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Alternation of nuclear phase in the filamentous basidiomycete, *Helicobasidium mompa*

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Variation in the number of nuclei and cellular ploidy were observed in eight strains of *Helicobasidium mompa*. The basidiospores, single-spore isolates and field-isolated strains were all dikaryons. The cellular ploidy, which was assessed by analyzing the fluorescence emitted by DAPI-stained nuclei, was unstable: monokaryotic strains derived from the original dikaryotic strains by successive subcultures were mainly tetraploid, although the original dikaryon was in most cases diploid. On the other hand, a dikaryotic strain derived by treatment with benomyl was haploid. These results suggest that diploid dikaryon is a normal nuclear phase of *H. mompa* in nature, and the alternation of ploidy may be due to a feature of the mating system of this fungus.

Key Words——cellular ploidy; cytology; fluorescent microscopy; haploidaization; *Helicobasidium*.

Mating between compatible monokaryons of the tetrapolar filamentous basidiomycete *Coprinus cinereus* (Schaeffer ex Fries) S. F. Gray involves dikaryotization and clamp-cell formation, followed by development of fruiting bodies, meiosis and basidiospore formation. The number of nuclei and their ploidy are generally stable during mycelial growth, except during development of fruiting bodies and meiosis (Wong and Gruen, 1977; Casselton and Olesnicky; 1998; Kües, 2000), though subculturing the bipolar filamentous basidiomycete *Pholiota nameko* (T. Ito) S. Ito et Imai frequently induces dedikaryotization of the dikaryotic mycelium (Masuda et al., 1995; Kumata et al., 1995). Moreover, strains containing both diploid and haploid nuclei have been reported for *Cryptococcus neoformans* (Sanfelice) Vuillemin, a bipolar basidiomycete yeast, and shifts in cellular ploidy occur widely (White and Tacobson., 1985; Tanaka et al 1999; Hata et al., 2000). These phenomena suggest that the number of nuclei of dikariotic mycelium in bipolar basidiomycetes is less stable than in tetrapolar basidiomycetes, regardless of whether the mating type locus is a multiple allele. This may be because certain mating-type genes associated with nuclear migration, present in tetrapolar basidiomycetes, are lacking in bipolar basidiomycetes (Casselton and Olesnicky 1998; Kües, 2000).

The filamentous basidiomycete *Helicobasidium mompa* Tanaka is a commercially important, soil-borne, root pathogen affecting a wide range of plant species. It is the causal agent of purple root rot disease, and host plants infected by the fungus quickly wither and die. In Japan, this fungal disease, which spreads rapidly and is very difficult to prevent, has done great damage to commercially grown grape vines and apple and pear trees. Unfortunately, despite its importance, little is known about the cytology and genetics of this fungus.

Investigation of the cellular ploidy of *H. mompa* is very important for understanding its pathogenesis and mating system. Pulsed-field electrophoresis is a good tool for differentiating chromosomes among strains, especially with respect to molecular size (Zolan and Pukkila, 1995), but it remains difficult to determine the number of chromosomes of this fungus, because of the difficulty of producing protoplasts. In this report, we describe the ploidy of several *H. mompa* strains and the occurrence of shifts in ploidy, which were examined by semiquantitative fluorescence microscopy after nuclear staining. This is the first report dealing with a cytological analysis of *H. mompa*; and in particular, mating type and the nuclear migration system of this fungus are discussed.

Material and Methods

Strains and culture conditions The fruiting body of *Helicobasidium mompa* developed on an apple tree was kindly provided by Prof. Yukio Harada (Hirosaki University, Japan). The basidiospores were collected by washing the fruiting body with sterile water and then subjected to microscopic analysis. The strains of *H. mompa* used in this study (Table 1) were originally obtained from Dr. Naoyuki Matsumoto (National Institute of Agro-Environmental Science, Japan) and maintained on 1/5 OMA medium (oat meal 12 g/L, agar 20 g/L). To isolate haploid strains, monokaryotic strains were treated with haploidizing reagents, benomyl (1.0 μg/ml), para-

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Table 1. *Helicobasidium mompa* strains used in this study.

Strain	Nuclear Phase	Characteristics	Source
V468	dikaryon	single basidiospore culture	Dr. Matsumoto
V1M	monokaryon	derived from original dikaryon strain V1	Dr. Matsumoto
V3M	monokaryon	derived from original dikaryon strain V3	Dr. Matsumoto
V4M	monokaryon	derived from original dikaryon strain V4	Dr. Matsumoto
V16	dikaryon	field-isolated original dikaryon strain	Dr. Matsumoto
V16M	monokaryon	derived from original dikaryon strain V16	Dr. Matsumoto
V16Mben1	dikaryon	derived from V16M strain by treatment with benomyl	This study
V243M	monokaryon	derived from original dikaryon strain V243	Dr. Matsumoto

-fluoro-DL-phenylalanine (100 μg/ml), and chloral hydrate (1.5 mg/ml), for 2 wk on 1/5 OMA agar plates. When a sector appeared, it was transferred along with the agar block to another 1/5 OMA plate and maintained.

Nuclear staining and measurement of fluorescence intensity *Helicobasidium mompa* strains were grown on 1/5 OMA at 25°C for 1 wk, after which the mycelium, along with a square agar block (15 × 15 mm), was transferred onto glass slide and stained with 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole) solution dissolved in 1 × PBS buffer (8 g NaCl, 0.2 g KCl, 2.9 g Na_2HPO_4, 0.2 g KH_2PO_4 /L). Images of the fluorescence emitted from by DAPI-stained nuclei were obtained using a Nikon ECLIPSE E600 fluorescence microscope, recorded with a Hamamatsu Photonics C4742-95 CCD camera, and stored on an Apple Macintosh G3 computer. The fluorescence intensities of the nuclei in the recorded images were measured using Argus-fish-PPC software (Hamamatsu Photonics, Hamamatsu Japan) according to the manufacturer's instructions. To minimize error within the recorded images, fluorescence emitted from the cytoplasm of DAPI-stained mycelia was normalized with respect to the average intensity in each image; total fluorescence intensity was then expressed in arbitrary units calculated per unit area.

Results and Discussion

Analysis of the number of nuclei in a single cell To better understand the cytological characteristics of *H. mompa*, the number of nuclei in single cells and their basidiospores were determined. Basidiospores that developed from the fruiting body of *H. mompa* on an apple tree (Fig. 1) and their DAPI-stained nuclei are shown in Fig. 2. Typical basidiospores each contained two nuclei. We also examined strain V468, which was a single-spore isolate, and strain V16, which was isolated from the field. In each of these strains, two nuclei were observed between two septa of the hyphae, indicating that the single-spore isolate and the strain from nature were both dikaryotic (Fig. 3A and B), and suggesting that dikaryoic hyphae germinate from dikaryotic basidiospores in a manner similar to that seen in *Agaricus bisporus* (Lange) Singer (Saksena et al., 1976).

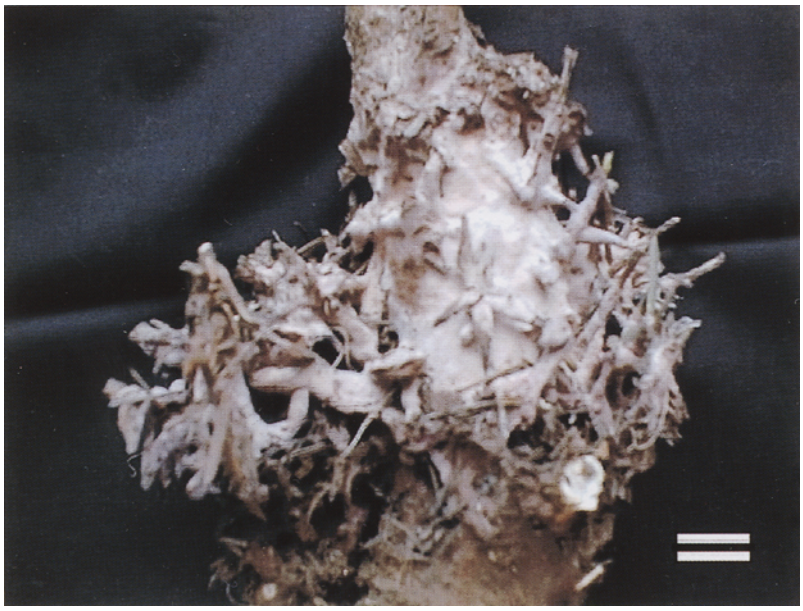

Fig. 1. Fruiting body of *H. mompa* on an apple tree. Scale bar: 1 cm

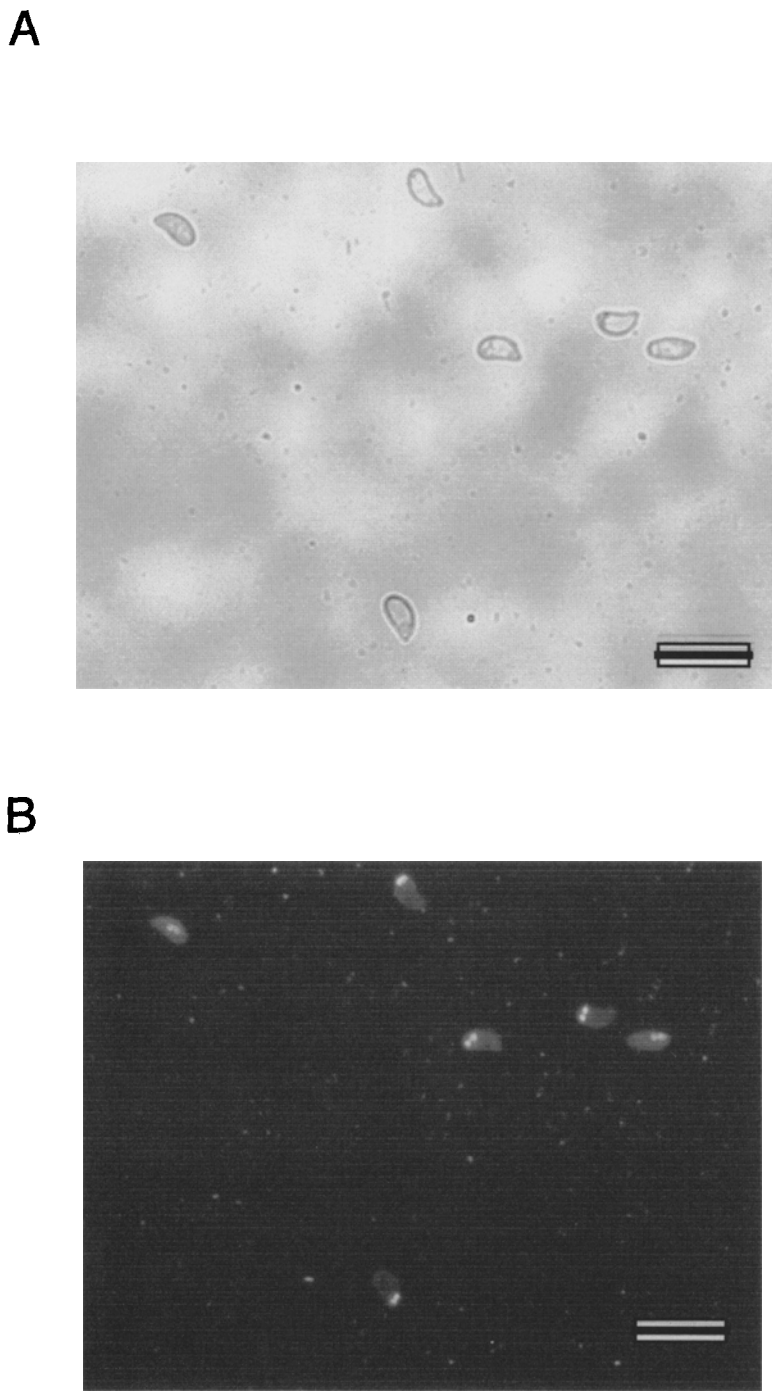

Fig. 2. Basidiospores of *H. mompa*. A, Light microscopy. B, Fluorescence microscopy after staining with DAPI. Scale bar: 10 μm.

Successive subcultures of strain V16, however, yielded a monokaryotic strain, which was named V16M (Fig. 3C). Such dedikaryotization was also observed in other strains, with monokaryotic strains V1M, V3M, V4M and V243M being derived by successive subcultures from the original dikaryotic strains V1, V3, V4 and V243, respectively. Similar dedikaryotization by subculture was previously reported in *P. nameko*, which is known to be a bipolar filamentous basidiomycete (Masuda et al. 1995; Kumata et al., 1995). Clamp-cell formation was not detected in any of the *H. mompa* strains used in this study, suggesting that *H. mompa* may be a bipolar filamentous basidiomycete that is unable to form clamp cells and maintain itself as a dikaryon. It seems likely, therefore, that *H. mompa* either lacks or is defective in one or more of the genes related to stabilization and migration of nuclei (e.g., the A and B genes) that are present in the tetrapolar filamentous basidiomycete *C.*

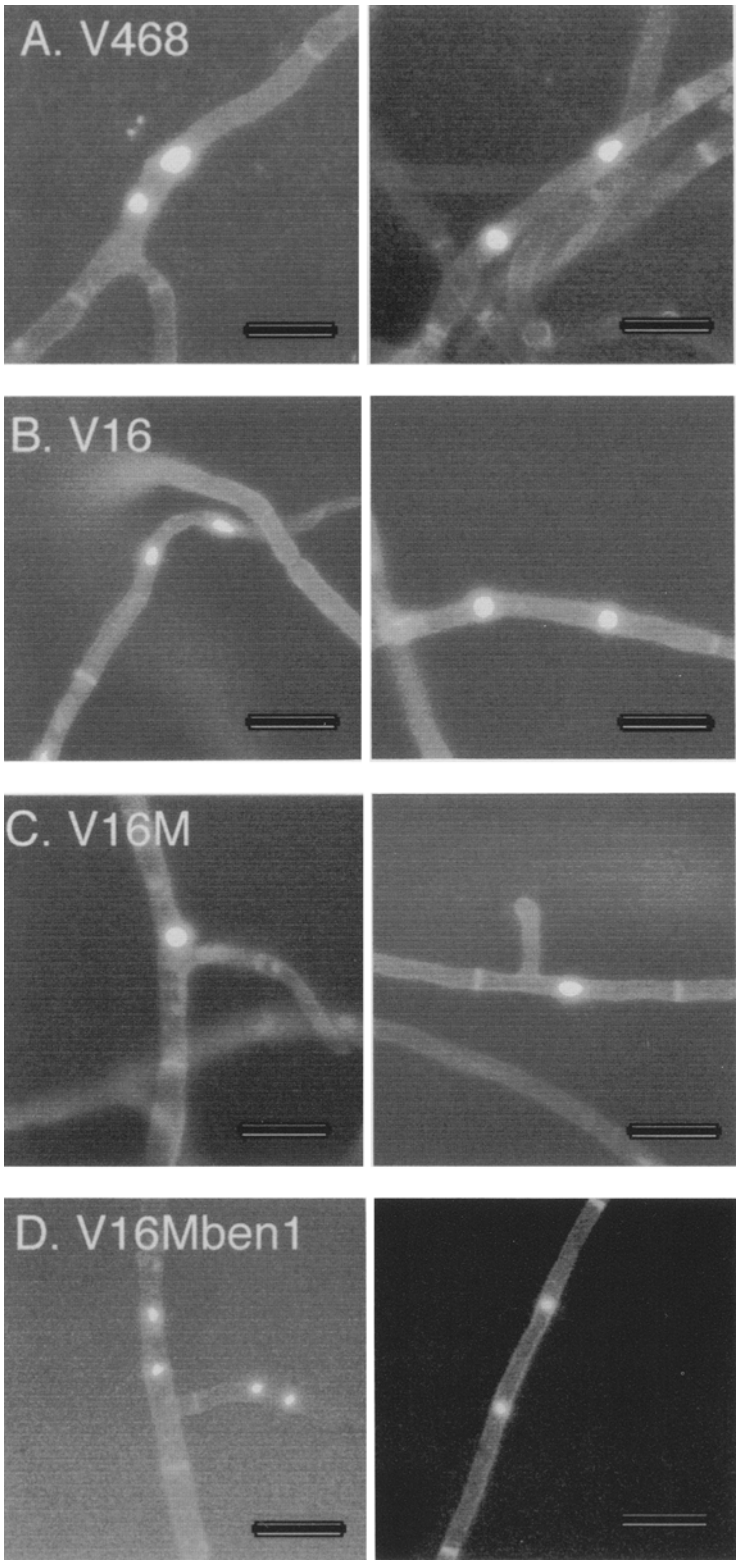

Fig. 3. Fluorescence microscopy of nuclei after staining with DAPI in *H. mompa* strains. A, V468 strain. B, V16 strain. C, V16M strain. D, V16Mben1 strain. Scale bar: 10 μm.

cinereus (Casselton and Olesnicky, 1998).

Haploidization To investigate whether any of the monokaryotic strains were haploid, strain V16M was treated on 1/5 OMA agar plates with haploidizing agents.

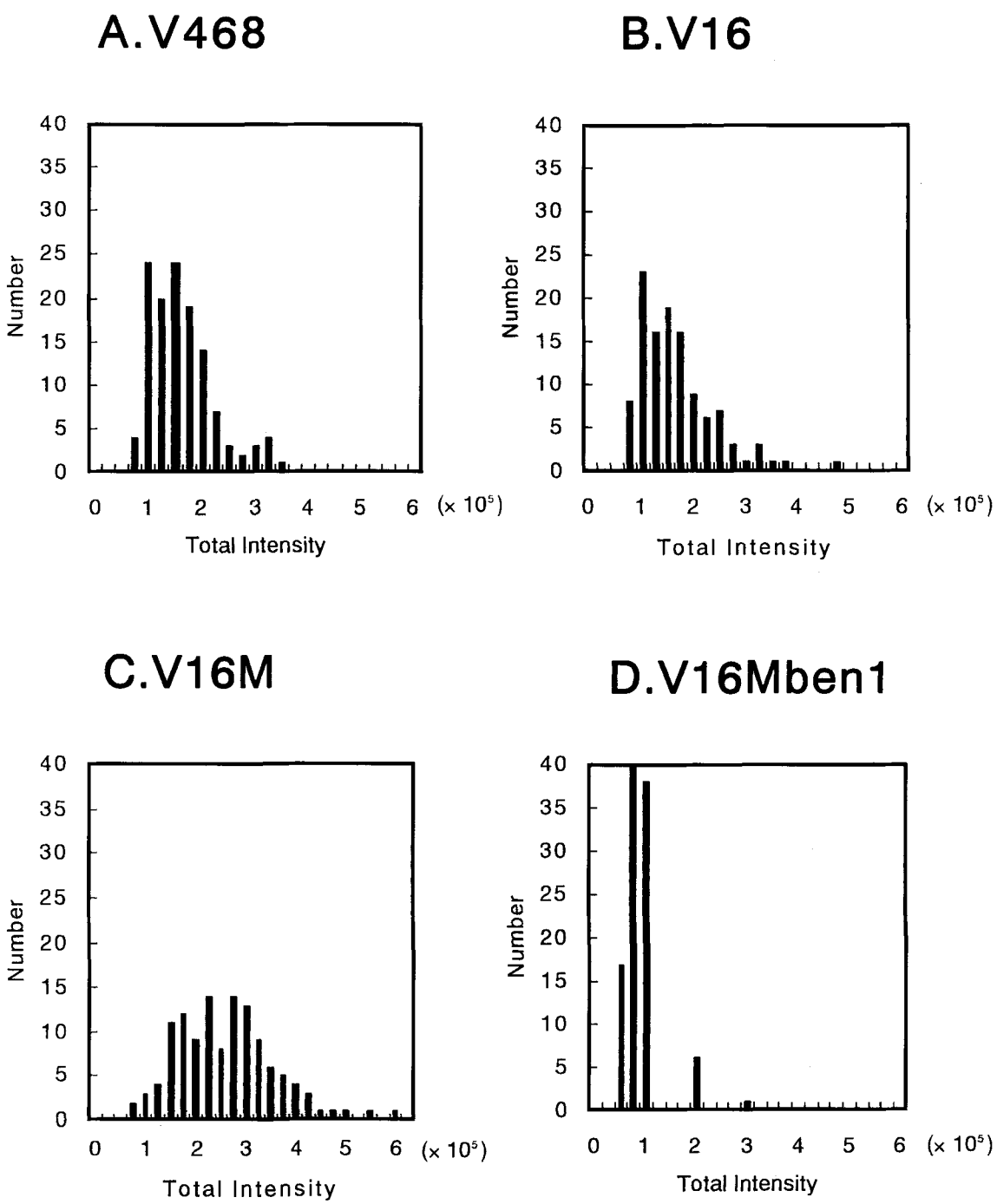

Fig. 4. Distribution of fluorescence intensity of DAPI-stained nuclei in strains of various ploidy. Total intensity is expressed in arbitrary units. A, strain V468; B, strain V16 strain; C, strain V16M; D, strain V16Mben1. Total numbers of nuclei analyzed are shown in Table 2.

Of these, benomyl (1 μg/ml) produced a sector in which the mycelium was colored brown and was narrower than that in the surrounding area (data not shown). A piece of mycelium from this sector was isolated and maintained on a 1/5 OMA plate as new strain, named V16Mben1. Fluorescence microscopic analysis revealed the newly derived strain to be dikaryotized (Fig. 3D), and its nuclei stained less intensely with DAPI than nuclei from strain V16M (Fig. 4C, D; Table 2), suggesting strongly that strain V16Mben1 was haploid. This result indicates that the monokaryotic strain V16M, derived from a dikaryotic strain, is polyploid (Table 2; Fig. 4C). It is also very likely that the dedikaryotization does not represent the loss of one of the two nuclei from the dikaryotic strain; rather the monokaryotic strain arose as a consequence of nuclear fusion.

Table 2. Average total intensity of nuclei stained with DAPI in strains of different ploidy.

Strain	No. of nuclei analyzed	Mean[a]	S.D.[b]	Ratio
V16M	122	248459	96169	1
V16	114	151865	70374	0.611
V16Mben	102	63509	44476	0.256
V468	125	152063	58316	0.612

[a] Total intensity is expressed in arbitrary units.
[b] Standard deviation.

Table 3. Average total intensity of nuclei stained with DAPI in different monokaryotic strains.

Strain	No. of nuclei analyzed	Mean[a]	S.D.[b]	Ratio
V16M	122	248459	96169	1
V1M	102	222543	87503	0.896
V3M	102	257920	133952	1.03
V4M	97	207390	92439	0.835
V243M	103	188959	62701	0.761

[a] Total intensity is expressed in arbitrary units.
[b] Standard deviation.

Fluorescence microscopic analysis of ploidy The ploidy of *H. mompa* strains was estimated by measuring the fluorescence intensity of their DAPI-stained nuclei. About 100 nuclei from each strain were imaged and analyzed; their distributions and average fluorescence intensities are shown in Fig. 4 and Table 2, respectively. The fluorescence intensity of the nuclei from strain V16Mben1 were only about 25% of that from strain V16M and nearly 60% of that from strain V16. The fluorescence for strain V16Mben1 ranged from about 0.5×10^5 to 1.5×10^5, whereas that from strain V16M was mainly from about 1.5×10^5 to 3.5×10^5, and that from strain V16 was mainly from about 1.0×10^5 to 2.0×10^5. Given that strain V16Mben1 is a haploid dikaryon, the respective fluorescence intensities of their nuclei indicate that strains V16 and V468 are mainly diploid and strain V16M is mainly tetraploid. However, the range of intensities of the nuclei in these strains was somewhat wider than that in strain V16Mben1. This suggests that ploidy is not stable in this fungus; that other types of cellular ploidy (e.g., haploid and triploid) may occur in putatively diploid and tetraploid strains; and that the nuclei in single-spore isolates are mainly diploid. These phenomena are consistent with the idea that, because clamp cell formation is absent in these fungi, shifts in cellular ploidy may occur in order for each nucleus to correctly migrate in the mycelium. If so, the diploid dikaryon may be a normal nuclear phase of *H. mompa* in nature.

The nuclear fluorescence intensity of nuclei in strain V468, a single spore isolate, showed a similar distribution pattern and mean value to the intensity. In addition, the distributions and average nuclear fluorescence intensities of other derived monokaryotic strains were very similar to those of strain V16M (Fig. 5; Table 3). One exception was strain V243M, but the difference in nuclear intensity in that strain reflects the fact that it is, in part, a diploid dikaryon. Thus, the shift in cellular ploidy and dedikaryotization due to nuclear fusion is characteristic of *H. mompa*, and might be a common feature in fungi that do not exhibit clamp cell fusion.

In addition to examining the presence of a polyploid strain and the instability of the cellular ploidy and number of nuclei, we isolated a putative haploid strain of *H. mompa*, but we were unable to derive monokaryons from all of the isolated dikaryon strains. At present, we do not know the reason why the cellular ploidy of some dikaryotic strains are unstable and others are stable, because the mating system in *H. mompa* is not yet completely understood.

Various specific mating-type genes regulate clamp cell formation and fusion and nuclear migration in tetrapolar basidiomycetes; these include the pheromone, pheromone receptor, and homeobox genes (Casselton and Olesnicky, 1998). Monokariotization of the dikaryotic mycelia of *P. nameko*, a bipolar basidiomycete, has been reported (Masuda et al., 1995), though *P. nameko* can produce clamp cells, and apparently there is no shift in ploidy in the monokariotized fungus. These phenomena suggest that the system for mating and nuclear migration in *H. mompa* differs from that in bipolar basidiomycetes.

A mating-type-like locus, homologous to that of the ascomycetes yeast *Saccharomyces cerevisiae*, has been cloned from the asexual pathogenic yeast *Candida albicans* (Hull and Johnson, 1999), and polyploidy has been reported for some strains (Hubbard et al., 1985; Mayer et al., 1992). Thus, the mating system of *H. mompa* may be similar to that of filamentous ascomycetes fungi, such as the yeasts (Coppin et al., 1997). Further study of the cellular and molecular biology of *H. mompa*, aimed at a better understanding of heterokaryosis and the mating system and nuclear migration of this fungus should help resolve these outstanding issues.

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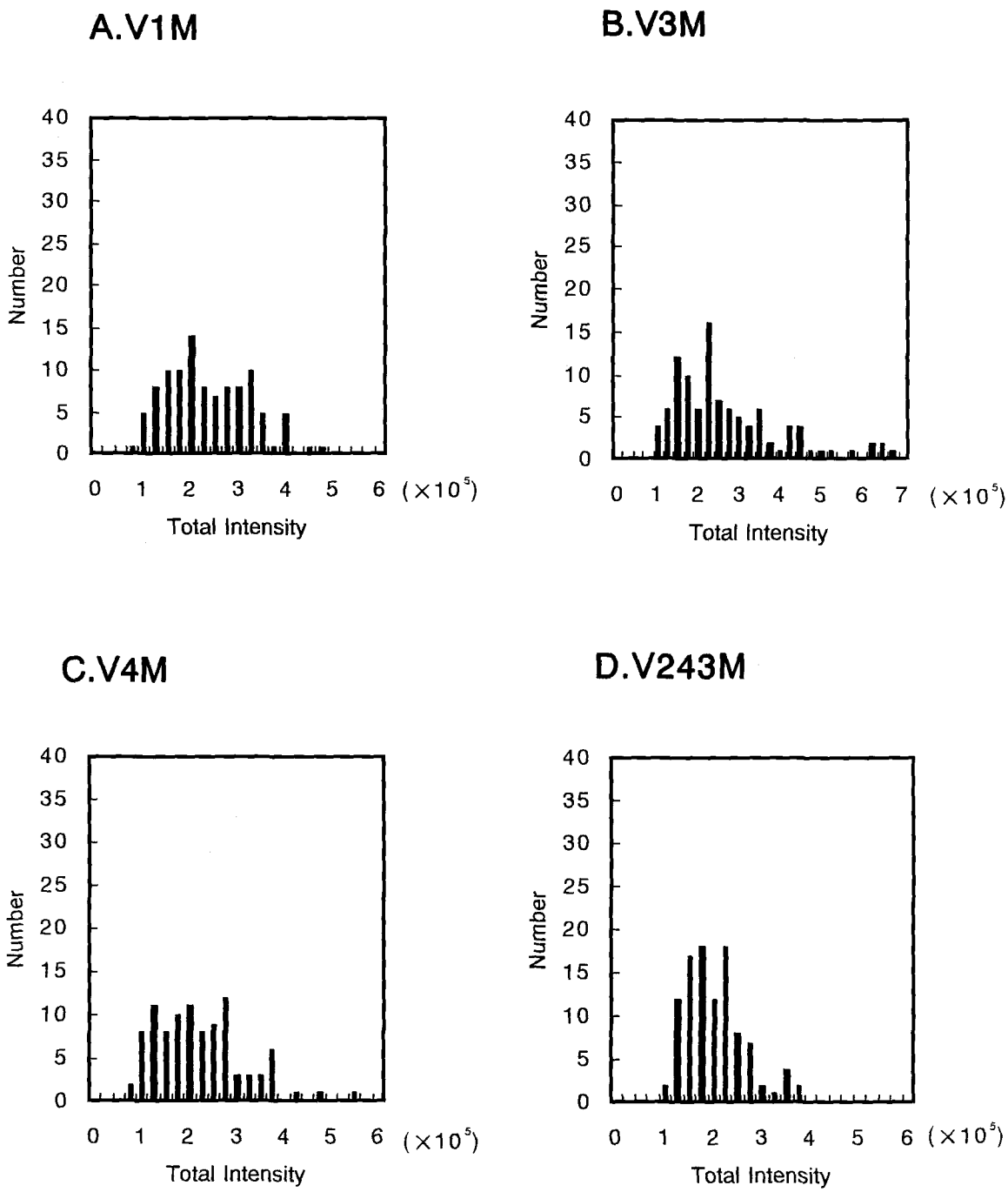

Fig. 5. Distribution of fluorescence intensities of DAPI-stained nuclei in derived monokaryon strains. Total intensity is expressed in arbitrary units. A, strain V1M; B, strain V3M; C, strain V4M; D, strain V243M. Total numbers of nuclei analyzed are shown in Table 3.

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