**

|                       |     |        |                                |        |   |
|-----------------------|-----|--------|--------------------------------|--------|---|
| Yeast extract .....   | 0.4 | g      | Yeast extract .....            | 1.0    | g |
| Glucose.....          | 4   | g      | Agar (if needed).....          | 15.0   | g |
| Distilled water ..... | 1   | L      | Filtered, aged sea water ..... | 1.0    | L |
| Agar.....             | 15  | g      |                                | pH 7.2 |   |
|                       |     | pH 5.0 |                                |        |   |

**21 SYS Medium**

|   |       |        |                       |              |   |
|---|-------|--------|-----------------------|--------------|---|
| Starch .....                                    | 20    | g      | Tryptone (Difco)..... | 2.0          | g |
| Yeast extract .....                             | 0.5   | g      | Beef extract .....    | 0.5          | g |
| Soytone.....                                    | 0.5   | g      | Yeast extract .....   | 0.5          | g |
| $\text{KH}_2\text{PO}_4$ .....                  | 0.1   | g      | Sodium acetate .....  | 0.2          | g |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ..... | 0.1   | g      | Agar (if needed)..... | 15.0         | g |
| $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ..... | 1     | mg     | Distilled water ..... | 1.0          | L |
| $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ..... | 1     | mg     |                       | pH 7.2 - 7.4 |   |
| Thiamine Hcl .....                              | 3     | mg     |                       |              |   |
| Nicotinamide.....                               | 0.005 | mg     |                       |              |   |
| Agar.....                                       | 15    | g      |                       |              |   |
| Distilled water .....                           | 1     | L      |                       |              |   |
|   |       | pH 5.2 |                       |              |   |

**330**

|   |     |                   |  |  |  |
|---|-----|-------------------|--|--|--|
| Methanol* .....                                 | 10  | ml                |  |  |  |
| Bacto-Yeast extract .....                       | 0.2 | g                 |  |  |  |
| $(\text{NH}_4)_2\text{SO}_4$ .....              | 3   | g                 |  |  |  |
| $\text{KH}_2\text{PO}_4$ .....                  | 1.4 | g                 |  |  |  |
| $\text{Na}_2\text{HPO}_4$ .....                 | 3   | g                 |  |  |  |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ..... | 0.2 | g                 |  |  |  |
| Ferric citrate .....                            | 30  | mg                |  |  |  |
| $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ..... | 30  | mg                |  |  |  |
| $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ..... | 5   | mg                |  |  |  |
| $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ..... | 5   | mg                |  |  |  |
| $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ..... | 0.5 | mg                |  |  |  |
| Agar .....                                      | 15  | g                 |  |  |  |
| Distilled water .....                           | 1   | L                 |  |  |  |
|   |     | Adjust pH to 7.0. |  |  |  |

\*Sterilize separately by filtration and add to other ingredients.

**331 SP5 medium**

|                       |     |   |
|-----------------------|-----|---|
| Casitone (Difco)..... | 9.0 | g |
|-----------------------|-----|---|

**332 Enriched Cytophaga agar**

|                       |      |              |
|-----------------------|------|--------------|
| Tryptone (Difco)..... | 2.0  | g            |
| Beef extract .....    | 0.5  | g            |
| Yeast extract .....   | 0.5  | g            |
| Sodium acetate .....  | 0.2  | g            |
| Agar (if needed)..... | 15.0 | g            |
| Distilled water ..... | 1.0  | L            |
|                       |      | pH 7.2 - 7.4 |

**333 Marine Cytophaga agar**

|                                |      |              |
|--------------------------------|------|--------------|
| Tryptone (Difco).....          | 2.0  | g            |
| Beef extract .....             | 0.5  | g            |
| Yeast extract .....            | 0.5  | g            |
| Sodium acetate .....           | 0.2  | g            |
| Agar (if needed).....          | 15.0 | g            |
| Filtered, aged sea water ..... | 1.0  | L            |
|                                |      | pH 7.2 - 7.4 |

**334 Flavobacterium columnare agar**

|                       |      |              |
|-----------------------|------|--------------|
| Tryptone (Difco)..... | 2.0  | g            |
| Beef extract .....    | 0.5  | g            |
| Yeast extract .....   | 0.5  | g            |
| Sodium acetate .....  | 0.2  | g            |
| Agar (if needed)..... | 10.0 | g            |
| Distilled water ..... | 1.0  | L            |
|                       |      | pH 7.2 - 7.4 |

**335 Medium for marine flexibacteria**

|                                |      |        |
|--------------------------------|------|--------|
| $\text{KNO}_3$ .....           | 0.5  | g      |
| Sodium glycerophosphate .....  | 0.1  | g      |
| Trace element solution* .....  | 1.0  | ml     |
| Tris .....                     | 1.0  | g      |
| Tryptone (Difco).....          | 5.0  | g      |
| Yeast extract .....            | 5.0  | g      |
| Agar (if needed).....          | 15.0 | g      |
| Filtered, aged sea water ..... | 1.0  | L      |
|                                |      | pH 7.0 |

## LIST OF MEDIA

**\* Trace element solution**

|  |       |    |
|--|-------|----|
| H <sub>3</sub> BO <sub>4</sub> .....                       | 2.85  | g  |
| MnCl <sub>2</sub> · 4H <sub>2</sub> O .....                | 1.80  | g  |
| FeSO <sub>4</sub> · 7H <sub>2</sub> O .....                | 1.36  | g  |
| CuCl <sub>2</sub> · 2H <sub>2</sub> O .....                | 26.90 | mg |
| ZnCl <sub>2</sub> .....                                    | 20.80 | mg |
| CoCl <sub>2</sub> · 6H <sub>2</sub> O .....                | 40.40 | mg |
| Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O ..... | 25.20 | mg |
| Sodium tartrate .....                                      | 1.77  | g  |
| Distilled water .....                                      | 1.0   | L  |

**336 Cytophaga (marine) medium**

|                                |        |   |
|--------------------------------|--------|---|
| Tryptone (Difco) .....         | 1.0    | g |
| Yeast extract .....            | 1.0    | g |
| Agar (if needed) .....         | 15.0   | g |
| Filtered, aged sea water ..... | 1.0    | L |
|                                | pH 7.2 |   |

**337 SP2 medium**

|                                |        |   |
|--------------------------------|--------|---|
| Casitone (Difco) .....         | 1.0    | g |
| Yeast extract .....            | 0.2    | g |
| Sodium acetate .....           | 0.02   | g |
| Agar (if needed) .....         | 15.0   | g |
| Filtered, aged sea water ..... | 1.0    | L |
|                                | pH 7.2 |   |

**338 Caulobacter medium**

|   |        |   |
|---|--------|---|
| Peptone .....                               | 2.0    | g |
| Yeast extract .....                         | 1.0    | g |
| MgSO <sub>4</sub> · 7H <sub>2</sub> O ..... | 0.2    | g |
| Agar .....                                  | 10.0   | g |
| Distilled water .....                       | 1.0    | L |
|   | pH 7.0 |   |

**339 Marine broth 2216**

|                                |                |   |
|--------------------------------|----------------|---|
| Marine broth 2216 (Difco) .... | 37.4           | g |
| Distilled water .....          | 1.0            | L |
|                                | pH unadjusted. |   |

**340 Marine agar 2216**

|                                |                |   |
|--------------------------------|----------------|---|
| Marine agar 2216 (Difco) ..... | 55.1           | g |
| Distilled water .....          | 1.0            | L |
|                                | pH unadjusted. |   |

**341 Medium for freshwater flexibacteria**

|   |        |    |
|---|--------|----|
| MgSO <sub>4</sub> · 7H <sub>2</sub> O ..... | 0.1    | g  |
| KNO <sub>3</sub> .....                      | 0.1    | g  |
| CaCl <sub>2</sub> · 2H <sub>2</sub> O ..... | 0.1    | g  |
| Sodium glycerophosphate .....               | 0.1    | g  |
| Trace element solution* .....               | 1.0    | ml |
| Tris .....                                  | 1.0    | g  |
| Thiamine .....                              | 1.0    | mg |
| Casamino acids (Difco) .....                | 1.0    | g  |
| Glucose .....                               | 1.0    | g  |
| Cobalamine .....                            | 1.0    | μg |
| Agar .....                                  | 10.0   | g  |
| Distilled water .....                       | 1.0    | L  |
|   | pH 7.5 |    |

\* See medium 335

**342 Nissui plate sheep blood agar G\***

\*Nissui Pharmaceutical Co. Ltd.

**702 IFO medium 702**

(for rehydration of dried culture)

|   |        |   |
|---|--------|---|
| Polypepton* .....                           | 10     | g |
| Yeast extract .....                         | 2      | g |
| MgSO <sub>4</sub> · 7H <sub>2</sub> O ..... | 1      | g |
| Distilled water .....                       | 1      | L |
|   | pH 7.0 |   |

\*Wako Pure Chemicals Ind. Ltd., Osaka, Japan.

## Scientific Papers, 1995–1996

### **Taxonomic status of *Streptomyces coelicolor* A3(2) and *Streptomyces lividans* 66**

K. Hatano, T. Tamura and T. Nishii

Actinomycetologica 8: 47–50 (1994)

### **DNA molecular hybridization of *Brachybacterium* –the method of photobiotinylated DNA**

C.-X. Fang<sup>1)</sup>, A. Yokota and Mariko Takeuchi

J. Adv. Biochem. Biophys. (1995)

1) School of Life Sciences, Wuhan University

### **The role of analysis of cellular lipids and DNA hybridization in taxonomic study of *Brachybacterium***

C.-X. Fang<sup>1)</sup>, A. Yokota and Mariko Takeuchi

Bull. Wuhan Univ. 41: 239–244 (1995)

1) School of Life Sciences, Wuhan University

### **Chemotaxonomic significance of polyamine distribution patterns in the *Flavobacterium*–*Cytophaga* complex and the related genera**

K. Hamana<sup>1)</sup>, Y. Nakagawa and K. Yamasato<sup>2)</sup>

Microbios 81: 135–145 (1995)

1) College of Medical Care and Technology, Gunma University

2) Institute of Applied Microbiology, The University of Tokyo

### **16S rRNA-based phylogenetic analysis of marine flavobacteria**

N. Hanzawa<sup>1)</sup>, S. Kanai<sup>1)</sup>, A. Katsuta<sup>1)</sup>, Y. Nakagawa and K. Yamasato<sup>2)</sup>

J. Marine Biotech. 3: 111–114 (1995)

1) Marine Biotechnology Institute, Kamaishi Lab.

2) Culture Collection Center, Tokyo University of Agriculture

### **A selective isolation method for *Actinomadura viridis* in soil**

M. Hayakawa<sup>1)</sup>, Y. Momose<sup>1)</sup>, T. Kajiura<sup>1)</sup>, T. Yamazaki<sup>1)</sup>, T. Tamura, K. Hatano and H. Nonomura<sup>1)</sup>

J. Ferment. Bioeng. 79: 287–289 (1995)

1) Department of Fermentation Technology, Faculty of Engineering, Yamanashi University

### ***Amauroascus purpureus*, a new species of the Amauroascaceae (Ascomycotina)**

T. Ito and A. Nakagiri

Mycotaxon 55: 347–352 (1995)

***Westerdykella globosa*, a proposal for a new combination**

T. Ito and A. Nakagiri  
Mycoscience 36: 361–363 (1995)

**Fern rust fungi (Uredinales) of Nepal and Pakistan**

M. Kakishima<sup>1)</sup>, I. Okane and Y. Ono<sup>2)</sup>  
In: Cryptogams of the Himalayas Vol. 3, Nepal and Pakistan. (ed. by M. Watanabe and H. Hagiwara), pp. 45–60. Nat. Sci. Mus., Tokyo (1995)  
1) Institute of Agriculture and Forestry, University of Tsukuba  
2) Faculty of Education, Ibaraki University

***Bacillus curdlanolyticus* sp. nov. and *Bacillus kobensis* sp. nov., which hydrolyze resistant curdlan**

Y. Kanzawa<sup>1)</sup>, A. Harada<sup>2)</sup>, Mariko Takeuchi, A. Yokota and T. Harada<sup>1)</sup>  
Int. J. Syst. Bacteriol. 45: 515–521 (1995)  
1) Kobe Women's University  
2) Faculty of Science, Osaka University

**Effects of leukemia inhibitory factor on the differentiation of astrocyte progenitor cells from embryonic mouse cerebral hemispheres**

Y. Nakagaito, T. Yoshida, M. Satoh and Masao Takeuchi  
Develop. Brain Res. 87: 220–223 (1995)

**Protocol for cloning of PCR amplified DNA, and preparation of single-stranded DNA and plasmid used as a sequencing template**

Y. Nakagawa  
Microbiol. Cult. Coll. 11: 23–30 (1995)

[in Japanese]

**Mangrove fungi**

A. Nakagiri  
Puranta 40: 27–35 (1995)

[in Japanese]

**Taxonomy and ecology of marine fungi —Marine fungi: their autecology and evolution—**

A. Nakagiri  
Nippon Kingakukai Kaiho 36: 115–120 (1995)

[in Japanese]



**Spermogonal and aecial stages of oat crown rust, *Puccinia coronata* var. *avenae*, in Japan**

I. Okane and M. Kakishima<sup>1)</sup>

Ann. Phytopathol. Soc. Jpn. 61: 391–395 (1995)

1) Institute of Agriculture and Forestry, University of Tsukuba

**Uredinales collected in Mt. Nanga Parbat area, Pakistan**

Y. Ono<sup>1)</sup> and I. Okane

In: Cryptogams of the Himalayas Vol. 3, Nepal and Pakistan. (ed. by M. Watanabe and H. Hagiwara), pp. 61–68. Nat. Sci. Mus., Tokyo (1995)

1) Faculty of Education, Ibaraki University

**Investigation into protective agent for halophilic bacteria subjected to liquid drying (L-drying)**

T. Sakane

Cryobiol. Cryotechnol. 41: 87–92 (1995)

[in Japanese]

**Distribution of 3-ketolactose formation among *Sphingomonas* spp. and other members of the alpha subclass of the *Proteobacteria***

T. Sakane, Mariko Takeuchi, A. de Bruyn<sup>1)</sup>, K. Kersters<sup>2)</sup> and A. Yokota

Int. J. Syst. Bacteriol. 45: 342–347 (1995)

1) Laboratorium voor Organische Chemie

2) Laboratorium voor Microbiologie

**Taxonomic study of the genus *Brachybacterium*: Proposal of *Brachybacterium conglomeratum* sp. nov., nom. rev., *Brachybacterium paraconglomeratum* sp. nov., and *Brachybacterium rhamnosum* sp. nov.**

Mariko Takeuchi, C.-X. Fang<sup>1)</sup>, and A. Yokota

Int. J. Syst. Bacteriol. 45: 160–168 (1995)

1) School of Life Sciences, Wuhan University

**Taxonomic study of bacteria isolated from plants: Proposals of *Sphingomonas rosa* sp. nov., *Sphingomonas pruni* sp. nov., *Sphingomonas asaccharolytica* sp. nov., and *Sphingomonas mali* sp. nov.**

Mariko Takeuchi, T. Sakane, M. Yanagi<sup>1)</sup>, K. Yamasato<sup>1)</sup>, K. Hamana<sup>2)</sup> and A. Yokota

Int. J. Syst. Bacteriol. 45: 334–341 (1995)

1) Institute of Applied Microbiology, The University of Tokyo

2) College of Medical Care and Technology, Gunma University

**Induction of NCAM expression in mouse olfactory keratin-positive basal cells in vitro**

M. Satoh and Masao Takeuchi

Develop. Brain Res. 87: 111–119 (1995)

**Platelet-like particles released by inhibition of DNA synthesis in the human-megakaryoblastic leukemia cell line, MEG-O1s**

Masao Takeuchi, H. Kuno, M. Satoh, T. Yoshida, M. Ogura<sup>1)</sup> and K. Takeuchi<sup>2)</sup>  
Tiss. Cult. Res. Commun. 14: 165–175 (1995)

1) Aichi Cancer Center Hospital

2) Osaka College of Health Science

**Four new species of the genus *Actinokineospora* : *Actinokineospora inagensis* sp. nov., *Actinokineospora globicatena* sp. nov., *Actinokineospora terrae* sp. nov., and *Actinokineospora diospyosa* sp. nov.**

T. Tamura, M. Hayakawa<sup>1)</sup>, H. Nonomura<sup>1)</sup>, A. Yokota and K. Hatano  
Int. J. Syst. Bacteriol. 45: 371–378 (1995)

1) Department of Fermentation Technology, Faculty of Engineering, Yamanashi University

**Five new species of the genus *Catenuloplanes*: *Catenuloplanes niger* sp. nov., *Catenuloplanes indicus* sp. nov., *Catenuloplanes atrovinosus* sp. nov., *Catenuloplanes castaneus* sp. nov., and *Catenuloplanes nepalensis* sp. nov.**

T. Tamura, A. Yokota, L.H. Huang<sup>1)</sup>, Toru Hasegawa and K. Hatano  
Int. J. Syst. Bacteriol. 45: 858–860 (1995)

1) Central Research Division, Pfizer Inc.

**Establishment of *Kineococcus*, *Luteococcus*, *Catenuloplanes*, and *Couchioplanes* as new actinomycete genera including new species and their relationships with related organisms**

T. Tamura  
Actinomycetologica. 9: 206–215 (1995)

**The phylogenetic relationships among species of the genus *Metschnikowia* Kamienski and its related genera based on the partial sequences of 18S and 26S ribosomal RNAs (*Metschnikowiaceae*)**

Y. Yamada<sup>1)</sup>, T. Nagahama<sup>1)</sup> and I. Banno  
Biosci. Biotech. Biochem. 59: 439–444 (1995)

1) Department of Agricultural Chemistry, Shizuoka University

**The phylogenetic relationships of Methanol-assimilating yeasts based on the partial sequences of 18S and 26S ribosomal RNAs: The proposal of *Komagataella* gen. nov. (*Saccharomycetaceae*)**

Y. Yamada<sup>1)</sup>, M. Matsuda<sup>1)</sup>, K. Maeda<sup>1)</sup> and K. Mikata  
Biosci. Biotech. Biochem. 59: 439–444 (1995)

1) Department of Agricultural Chemistry, Shizuoka University

**The phylogenetic relationships of the Q9-equipped, hat-shaped ascospore-forming species of the *Yamadazyma* Billon-Grand (Saccharomycetaceae) based on the partial sequences of 18S and 26S ribosomal RNAs**

Y. Yamada<sup>1)</sup>, T. Suzuki<sup>1)</sup>, M. Matsuda<sup>1)</sup> and K. Mikata

Biosci. Biotech. Biochem. 59: 445-450 (1995)

1) Department of Agricultural Chemistry, Shizuoka University

**The phylogenetic relationships of *Pichia jadinii*, formerly classified in the genus *Hansenula* and related species based on the partial sequences of 18S and 26S ribosomal RNAs (Saccharomycetaceae)**

Y. Yamada<sup>1)</sup>, M. Matsuda<sup>1)</sup> and K. Mikata

Biosci. Biotech. Biochem. 59: 518-520 (1995)

1) Department of Agricultural Chemistry, Shizuoka University

**The phylogeny of *Williopsis salicorniae* Hinzelin, Kurtzman et Smith based on the partial sequences of 18S and 26S ribosomal RNAs (Saccharomycetaceae)**

Y. Yamada<sup>1)</sup>, J. Yano<sup>1)</sup>, M. Matsuda<sup>1)</sup>, T. Higashi<sup>1)</sup> and K. Mikata

Biosci. Biotech. Biochem. 59: 945-948 (1995)

1) Department of Agricultural Chemistry, Shizuoka University

**The phylogeny of *Yamadazyma ohmeri* (Etchells et Bell) Billon-Grand based on the partial sequences of 18S and 26S ribosomal RNAs: The proposal of *Kodamaea* gen. nov. (Saccharomycetaceae)**

Y. Yamada<sup>1)</sup>, T. Suzuki<sup>1)</sup>, M. Matsuda<sup>1)</sup> and K. Mikata

Biosci. Biotech. Biochem. 59: 1172-1174 (1995)

1) Department of Agricultural Chemistry, Shizuoka University

**The phylogenetic relationships of *Eeniella nana* Smith, Batenburg-van der Vegte et Scheffers based on the partial sequences of 18S and 26S ribosomal RNAs (Candidaceae)**

Y. Yamada<sup>1)</sup>, M. Matsuda<sup>1)</sup> and K. Mikata

J. Ind. Microbiol. 14: 456-460 (1995)

1) Department of Agricultural Chemistry, Shizuoka University

**The phylogenetic relationships of species of the genus *Kluyveromyces* van der Walt (Saccharomycetaceae) deduced from the partial sequences of 18S and 26S ribosomal RNAs**

S. Ando<sup>1)</sup>, K. Mikata, Y. Tahara<sup>2)</sup> and Y. Yamada<sup>2)</sup>

Biosci. Biotech. Biochem. 60: 1063-1069 (1996)

1) United Graduate School of Agricultural Science, Gifu University

2) Department of Agricultural Chemistry, Shizuoka University

**Constructed wetlands; Wastewater treatment systems using purification mechanisms**

**in nature**

K. Hatano

Bioscience &amp; Industry 54: 23-25 (1996)

[in Japanese]

***Bacillus ehimensis* sp. nov. and *Bacillus chitinolyticus* sp. nov., new chitinolytic members of the genus *Bacillus***K. Kuroshima, T. Sakane, R. Takata<sup>1)</sup> and A. Yokota<sup>2)</sup>

Int. J. Syst. Bacteriol. 46: 76-80 (1996)

1) Department of Agriculture, Ehime University

2) Institute of Molecular and Cellular Biosciences, The University of Tokyo

**Transfer of "*Pseudomonas riboflavina*" (Foster 1944), a gram-negative, motile rod with long-chain 3-hydroxy fatty acids, to *Devosia riboflavina* gen. nov., sp. nov., nom. rev.**Y. Nakagawa, T. Sakane and A. Yokota<sup>1)</sup>

Int. J. Syst. Bacteriol. 46: 16-22 (1996)

1) Institute of Molecular and Cellular Biosciences, The University of Tokyo

**Emendation of the genus *Cytophaga* and transfer of *Cytophaga agarovorans* and *Cytophaga salmonicolor* to *Marinilabilia* gen. nov.: phylogenetic analysis of the *Flavobacterium*-*Cytophaga* complex**Y. Nakagawa and K. Yamasato<sup>1)</sup>

Int. J. Syst. Bacteriol. 46: 599-603 (1996)

1) Institute of Applied Microbiology, The University of Tokyo

**Emendation of the genus *Planococcus* and transfer of *Flavobacterium okeanokoites* Zobell and Upham 1944 to the genus *Planococcus* as *Planococcus okeanokoites* comb. nov.**Y. Nakagawa, T. Sakane and A. Yokota<sup>1)</sup>

Int. J. Syst. Bacteriol. 46: 866-870 (1996)

1) Institute of Molecular and Cellular Biosciences, The University of Tokyo

**Taxonomy and ecology of *Dactylella iridis*: Its redescription as an entomogenous and nematode-capturing hyphomycete**

A. Nakagiri and T. Ito

Mycoscience 37: 81-89 (1996)

**Biodiversity and ecology of the oomycetous fungus, *Halophytophthora***A. Nakagiri, S.Y. Newell<sup>1)</sup>, T. Ito, T.K. Tan<sup>2)</sup> and C.L. Pek<sup>2)</sup>

In: Biodiversity and the Dynamics of Ecosystems, DIWPA Series Vol. 1.

(ed. by I.M. Turner, C.H. Diong, S. S. L. Lim and P. K. L. Ng), pp. 273-280.

The International network for DIVERSITAS in Western Pacific and Asia. (1996)

- 1) Marine Institute, University of Georgia
- 2) School of Biological Sciences, National University of Singapore

***Discostroma tricellulare*, a new endophytic ascomycete with a *Seimatosporium* anamorph isolated from *Rhododendron***

I. Okane, A. Nakagiri and T. Ito  
 Can. J. Bot. 74: 1334-1344 (1996)

***Leucobacter komagatae* gen. nov., sp. nov., a new aerobic gram-positive, nonsporulating rod with 2,4-diaminobutyric acid in the cell wall**

Mariko Takeuchi, N. Weiss<sup>1)</sup>, P. Schumann<sup>2)</sup> and A. Yokota<sup>2)</sup>  
 Int. J. Syst. Bacteriol. 46: 967-971 (1996)

- 1) DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen
- 2) Institute of Molecular and Cellular Biosciences, The University of Tokyo

**An effective production of optically active amino acids**

S. Tokuyama<sup>1)</sup> and K. Hatano  
 Bioscience & Industry 54: 790-792 (1996)  
 1) Faculty of Agriculture, Shizuoka University

[in Japanese]

**Should *Petasospora* Boiden et Abadie (Saccharomycetaceae) be retained ? —The phylogeny based on the partial sequences of 18S and 26S ribosomal RNAs**

Y. Yamada<sup>1)</sup>, T. Higashi<sup>1)</sup> and K. Mikata  
 Biosci. Biotech. Biochem. 60: 818-822 (1996)

- 1) Department of Agricultural Chemistry, Shizuoka University

**The phylogeny of species of the genus *Saccharomycopsis* Schiönnig (Saccharomycetaceae) based on the partial sequences of 18S and 26S ribosomal RNAs**

Y. Yamada<sup>1)</sup>, M. Matsuda<sup>1)</sup> and K. Mikata  
 Biosci. Biotech. Biochem. 60: 1303-1307 (1996)

- 1) Department of Agricultural Chemistry, Shizuoka University

## Presentation of Papers at Scientific Meetings, 1995–1996

### **Seminar of Society for Actinomycetes Japan (Feb. 15, 1995)**

K. Hatano

The present situations of streptomycete taxonomy and criteria of application for a Japanese patent

### **95th General Meeting of American Society for Microbiology (May, 1995, Washington D.C.)**

A. Yokota<sup>1)</sup>, D. de Briel<sup>2)</sup>, Mariko Takeuchi, P. Riegel<sup>2)</sup>, Y. Piemont<sup>2)</sup> and H. Monteil<sup>2)</sup>

Taxonomy of corynform CDC group A and relatives

1) Institute of Molecular and Cellular Biosciences, The University of Tokyo

2) Institut e of Bacteriology, Faculty of Medicine, University of Lous Pasteur

### **Mycological Society of Japan (May, 1995, Tokyo)**

T. Ito and A. Nakagiri

A mycofloral study on mangrove mud in Okinawa

K. Mikata and Tatsuo Hasegawa

New species of *Metcshnikowia*

A. Nakagiri

Taxonomy and ecology of marine fungi –Marine fungi: autecology and their evolution–

I. Okane, A. Nakagiri and T. Ito

A new species of *Discostroma* with *Seimatosporium*-like anamorph, isolated from leaves of *Rhododendron*

### **The Annual Meeting of the Society for Actinomycetes Japan (June, 1995, Tokyo)**

T. Tamura, M. Hayakawa<sup>1)</sup> and K. Hatano

Proposal of a new actinomycete with motile spore

1) Department of Fermentation Technology, Faculty of Engineering, Yamanashi University

T. Tamura

Proposal of new genus and new species of actinomycetes and related organisms

### **6th International Marine Mycology Symposium (July, 1995, Portsmouth)**

A. Nakagiri, S.Y. Newell<sup>1)</sup> and T. Ito

Growth and reproduction of *Halophytophthora* species in mangrove ecosystems

1) Marine Institute, University of Georgia

A. Nakagiri and T. Ito

Morphology and taxonomy of spathulosporaceous fungi

**Japan Society for Culture Collections** (July, 1995, Gifu)

K. Kuroshima and T. Sakane

Preservation of bacteria by L-drying: Viabilities of L-dried bacteria after preservation for 20 years

**Japan Society for Bioscience, Biotechnology and Agrochemistry**

(August, 1995, Sapporo)

K. Hatano

Constructed wetlands; Wastewater treatment systems using purification mechanisms in nature

Y. Kanzawa<sup>1)</sup>, Mariko Takeuchi, A. Yokota<sup>2)</sup> and T. Harada<sup>1)</sup>

*Bacillus curdlanolyticus* sp. nov. and *Bacillus kobenensis* sp. nov., which hydrolyze resistant curdlan

1) Kobe Women's University

2) Institute of Molecular and Cellular Biosciences, The University of Tokyo

Y. Nakagawa, T. Sakane and A. Yokota<sup>1)</sup>

*Raromonas riboflavina* gen. nov., sp. nov., nom. rev.: a new gram-negative, motile rod with long chain 3-hydroxy fatty acids

1) Institute of Molecular and Cellular Biosciences, The University of Tokyo

Mariko Takeuchi, A. Yokota<sup>1)</sup> and T. Sakane

Taxonomic significance of cell-wall peptidoglycan type in Actinobacteria

1) Institute of Molecular and Cellular Biosciences, The University of Tokyo

A. Yokota<sup>1)</sup>, Mariko Takeuchi and D. de Briel<sup>2)</sup>

Taxonomic study of CDC group coryneform bacteria

1) Institute of Molecular and Cellular Biosciences, The University of Tokyo

2) Institut e of Bacteriology, Faculty of Medicine, University of Lous Pasteur

Y. Yamada<sup>1)</sup>, T. Higashi<sup>1)</sup> and K. Mikata

Should *Petasospora* Baidin et Abadie be retained ? - Phylogeny based on the partial rRNA Sequences.

1) Department of Agricultural Chemistry, Shizuoka University

Y. Yamada<sup>1)</sup>, M. Matsuda<sup>1)</sup> and K. Mikata

Phylogeny of *Saccharomycopsis* yeasts based on partial rRNA sequences

1) Department of Agricultural Chemistry, Shizuoka University

Y. Yamada<sup>1)</sup>, J. Yanol<sup>1)</sup>, M. Suzuki<sup>1)</sup>, M. Matsuda<sup>1)</sup> and K. Mikata

Phylogeny of *Issatchenkia* and *Ambrosiozyma* yeasts based on partial rRNA sequences

1) Department of Agricultural Chemistry, Shizuoka University

**Japan Society for Cryobiology and Cryotechnology** (August, 1995, Sapporo)

T. Sakane

Investigation into protective agent for halophilic bacteria subjected to L-drying

**3rd Meeting on Microbial Interrelation** (Sept. 1995, Hiroshima)

A. Nakagiri

*Dactylella iridis*: an entomogenous, nematophagous and aquatic hyphomycete

**Japan Society for Cell Biology** (October, 1995, Sendai)

Y. Nakagaito, T. Yoshida, M. Satoh, and Masao Takeuchi

Effect of leukemia inhibitory factor (LIF) on the differentiation of astrocyte progenitor cells

K. Takeuchi<sup>1)</sup>, H. Kuno, M. Satoh, T. Yoshida, and Masao Takeuchi

Particle liberation from human megakaryoblastic leukemia cells (MEG-O1)

1) Osaka College of Health Science

**Annual Meeting on Microbial Taxonomy** (October, 1995, Kawaguchi-ko)

Mariko Takeuchi and A. Yokota<sup>1)</sup>

Taxonomic significance of chemotaxonomic features in Actinobacteria

1) Institute of Molecular and Cellular Biosciences, The University of Tokyo

**1st International workshop for DIVERSITAS in Western Pacific and Asia (DIWPA)**  
(Dec., 1995, Singapore)

A. Nakagiri, S.Y. Newell<sup>1)</sup>, T. Ito and T.K. Tan<sup>2)</sup>

Biodiversity and ecology of *Halophytophthora*

1) Marine Institute, University of Georgia

2) School of Biological Sciences, National University of Singapore

**Japan Society for Bioscience, Biotechnology, and Agrochemistry** (April, 1996, Kyoto)

Y. Nakagawa, T. Sakane and A. Yokota<sup>1)</sup>

Whether rod or coccus is not a generic criterion: emendation of the genus *Planococcus* and transfer of *Flavobacterium okeanokoites* Zobell and Upham 1944 to the



genus *Planococcus* as *Planococcus okeanokoites* comb. nov.

1) Institute of Molecular and Cellular Biosciences, The University of Tokyo

K. Ueda and K. Mikata

Phylogenetic relationships among species of *Dipodascus* and *Galactomyces* from 18S rDNA sequences

Y. Yamada<sup>1)</sup>, K. Hosino<sup>1)</sup>, Y. Tahara<sup>1)</sup> and K. Mikata

Phylogeny of *Pichia* yeasts based on partial rRNA sequences

1) Department of Agricultural Chemistry, Shizuoka University

Y. Yamada<sup>1)</sup>, T. Higashi<sup>1)</sup>, Y. Tahara<sup>1)</sup> and K. Mikata

Phylogeny of *Arthroascus*, *Botryoascus* and *Hyphopichia* yeasts based on partial rRNA sequences

1) Department of Agricultural Chemistry, Shizuoka University

#### **Mycological Society of Japan** (May, 1996, Tsukuba)

T. Ito and A. Nakagiri

Mycoflora of the rhizospheres of mangrove trees

A. Nakagiri and T. Ito

A new spathulosporealean fungus inhabiting a marine red alga, *Amphiroa zonata* (Corallinaceae)

I. Okane, A. Nakagiri and T. Ito

Flora of endophytic fungi in leaves of Ericaceae

#### **The Japanese Tissue Culture Association** (May, 1996, Hiroshima)

T. Yoshida, Y. Nakagaito, M. Satoh, and Masao Takeuchi

Differentiation of astrocyte progenitor cells that proliferate in the presence of EGF

H. Kuno, H. Ikeda<sup>1)</sup>, Masao Takeuchi, and T. Yoshida

Method for detection of retroviruses in cell lines at cell banks

1) Health Science Research Resources Bank

#### **The Annual Meeting of the Society for Actinomycetes Japan** (June, 1996, Unazuki)

K. Hatano and T. Nishii

Reclassification of whorl-forming streptomycetes

T. Tamura, M. Hayakawa<sup>1)</sup> and K. Hatano

Proposal of a new actinomycete with motile spore

1) Department of Fermentation Technology, Faculty of Engineering, Yamanashi University

**Japan Society for Culture Collections** (June, 1996, Osaka)

H. Ikeda<sup>1)</sup>, K. Tsuzurahara<sup>2)</sup>, H. Kuno, T. Yoshida, Masao Takeuchi, and K. Hashimoto<sup>3)</sup>

Preservation of microorganisms carrying recombinant DNA by L-drying

1) Health Science Research Resources Bank

2) Japan Health Sciences Foundation

3) National Institute for Health

T. Ito and A. Nakagiri

Viability of frozen cultures of basidiomycetes after fifteen-year storage

K. Mikata

Preservation of yeast cultures by L-drying: Viability of after 15 years storage

Mariko Takeuchi

Taxonomic study of *Sphingomonas* species

**Asian International Mycological Congress '96** (July, 1996, Chiba)

T. Ito

Preservation and maintenance of fungus cultures at the Institute for Fermentation, Osaka (IFO)

A. Nakagiri and T. Ito

Ecology of a versatile fungus, *Dactylella iridis*

**8th International Congress for Culture Collections** (August, 1996, Veldhoven)

T. Ito

Preservation of fungal cultures at the Institute for Fermentation, Osaka (IFO)

T. Ito

Preservation of fungus cultures by L-drying at the Institute for Fermentation, Osaka (IFO)

Masao Takeuchi, H. Kuno, H. Ikeda<sup>1)</sup>, and T. Yoshida

Detection of reverse transcriptase in cell cultures by polymerase chain reaction

1) Health Science Research Resources Bank

Masao Takeuchi

Recent activities of the IFO culture collection

**Japan Society for Cell Biology** (October, 1996, Kyoto)

Y. Nakagaito, M. Satoh, H. Kuno, Masao Takeuchi, A. Hakura<sup>1)</sup>, and T. Yoshida

Establishment of an EGF-dependent, neural progenitor cell line

1) Research Institute for Microbial Diseases, Osaka University

**The Pharmaceutical Society of Japan, Kinki Branch** (October, 1996, Hirakata)

M. Sakaguchi<sup>1)</sup>, K. Maruyama<sup>1)</sup>, E. Matsumura<sup>1)</sup>, M. Satoh, and Masao Takeuchi

Neurotrophic effect of  $\beta$ -casomorphin-5 in a mouse neuroblastoma cell line

1) Laboratory of Cell Biology, Osaka University of Pharmaceutical Sciences

**Annual Meeting on Microbial Taxonomy** (Nov. 1996, Kyoto)

T. Tamura

Phylogenetic study of actinomycetes based on 16S rRNA sequences

K. Ueda and K. Mikata

Phylogeny of *Dipodascus*, *Galactomyces* and *Geotrichum* and sequence heterogeneities among 18S rDNA operons

**3rd Internet World Congress on Biomedical Sciences** (December, 1996, Internet)

Rapid detection of retrovirus-derived reverse transcriptase in cell culture supernatant

H. Kuno, H. Ikeda<sup>1)</sup>, Masao Takeuchi, and T. Yoshida

1) Health Science Research Resources Bank

## Miscellaneous Scientific Papers, 1995–1996

R. Harasawa, H. Mizusawa, and Masao Takeuchi. 1995. Simple detection of the contamination in animal cell cultures. *Protein, Nucleic Acid, Enzyme* 40, 2361. [in Japanese]

Masao Takeuchi and T. Yoshida. 1995. Protocols for detection of microbial contamination in animal cell cultures. In: *Sosikibaiyou-no-gijyutu*, 3rd edition, (Kisohen). p 77–81. Asakura Shoten, Tokyo. [in Japanese]

A. Nakagiri. 1996. Labyrinthulids. In: *Report of Studies on Cellular Characteristics and Phylogeny of Chromista*. (ed. by I. Inouye), pp. 97–99. Kagaku Kenkyu-hi Kenkyu Seika Houkokusho (06304007). [in Japanese]

A. Nakagiri. 1996. Chap. 5. Umi-no-seibutsuso-no-seiritsu, 1. Kinrui. In: *Chibaken-no-Shizenshi*, Vol. 1 *Chibaken-no-Shizen* (ed. by M. Okiyama), pp. 199–205. Chiba Historical Materials Research Foundation. [in Japanese]

A. Nakagiri. 1996. Chap. 10. Umi-no-kinnrui, shokubutsu, 2. Kinrui. In: *Chibaken-no-Shizenshi*, Vol. 1 *Chibaken-no-Shizen* (ed. by M. Okiyama), pp. 409–416. Chiba Historical Materials Research Foundation. [in Japanese]

## Distributive Regulations

The Japanese government prohibits the transport of the following strains as plant pathogenic materials (IFO class A).

| Name of taxon                     | IFO No.     |
|-----------------------------------|-------------|
| Bacteria                          |             |
| <i>Burkholderia cepacia</i>       | 3739, 14074 |
| <i>Curtobacter flaccumfaciens</i> | 12156       |
| <i>Erwinia amylovora</i>          | 12687       |
| <i>Rhodococcus fascians</i>       | 12155       |
| <i>Streptomyces ipomoea</i>       | 13050       |
| Fungi                             |             |
| <i>Ceratocystis fagacearum</i>    | 7528        |
| <i>Ceratocystis ulmi</i>          | 6128        |
| <i>Coryneum carpophilum</i>       | 5908, 6910  |
| <i>Diplodia maydis</i>            | 6115        |
| <i>Nematospora coryli</i>         | 0658, 1220  |

The IFO suspends to export the following strains according to the Japanese law.

| Name of taxon                  | IFO No.   |
|--------------------------------|---|
| Fungi                          |   |
| <i>Aspergillus flavus</i>      | 30180   |
| <i>Aspergillus parasiticus</i> | 4082, 4301, 30179   |
| <i>Aspergillus taxicarius</i>  | 30108, 30109, 30110, 30111, 31250   |
| <i>Cochliobolus miyabeanus</i> | 4870, 5277, 5844, 6631, 7503, 9633  |
| <i>Pyricularia grisea</i>      | 5280, 6694, 7286, 7287, 9349, 9480,<br>30511, 30512, 30513, 30514, 30515,<br>30516  |
| <i>Pyricularia oryzae</i>      | 5279, 5994, 6193, 8771, 8773, 8774,<br>8775, 30517, 30518, 30519, 30520,<br>30625, 30726, 30727, 30729, 30730,<br>30731, 30732, 30733, 30734, 30735,<br>30736, 31175, 31176, 31177, 31178 |

## Corrections

In the issue of IFO Research Communications No. 17, the following corrections should be made.

| Page | Line | Type                     | Should read       |
|------|------|--------------------------|-------------------|
| 11   | 24   | 223,000                  | 22,300            |
| 18   | 7    | (1981)                   | (1881)            |
| 140  | 1    | <i>RTenibacterium</i>    | Renibacterium     |
| 142  | 8    | <i>Vibrio panaeicida</i> | Vibrio penaeicida |
| 143  | 21   | <i>arnobacterium</i>     | Carnobacterium    |

発酵研究所研究報告 第18号

平成9年3月1日 印刷

平成9年3月9日 発行 定価(含消費税) 1,300円

編集委員 上田 久美子、坂根 健、佐藤 邦子、  
武内 真理子、田村 朋彦、中垣内 弓子、  
中桐 昭、吉田 東歩

編集責任者 吉田 東歩

発行人 竹内 昌男

発行所 財団法人 発酵研究所  
大阪市淀川区十三本町2丁目17番85号

TEL. 06-300-6555

FAX. 06-300-6814

印刷所 日本印刷出版株式会社  
大阪市福島区吉野1丁目2番7号