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

Kenichi Nishizawa · Yoshikiko Amano · Kouichi Nozaki
Nami Hosokawa · Masahiro Shiroishi · Takahisa Kanda

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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.

**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, 95g dry wt; rice bran, 85g dry wt; water, 300g/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 (5mm in diameter). The disks were inoculated into 40ml 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.
Preparation of cell-free extracts

Mycelia were collected by filtration, washed with deionized distilled water, suspended in 0.05M Tris-HCl buffer (pH 7.0), and then homogenized at 18 000rpm for 3min with an Ultra Turrax homogenizer (Jahnke & Kunkel, Staufen, Germany) in an ice bath. The homogenate was centrifuged at 24 000g at 4°C for 30min, and the resulting supernatant was used immediately for isozyme analysis.

Polyacrylamide gel electrophoresis and staining procedure

Polyacrylamide slab gel electrophoresis was carried out using a apparatus from Atoo (Tokyo, Japan). Enzymes were separated on a 7.5% slab gel (80/11003 80/11003 1mm) 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 200mg 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 arithmetic 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, 300g/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 1200lx alternated with the dark every