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FULL PAPER

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Some characteristics of three groups in *Flammulina velutipes* classified by analysis of esterase isozymes

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Abstract Analysis of isozymes was carried out against wild and cultivated commercial stocks of *Flammulina velutipes* to analyze their genetic differences. Esterase isozymes from *F. velutipes* showed many bands and variations among the different stocks on the gel. The stocks of *F. velutipes* in Japan were largely classified into three groups (tentatively named groups A, B, and C) according to the cluster analysis of esterase isozymes. Some characteristics of the three groups were examined. Group C was characterized by a larger spore size, slower spawn running, and a paler pileus color than groups A and B. Furthermore, group B showed a smaller spore size, slower spawn running, and paler pileus color than group A.

Key words Analysis of isozyme · Cluster analysis · Esterase · *Flammulina velutipes*

Introduction

Electrophoretic comparison of proteins and enzymes has been used for studies of developmental processes and classification of basidiomycetes. Fruitful results have been reported concerning identification and discrimination of mushrooms such as *Coprinaceae* (Kitamoto et al. 1986), *Lentinula edodes* (Berk.) Pegler (Okunishi et al. 1979), and *Agaricus bisporus* (J. Lange) Imbach (Wang et al. 1991). In addition, Royse and May (1982) studied the isozymes of four enzymes for classifying stocks of *Agaricus brunnescens* Peck (= *Agaricus bisporus*). Ohmasa and Furukawa (1986) also reported that distribution analysis of the isozymes was useful for the discrimination of *Lentinula edodes*. However, there are few reports about the relationship between the results of discrimination of basidiomycetes by analysis of isozymes and their characteristics. This article concerns classification of various stocks of *Flammulina velutipes* (Curt.: Fr.) Sing. and the differences in some characteristics of each group classified by analysis of the isozymes.

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Materials and methods

Organisms

The stocks of *F. velutipes* used in this study are listed in Table 1. Most of the stocks were wild strains, and we collected these fruiting bodies ourselves during 1983–1999. Pure cultures were prepared by isolation from the tissues. All these stocks were maintained on potato dextrose agar medium (PDA) (Nissui Seiyaku, Tokyo, Japan). The mycelia of each stock had the capability of fruiting body formation and produced fruiting bodies easily in the cedar sawdust–rice bran medium (sawdust, 95 g dry wt; rice bran, 85 g dry wt; water, 300 g/800-ml bottle), which is generally used in commercial bottles for enokitake cultivation.

Culture conditions

Mycelia of each stock were cultured at 20°C on PDA medium in a 9-cm Petri dish. After covering the mycelia on the surface of the medium, the mycelia disks were prepared by punching with a cork borer (5 mm in diameter). The disks were inoculated into 40 ml MYS medium (0.1% malt extract, 0.05% yeast extract, 0.7% soyton) in 300-ml Erlenmeyer flasks and were incubated at 20°C for 20 days in the dark under static conditions.

Table 1. Stocks of *Flammulina velutipes*

Origin[a]	Stock no.
Japan	
Iwate Pref.	Iw-1, 2
Akita Pref.	Ak-1
Yamagata Pref.	Ym-1–3
Fukushima Pref.	Fk-1
Niigata Pref.	Ni-1
Toyama Pref.	Ty-1
Nagano Pref.	Na-1–123
Yamanashi Pref.	Ya-1
Tochigi Pref.	Tc-1–3
Ibaraki Pref.	Ib-1–4
Nara Pref.	Nr-1, 2
Tottori Pref.	Tt-1–4
Taiwan	
Shanlinshi	Tw-1
Alishan	Tw-2, 3
China	
Shanghai	Ch-1–6
Korea	
Suweon	Kr-1
Yesan	Kr-2
Thailand	
Chiang Mai	Ti-1
Norway	
Raenseter	Nw-1, 2
IFO[b]	IFO-1–7
Cultivated commercial stock[c]	CCS-1–19
Total	186

[a] All these stocks were collected by the authors from 1983 to 1999; pure cultures were isolated from the tissue. All these stocks were maintained on potato dextrose agar medium (Nissui Seiyaku)

[b] Institute for Fermentation, Osaka: IFO-1; IFO 7046, IFO-2; IFO 7777, IFO-3; IFO 30489, IFO-4; IFO 30494, IFO-5; IFO 30601, IFO-6; IFO 30875, IFO-7; IFO 30905

[c] All stocks were/or are cultivated in Nagano Prefecture: CCS-1; HATSUYUKI, CCS-2; SHINANO2, CCS-3; NAKANO, CCS-4; N2, CCS-5; K1 CCS-6; R2, CCS-7; JOSHO, CCS-8; INA, CCS-9; NAKANOJA, CCS-10; NAKANOJB CCS-11; TK, CCS-12; YOMASE1, CCS-13; HOKUTOM-50, CCS-14; SHINANO4 CCS-15; SHINANO6, CCS-16; SHINANO7, CCS-17; GARYU2, CCS-18; GARYU5 CCS-19; GARYU3

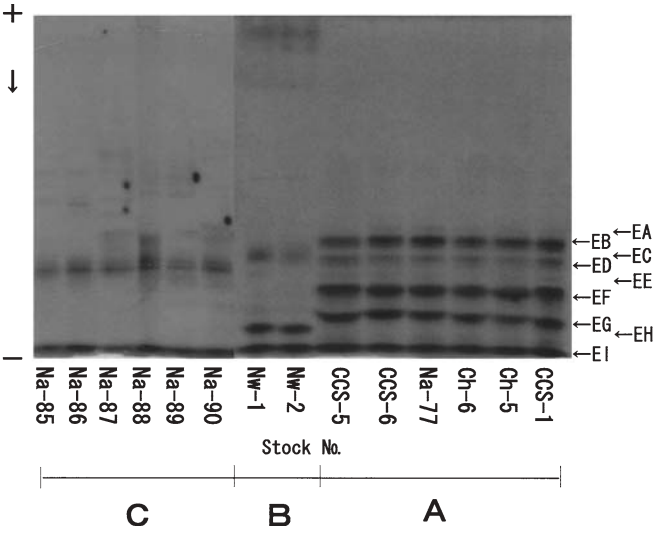

Fig. 1. Esterase isozyme patterns of the test stocks in *Flammulina velutipes*

Preparation of cell-free extracts

Mycelia were collected by filtration, washed with deionized distilled water, suspended in 0.05 M Tris-HCl buffer (pH 7.0), and then homogenized at 18000 rpm for 3 min with an Ultra Turrax homogenizer (Jahnke & Kunkel, Staufen, Germany) in an ice bath. The homogenate was centrifuged at 24000 *g* at 4°C for 30 min, and the resulting supernatant was used immediately for isozyme analysis.

Polyacrylamide gel electrophoresis and staining procedure

Polyacrylamide slab gel electrophoresis was carried out using an apparatus from Atoo (Tokyo, Japan). Enzymes were separated on a 7.5% slab gel (80 × 80 × 1 mm) using Tris-HCl buffer (pH 8.9, in gel) and Tris glycine buffer (pH 8.3, in electrode vessels) at constant 20 mA/gel in a 4°C cooling cabinet. Bromophenol blue (BPB) was used as a front marker. The extract of 0.02 ml (equivalent to each 200 mg dry mycelium/ml) prepared by the aforementioned procedure was applied to each slot of the gel. After electrophoresis, slab gels were removed from the glass cabinet, rinsed with cold distilled water, and incubated with appropriate staining mixtures for the activity staining of various enzymes. The staining and incubation conditions were those described by Kitamoto et al. (1986) for esterase (Est) and acid phosphatase (Acp), by Ohmasa and Furukawa (1986) for malate dehydrogenase (MDH) and leucin aminopeptidase (Lap), and by Sekine et al. (1994) for peroxidase (Px).

Cluster analysis

The distance values were defined as the dice coefficient between pairs of different banding patterns of enzymes. The dice coefficient was calculated by the following equation: $S = 1 - 2Nab/(Na + Nb)$ (where S is the dice coefficient; Nab the number of common bands; and Na and Nb, the total numbers of bands, respectively). The dendrogram based on the distance values was constructed by unweighted pair groups with arithmetric averaging (UPGMA) analysis using the computer package PHYLIP (Felsenstein 1994).

Some physiological and morphological characteristics

Linear growth of the mycelia of test stocks on PDA medium at 25°C was observed as one of the physiological characteristics. Fruiting bodies were obtained for the observation of some morphological characteristics by commercial bottle cultivation as follows: spawn running was performed in cultivation with 800-ml bottles containing the sawdust and rice bran medium (cedar sawdust, 95 g dry wt; rice bran, 85 g dry wt; water, 300 g/bottle) at 18°C and 60% relative humidity (RH) in the dark for 21 days. After incubation the bottles were placed at 15°C and 90% RH under intermittent illumination with 1200 lx alternated with the dark every

30 min for the initiation and development of the fruiting bodies. After fruiting bodies were matured, they were harvested; we then measured fruiting body yield, stipe length, and diameter and observed the shape and color of pilei. The size of basidiospores was measured under a microscope (Olympus BH-2).

Results and discussion

Analysis of isozymes

In a preliminary experiment, malate dehydrogenase (MDH), esterase (Est), leucin aminopeptidase (Lap), acid phosphatase (Acp), and peroxidase (Px) isozymes were examined for several stocks that have different morphological characteristics (data not shown). Among isozyme patterns of different stocks used in this study, these enzymes except for esterase did not show distinct differences in the number and position of bands by activity staining. The esterase isozyme patterns were quite different among the test stocks of *F. velutipes*. It is considered that esterase isozyme analysis is most useful for the discrimination of *F. velutipes* stock cultures. Therefore, the analysis of EST isozymes was carried out for 186 stocks. Figure 1 shows typical electrophoresis patterns of EST isozymes. Nine main bands (EA–EI) were observed with some faint bands. From these results, 186 stocks seemed to classify into three groups (A, B, and

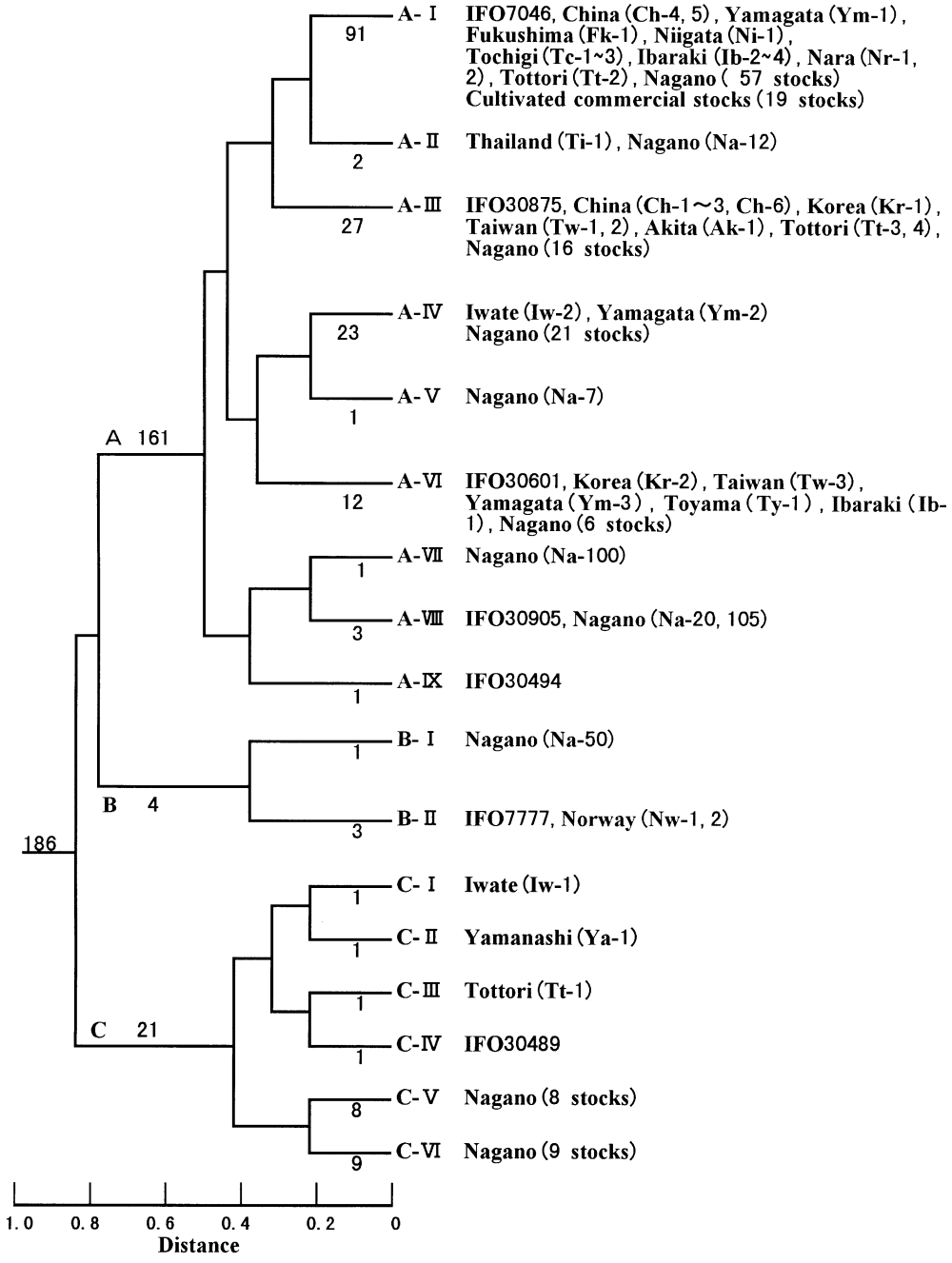

Fig. 2. Dendrogram of *F. velutipes* from esterase isozyme patterns. The dendrogram, based on the distance values, was constructed by unweighted pair groups with arithmetric averaging (UPGMA) analysis using the computer package PHYLIP (Felsenstein 1988)

C) according to the distribution of the nine bands. EB and ED bands were observed in all stocks of the group A, whereas the EH band was peculiar to group B. The EE band was also specific to group C, and the EI band was common to all stocks.

Cluster analysis

To classify the 186 stocks, the similarity of esterase isozyme patterns about the nine bands was evaluated by cluster analysis (Fig. 2). From this analysis, 186 stocks were largely divided into three groups (A, B, and C). Of these, 161 (87%) stocks belonged to group A, including all 19 stocks of commercial cultivars and 17 stocks from China, Korea, Taiwan, Thailand, and IFO. On the other hand, 4 stocks (IFO 7777, Nw-1, Nw-2, and Na-50) belonged to group B. One stock of these was from Nagano Prefecture in Japan and the others were from North Europe (IFO 7777 was of Swedish origin; Yokoyama 1991). In addition, 21 stocks belonged to group C, including each of the stocks from Iwate, Yamanashi, and Tottori Prefectures, respectively, and the other 17 stocks from Nagano Prefecture and IFO 30489. It was also reported that the stocks of *L. edodes* (Ohmasa and Furukawa 1986; Fukuda and Tokimoto 1991) and *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (Matsumoto et al. 1995) showed several isozyme patterns by esterase isozyme analysis. In the natural populations of *L. edodes* and *P. ostreatus*, the results of the cluster analysis derived from the isozyme patterns were coincident with geographic distances and genetic differences (Fukuda and Tokimoto 1991; Matsumoto et al. 1995). However, in the natural population of *F. velutipes*, the cluster analysis did not coincide with geographic distances. The commercial stocks of Nagano Prefecture were indistinguishable by esterase isozyme patterns, as these stocks belonged to the same cluster of group A.

Differences in some characteristics of test stocks

It was reported that the size and shape of spores were important characters in *Flammulina* (Bas 1983). We observed the size of the basidiospores of the groups A, B, and C to be $7 \pm 0.2 \times 3 \pm 0.14\,\mu m$, $5.5 \pm 0.31 \times 4 \pm 0.21\,\mu m$, and $9 \pm 0.25 \times 4 \pm 0.22\,\mu m$, respectively (Fig. 3). The shape of the ellipse was distinctly different among each group. Imazeki and Hongo (1987) reported that the size of basidiospores of *F. velutipes* was $5–7.5 \times 3–4\,\mu m$, and these values were the same as those of group A and B in this experiment. However, the spore size of group C was larger than these values reported previously. From this result, it is suggested that the group C may be a specific group.

Some characteristics of the *Flammulina* groups were also examined in yields of fruiting bodies, diameter, shape, and the color of pilei in addition to the days required for spawn running. Some characteristics such as the days required for spawn running and pileus color differed among groups. The days required for spawn running and pileus color of groups A, B, and C were 18.5 ± 1.6 days light brown to brown; 19.6 ± 0.7 days light yellow to light brown; and 20.5 ± 1.0 days pale yellow, respectively. Efficient comparison of dikaryotic stocks for their agronomic traits can be achieved with the use of principal component analysis (Kinugawa 1993). A Z-score diagram from the principal component analysis of some phenotypic values of *F. velutipes* is shown in Fig. 4. The slower spawn running was scattered from right to middle in the Z-score diagram and the paler pileus color was from top to bottom. The stocks used in this study were scattered diagonally from right upper to middle bottom. Stocks of group A were scattered at middle bottom (the second quadrant), stocks of group C were at right upper (the first quadrant), and stocks of group B were in the middle of groups A and C. From these results, it was characterized in group C that the spawn running was slower and pileus color was paler than that of group B, and in group B that spawn running was slower and pileus color was paler than that of group A.

Investigators have described additional taxa within the genus based on morphology or mating studies (Klan and

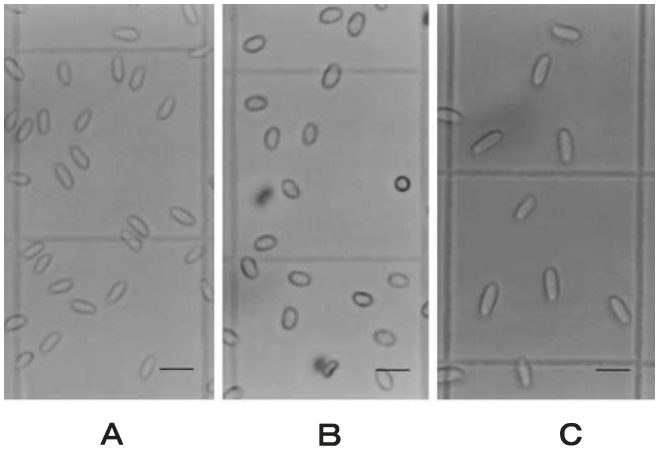

Fig. 3. Three type of basidiospores of *F. velutipes.* **A** $7 \times 3\,\mu m$ on average; **B** $5.5 \times 4\,\mu m$ on average; **C** $9 \times 4\,\mu m$ on average. *Bars* $10\,\mu m$

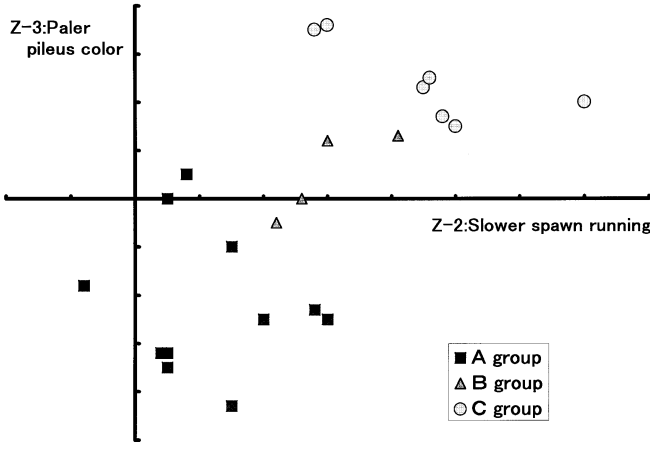

Fig. 4. Z-score diagram from principal component analysis of some phenotypic values of *F. velutipes.* Z2–Z3, Cumulative proportion of total variance is 23%

Baudisova 1992; Bas 1995; Petersen et al. 1999). In Europe and North America, the genus *Flammulina* Karst. included more than ten species (Methven et al. 2000). However, there have been few reports about the genus *Flammulina* Karst. in Japan. From our results, *F. velutipes* in Japan were classified at least into three groups. Further research on morphology and mating characteristics might provide more information about Japanese *Flammulina*.

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