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Cell form, function and lineage in the hymenia of *Coprinus cinereus* and *Volvariella bombycina*

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A hymenial cell initial was binucleate in *Coprinus cinereus* but uninucleate in *Volvariella bombycina*. Paraphyses and facial cystidia remained binucleate in *C. cinereus*, but marginal cystidia were the apical cells of branches from the multinucleate gill trama, becoming swollen when gills pulled away from their connection to the stipe, and were multinucleate. In *V. bombycina*, all mature cystidia were multinucleate but as a result of multiple mitoses. Meiotic DNA synthesis, as determined by fluorescence microspectrophotometry on DAPI-stained nuclei, was found to take place before karyogamy in *C. cinereus* and possibly after karyogamy in *V. bombycina*. The nature of fungal cell differentiation is discussed, and comparisons are made with published information about hymenium structure of *Agaricus bisporus*.

The gill of *Coprinus* is composed of highly differentiated cells with what appear to be distinct and recognizable functions – the morphological differentiation signalling the functional differentiation. But in many agarics such morphological differentiation is lacking, and the less obvious functions have to be deduced from other features. For example, we have recently demonstrated that a large proportion of the basidia remain in meiotic prophase throughout the lifetime of *Agaricus bisporus* (Lange) Imbach basidiomes (Allen, Moore & Elliott, 1992), implying that basidia in an arrested meiotic prophase serve a structural function in this species. In other species this function is served by sterile non-meiotic cells, none more recognizable than the paraphyses of *Coprinus*. These latter arise as branches from cells immediately beneath basidia and insert into the young hymenium (Rosin & Moore, 1985*b*), providing an example of differentiation in a defined temporal sequence. The analysis of the *Agaricus* hymenium by Allen *et al.* (1992) raises the question of how best to characterize hymenial elements, since their functions involve both the form of the cell and the behaviour of the nuclei. In this work we combine analysis of cell morphology and nuclear characteristics to compare ontogeny and nuclear behaviour in components of the highly differentiated hymenium of *C. cinereus* (Schaeff.: Fr.) S. F. Gray, on the one hand with morphologically similar cells produced as a wound response when primary gills in *Coprinus* are torn away from the stipe, and on the other hand with the hymenium of the homothallic species *Volvariella bombycina* (Schaeff.: Fr.) Sing.

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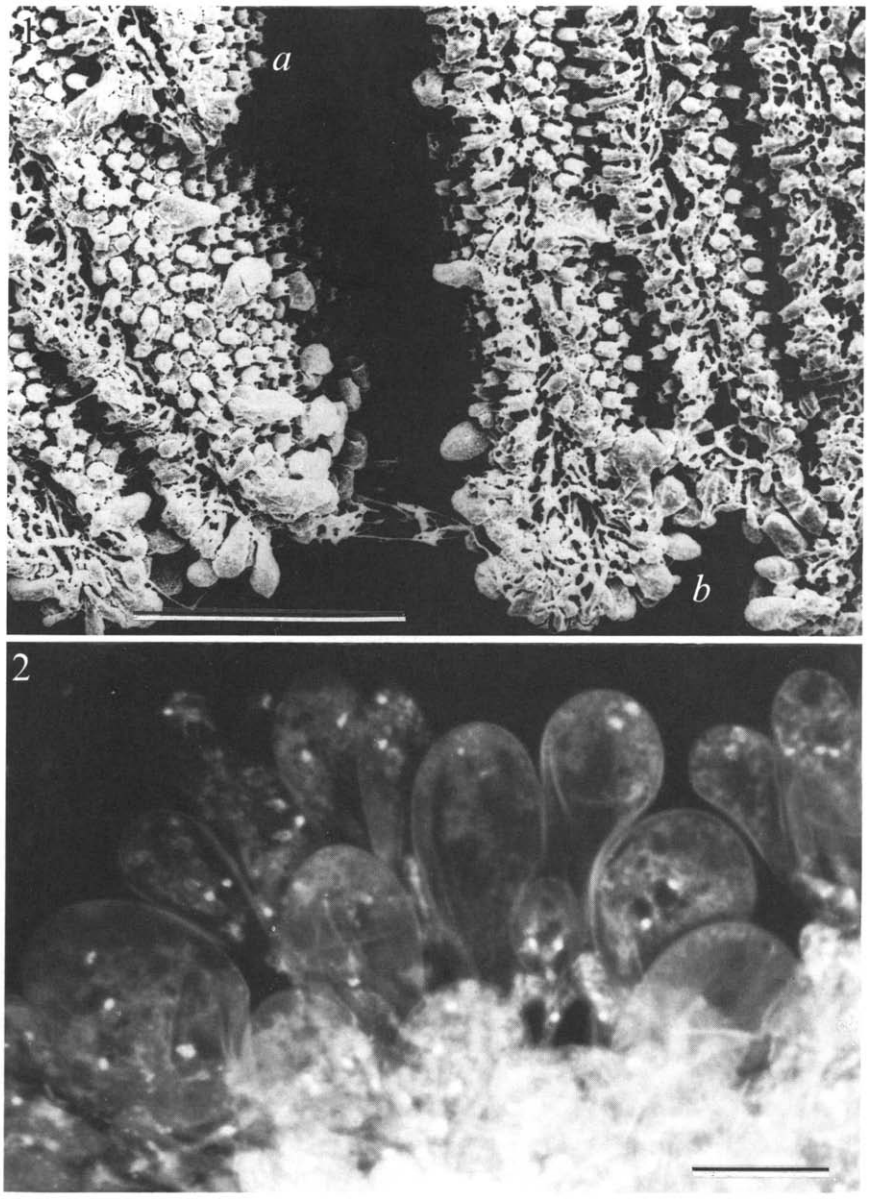
METHODS AND MATERIALS

Coprinus cinereus strain Meathop and *Volvariella bombycina* strain Vo-1 were maintained on MY medium (Rao & Niederpruem, 1969) and incubated at 26 °C. Fruiting was induced by illuminating a fully colonized plate with a 12 h dark/12 h light cycle.

Nuclei in squashed gills were stained with silver (Pukkila & Lu, 1985) or propionic/iron alum/haematoxylin (Henderson & Lu, 1968) and examined with bright-field microscopy, or stained with 4′,6-diamidino-2-phenylindole (DAPI) or ethidium bromide (EB) (Chiu & Moore, 1988*b*) and examined by fluorescence microscopy. DAPI was dissolved in Na/K phosphate buffer (pH 6·9) and used to stain a live (i.e. unfixed) squash preparation of a small piece of gill. The specimen was then mounted in 50% (v/v) aqueous glycerol to retard fading during observation. Nuclear DNA content was determined by measuring the intensity of the DAPI-stained nuclei with a Nikon Microphot epifluorescence microscope equipped with a photometer system. The photometer was zeroed on a field of view free of cells. Some specimens were chemically processed (Chiu & Chang, 1987) and coated with gold before examination with a Cambridge 200 SEM. Analysis of variance (ANOVA) was used to detect any significant difference among nuclear DNA contents at different developmental stages (at $P < 0{\cdot}05$), then the t test was applied to test the significance of differences between two means (at $P < 0{\cdot}05$).

RESULTS AND DISCUSSION

Coprinus cinereus and *Volvariella bombycina* have been used to study many aspects of basidiome morphogenesis (Reijnders,

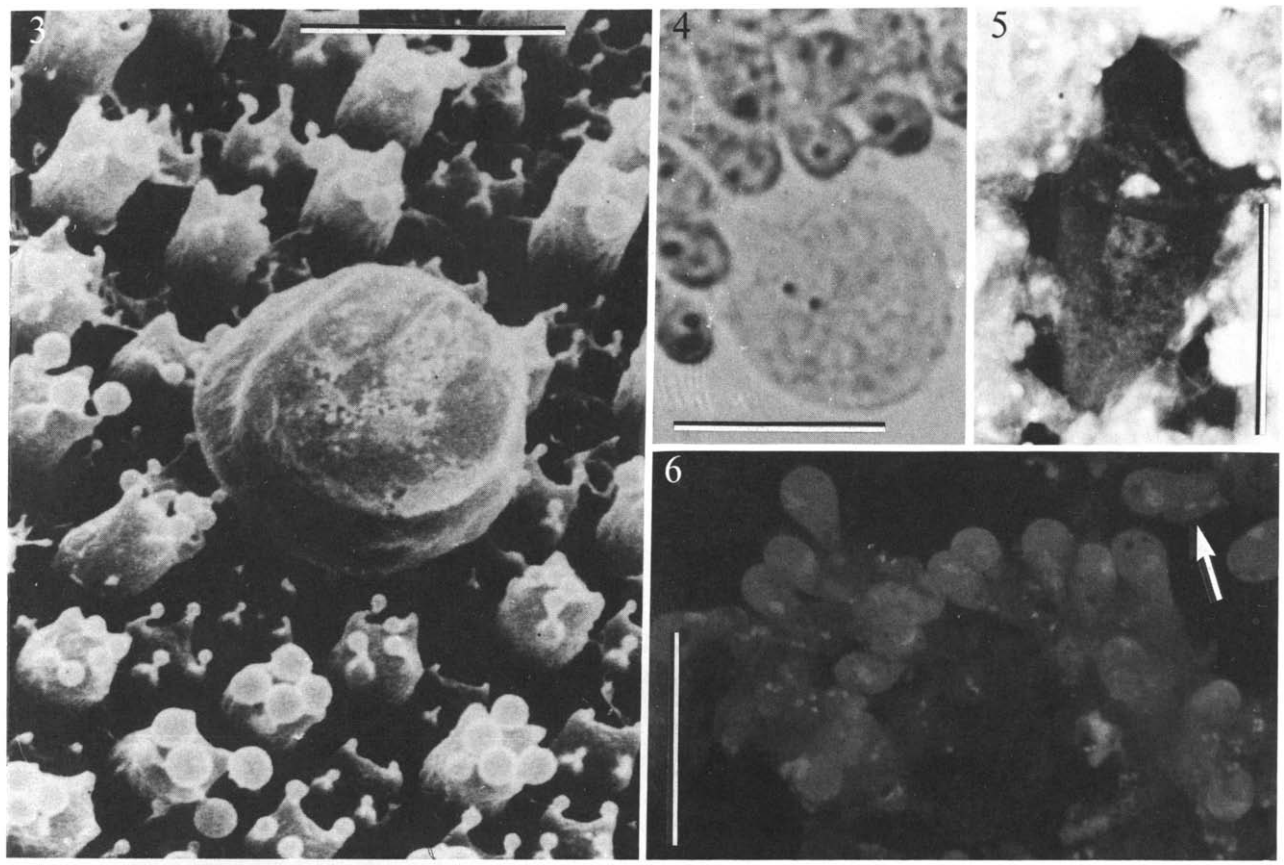


Figs 1 and 2. Marginal cystidia of *Coprinus cinereus*. **Fig. 1**. Scanning electron micrograph showing a gill of lesser rank with entire margin (*a*) in contrast with the primary gill having well-differentiated marginal cystidia (*b*) at sporulation stage of *Coprinus cinereus* (scale bar, 100 μm). **Fig. 2**. Fluorescent micrograph showing multinucleate marginal cystidia stained by ethidium bromide (scale bar, 8 μm).

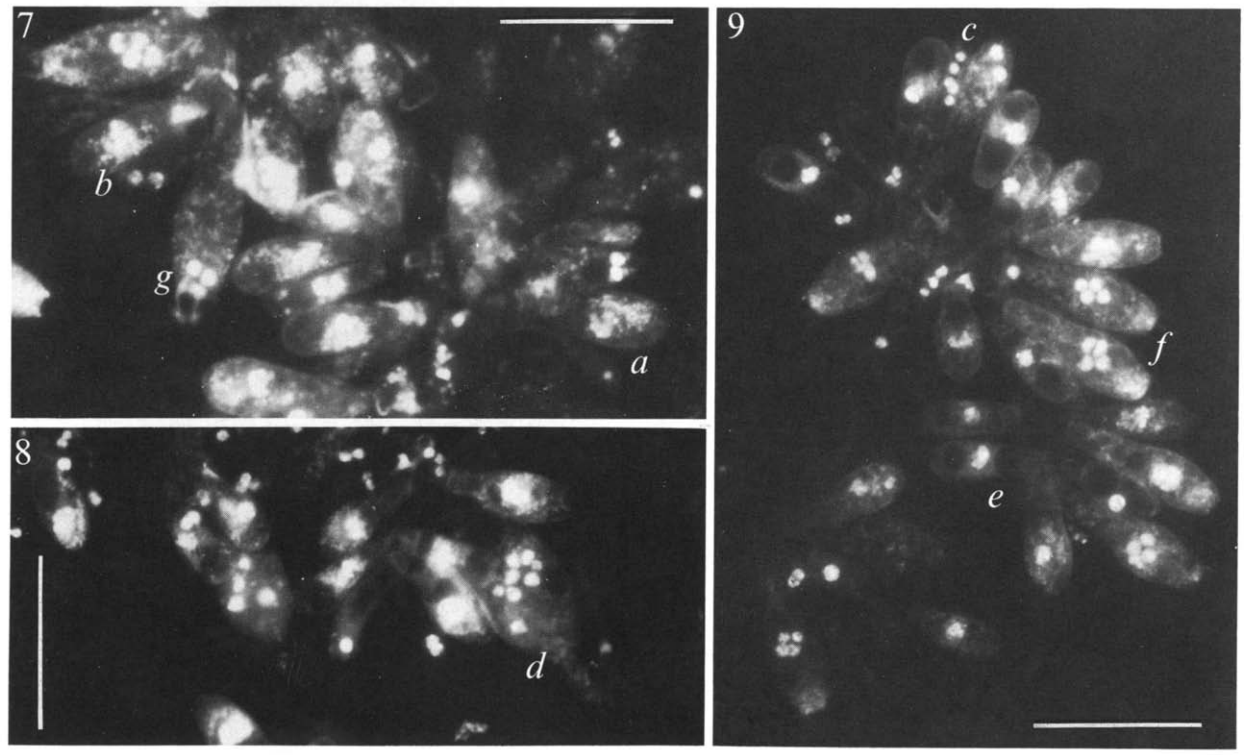
1963, 1979; Moore, 1984; Chiu, Moore & Chang, 1989; Chiu & Moore, 1990*b*,*c*; Lu, 1991) with recent attention concentrated especially on hymenophore and hymenium differentiation (Rosin & Moore, 1985*a*, *b*; Horner & Moore, 1987), particularly basidia (Lu, 1974, 1982; Oishi, Uno & Ishikawa, 1982; Chiu & Chang, 1987; Kanda *et al.*, 1989; Chiu & Moore, 1988*a*, 1990*a*; Kanada *et al.*, 1990).

In *C. cinereus*, gills are of two types: primary gills are formed first and bridge between the pileus context and the central stipe, their internal tramal hyphae being continuous with stipe hyphae; the other type arises later, and has a free edge (Reijnders, 1979; Moore, 1987). Marginal cystidia appear when primary gills pull away from the central stipe as the cap starts to expand (Fig. 1). Thus, marginal cystidia arise from the gill trama and many were multinucleate (Fig. 2) like the tramal cells (Lu, 1974). In contrast, a hymenial cell initial was binucleate and gave rise to binucleate paraphyses and solitary binucleate facial cystidia (Figs 3, 4). Uninucleate and multinucleate facial cystidia were seen, but only rarely (Figs 5, 6). In *Volvariella bombycina*, hymenial cell initials were uninucleate at first (Fig. 7) and then binucleate (Figs 7–10) in contrast to the multinucleate tramal hyphae (Figs 11, 12). A binucleate hymenial cell could differentiate into a multinucleate cystidium through mitotic divisions (Figs 8, 9) or be committed to become a basidium.

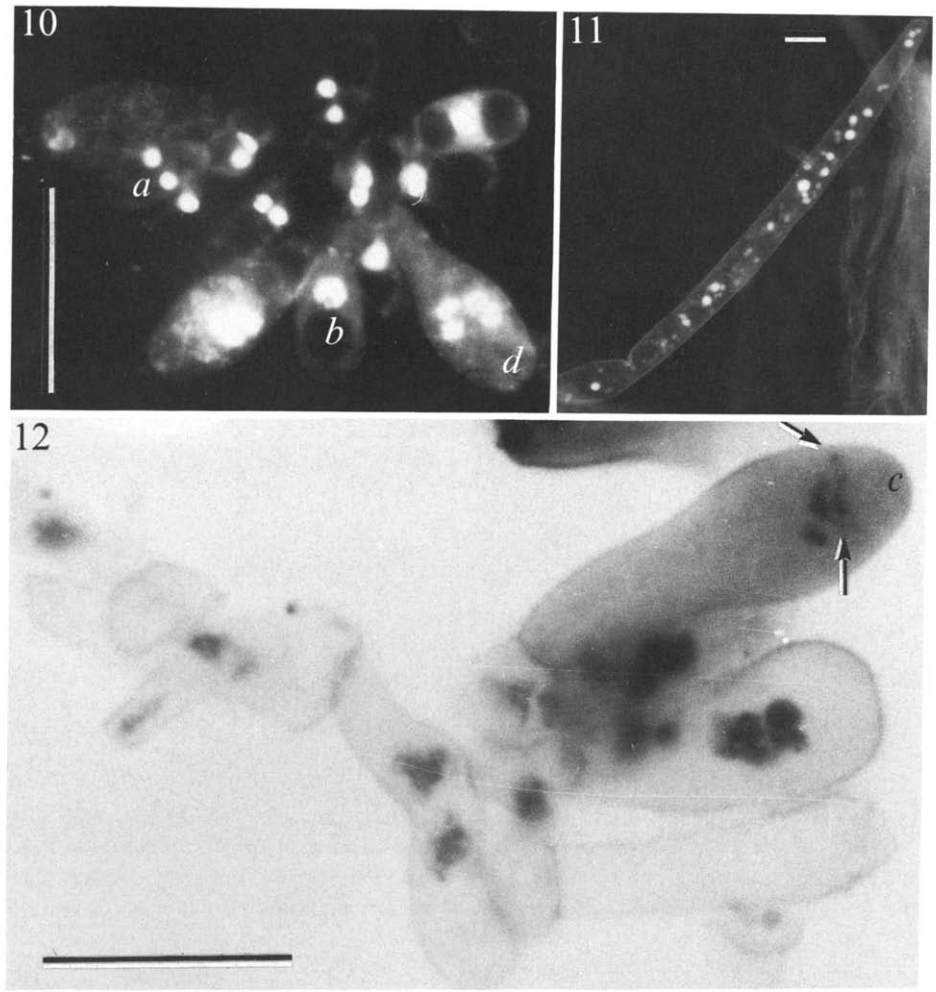
Nuclear behaviour in basidia was similar in both *C. cinereus* and *V. bombycina* (Lu, 1974; Chiu & Chang, 1987), but the time for DNA duplication differed (Table 1). Comparison of these data must bear in mind that meiosis is well synchronized in *C. cinereus* but is unsynchronized in *V. bombycina*. In particular, we should emphasize that a regular $n:2n:4n$ ratio (as a direct reflection of karyogamy first, followed by DNA replication) is not expected in these data for two reasons. First, the area of measurement of the photometer covers both conjugate nuclei



Figs 3–6. Facial cystidia of *Coprinus cinereus* (scale bars, 20 μm). **Fig. 3**. Scanning electron micrograph showing a solitary cystidium in the surface view of the young hymenium. Basidia in *C. cinereus* are dimorphic, long and short (Orton & Watling, 1979), and the long ones are slightly more advanced in their development than the short. **Fig. 4**. Light micrograph stained by silver nitrate to reveal the binucleate condition. **Figs 5 and 6**. Fluorescent micrographs stained by ethidium bromide revealing uninucleate and multinucleate conditions. The arrow indicates a binucleate hymenial cell initial.



Figs 7–9. Fluorescent micrographs showing hymenial cells of *Volvariella bombycina* stained by DAPI (scale bar, 20 μm). *a*, Uninucleate hymenial cell initial; *b*, binucleate hymenial cell; *c*, multinucleate young cystidium; *d*, multinucleate mature cystidium; *e*, prophase I basidium; *f*, interphase II basidium; *g*, basidium with nuclear migration into basidiospore.



Figs 10–12. Gill cells of *Volvariella bombycina*. **Fig. 10**. Fluorescent micrograph showing hymenial cells stained by DAPI (scale bar, 20 μm). **Fig. 11**. Fluorescent micrograph showing multinucleate gill trama stained by EB (scale bar, 50 μm). **Fig. 12**. Light micrograph showing subhymenial cells stained by propionic-iron alum-haematoxylin. *a*, binucleate hymenial cell; *b*, prophase I basidium; *c*, anaphase I basidium; *d*, interphase II basidium. The arrow indicates a spindle pole body (scale bar, 20 μm).

Table 1. Changes in the DNA contents of DAPI-stained nuclei in *Coprinus cinereus* and *Volvariella bombycina*

	Fluorescence intensity (arbitrary units)			
	Coprinus cinereus		*Volvariella bombycina*	
Stage	Sample size	Mean ± SD	Sample size	Mean ± SD
Tramal, 1 nucleus	ND	ND	29	6·9 ± 4·1
Subhymenial, 2 nuclei	18	17·5 ± 7·4	ND	ND
Hymenial, 2 nuclei	41	29·2 ± 9·5	45	26·3 ± 8·9
Prophase I, nucleus	125	29·9 ± 12·1	197	29·7 ± 7·5
Interphase II, 4 nuclei	36	46·2 ± 13·7	93	36·6 ± 10·3
Basidiospore, 1 nucleus	ND	ND	70	11·8 ± 6·8

ND, Not determined.

in the regularly dikaryotic cells of the subhymenial hyphae in *C. cinereus*, so a fluorescence intensity equivalent to *n* is not represented here. Second, in *V. bombycina* there is no opportunity to measure 2*n* reliably because a dikaryon with conjugate haploid nuclei is not formed, and hymenium cells with two nuclei include cystidia and basidia in different stages of meiosis.

For *C. cinereus*, the ANOVA test showed significant differences in mean DNA contents for pairwise comparison of at least two developmental stages ($P > 0{\cdot}05$) among subhymenial binucleate (= prekaryogamy), prophase I and interphase II stages, but the *t* test showed there was no significant difference the mean DNA contents at prekaryogamy and prophase I stages ($P = 0{\cdot}3$–$0{\cdot}5$). The results of a χ^2 test for goodness of fit for the hypothesis that DNA content doubles between subhymenial binucleate stage and hymenial binucleate stage also gave $P = 0{\cdot}3$–$0{\cdot}5$. Taken together, these analyses confirm that DNA synthesis occurs before karyogamy in the heterothallic *C. cinereus* (Lu, 1982; Kanada *et al.*, 1990).

For *V. bombycina*, the high DNA value at the binucleate hymenial cell stage (Table 1) reflects the mean DNA content per cell in a population of binucleate basidia *and* cystidia (the latter undergoing multiple mitoses). Analysis of variance showed significant differences between means in at least two stages among hymenial binucleate, prophase I and interphase II stages in *V. bombycina*. The *t* test found a significant difference in the mean DNA content between the binucleate and prophase I stages. A χ^2 test comparing the DNA contents

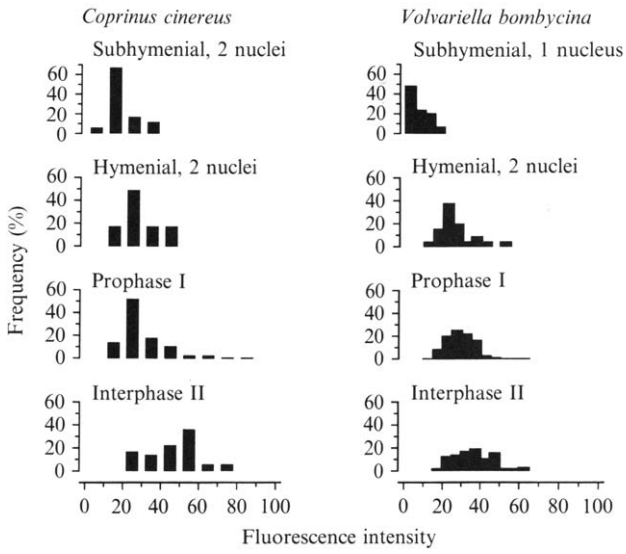


Fig. 13. Frequency histograms showing the distributions of fluorescence intensity measurements among cytologically recognizable cell types of the gill tissues of *Coprinus cinereus* and *Volvariella bombycina*.

at the uninucleate stage and the prophase I nucleus stage indicates a good fit for the proposition that DNA synthesis has taken place. Thus, a uninucleate hymenial cell initial (Fig. 7) becomes binucleate (Figs 7–10) by mitosis in *V. bombycina* as in the monokaryotic fruiting mutant of *C. cinereus* (Oishi, Uno & Ishikawa, 1982). Although DNA synthesis has been shown to take place at the prophase I stage in homothallic species which undergo synchronous meiosis (Oishi *et al.*, 1982; Bayman & Collins, 1990), meiosis in *V. bombycina* is asynchronous, consequently the population of binucleate hymenial cells is a mixed one, comprising basidia and cystidia, so the exact timing of the meiotic DNA synthesis cannot be determined. Nevertheless, examination of the frequency distributions of fluorescence intensities of the various cell types during hymenium development in the two species examined (Fig. 13) would favour the view that meiotic DNA synthesis took place at prophase I in *V. bombycina*. It is notable in Fig. 13 that the prekaryogamy and prophase I frequency distributions are virtually identical in *C. cinereus*. In this species meiotic DNA synthesis occurs before karyogamy, so these two sets of observations are measuring the same content of DNA in a synchronous population of cells at two successive stages of development. On the other hand, in *V. bombycina* the frequency distribution of hymenial cells with two nuclei shows two peaks, while the distribution of prophase I nuclei is a single broad peak. This would be consistent with the latter being a population of cells which are in various stages of the meiotic DNA replication cycle.

DNA synthesis occurred immediately postmeiotically in the 4-nucleus stage before sporulation (Table 1), resulting in formation of bi- or even tri-nucleate basidiospores in *C. cinereus* (75%) and in *V. bombycina* (12%) (Chiu & Chang, 1987).

A cell described as a basidium is quite clearly characterized by karyogamy, meiosis and the formation of basidiospores. In other words, application of the nomenclature involves consideration of the past and future behaviour of the cell. Usually, other descriptive terms, like paraphysis, sterile element or cystidium, are applied on the basis of the immediate morphology and/or position of the cell without reference to its ontogeny or fate. Ontogeny and fate are important considerations, because if we are to understand the mechanisms of differentiation and morphogenesis the descriptions of developmental pathways must be precise. Studies like this one demonstrate that the nuclear condition is an important component of cell morphology and that ontogeny is an important aspect of any description of cell differentiation. A hymenial cell initial may be pluripotent, but the varied differentiation pathways open to it involve commitment to expression of different sets of characters at different stages in development to provide the hymenium with cells with appropriate functional characteristics.

Some cell lineages lack the capacity to express morphologies developed by others. In the hymenium of *Agaricus* the 'epidermal pavement' which provides the structural support for basidia is made up of basidioles in an arrested meiotic state. Even after many days' existence, when the fruit body was close to senescence, 30–70% of the basidioles were in meiotic prophase (Allen *et al.*, 1992). This is not wastage of reproductive potential but constructive use (one is tempted to say imaginative use) of one differentiation pathway to serve two distinct but essential functions. *Coprinus* illustrates the other extreme in having a highly differentiated cell type – the paraphysis – with which to construct the epidermal pavement. These cells arise after the numerically static basidiole population commits to meiosis, branching from beneath the basidia and forcing their way into the hymenium (Rosin & Moore, 1985*b*). At maturity individual basidia are surrounded by about 5 paraphyses; thus, more than 80% of the hymenial cells serve a structural function. *Agaricus* and *Coprinus* hymenophore tissues reach essentially the same structural composition by radically different routes.

Some cell lineages approach the same ultimate morphology through different routes. Both *C. cinereus* and *V. bombycina* have facial and marginal cystidia. Both types of cystidium in *V. bombycina* are established when the hymenium is first laid down and, apart from location, their differentiation states and ontogeny appear to be identical. Facial cystidia in *C. cinereus* are also established as components of the very first population of dikaryotic hyphal tips which form hymenial tissue (Rosin & Moore, 1985*b*; Horner & Moore, 1987) and were mostly binucleate as a result. Marginal cystidia in *C. cinereus* were the apical cells of branches from the multinucleate gill trama, which became swollen to repair the injury caused when primary gills pulled away from the stipe; marginal cystidia retained the multinucleate character of their parental hyphae. Basidia provide more examples of 'convergent development'. A hymenial cell initial was binucleate in *C. cinereus* but uninucleate in *V. bombycina*, leading to inevitable differences in the course of the meiotic division, which were magnified by the fact that DNA synthesis took place before karyogamy in *C. cinereus* but after karyogamy in *V. bombycina*. Yet the two basidia exhibit remarkably similar morphologies and clearly serve the same function.

Do fungal differentiation pathways exhibit 'fuzzy logic'? Discussion of fungal cell differentiation often involves use of

the word 'switch' in phrases which imply wholesale diversion at some point between alternative developmental pathways. There are now many examples which suggest that fungal cells behave as though they assume a differentiation state even when all conditions of that state have not been met. Rather than rigidly following a prescribed sequence of steps, the differentiation pathways discussed here seem to be based on the application of rules which allow considerable latitude in expression; decisions between developmental pathways seem to be able to cope with a degree of uncertainty. Facial cystidia of *C. cinereus* are generally binucleate, reflecting their origin and the fact that they are sterile cells, yet occasional examples can be found of cystidia in which karyogamy has occurred (Fig. 5). This suggests that entry to the cystidial pathway of differentiation does not totally preclude expression of at least the start of the nuclear differentiation pathway characteristic of a different cell type. Equally, the fact that a large fraction of the basidiole population of *A. bisporus* remains in arrested meiosis indicates that entry to the meiotic division pathway does not guarantee sporulation. The literature contains many other examples. Watling (1971) observed some cystidia bearing hyphal outgrowth looking like sterigmata in a spontaneous fruit-body variant of *Psilocybe merdaria* (Fr.) Ricken, while Schwalb (1978) reported that basidia of a temperature-sensitive mutant of *Schizophyllum commune* Fr. not only aborted meiosis but also produced elongated sterigmata at the restrictive temperature. A spore-deficient mutant of *Lentinula edodes* (Berk.) Pegler [syn. *Lentinus edodes* (Berk.) Sing.] produced some abnormal basidia bearing both a hyphal outgrowth and basidiospores (Hasebe, Murakami & Tsuneda, 1991). We have induced similar abnormal growth in basidia by transplanting gills to agar medium containing some metabolic inhibitors (Chiu & Moore, 1988*b*, 1990*a*). Although these explantation experiments have been discussed mainly for their value in understanding commitment to the basidium differentiation pathway, it is equally important that all other cells of the hymenium and hymenophore showed no commitment, immediately reverting to hyphal growth on explantation as though they have an extremely tenuous grasp on their state of differentiation. That these cells do not default to hyphal growth *in situ* implies that their state of differentiation is somehow continually reinforced by some aspect of the environment of the tissue which they comprise.

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