Segregation of genotypically diverse progeny from self-fertilized haploids of the Chinese straw mushroom, *Volvariella volvacea*

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The electrophoretic karyotype of the Chinese straw mushroom, *Volvariella volvacea*, was determined. The haploid strain V34 of *V. volvacea* has 15 chromosomes ranging in size from 1·4 to 5·1 Mb. No chromosomal polymorphism in terms of size and number was seen in either of two growth stages: vegetative mycelia or fruit-body gill tissues. DNA fingerprints were prepared by the arbitrarily-primed polymerase chain reaction. Those obtained from different stages during fruit-body morphogenesis were identical. However, variation in DNA fingerprints was evident in protoplast regenerants derived from the same vegetative mycelium. Thus the haploid mycelium of strain V34 is heterokaryotic but the bulk genotype is stable during fruit-body development. F1 and F2 progenies germinated from the haploid, uninucleate basidiospores from self-fertilized fruit bodies also regularly segregate a range of mycelial morphological variants as well as phenotypic variation revealed by DNA fingerprints. We overview the known mechanisms to generate genetic variation and propose a novel mechanism that could account for the 1:1 segregation ratio of self-fertile to self-sterile progeny regularly obtained from selfed *Volvariella volvacea* fruit bodies.

Most of our understanding of basidiomycete genetics is owed to studies on the mushrooms *Coprinus cinereus* and *Schizophyllum commune*. As data accumulate on species of applied significance, exceptions mount to the 'rules' established by these two experimental organisms. For example, *Agaricus bisporus* usually forms only two basidiospores per basidium, each containing two meiotic daughter nuclei. This is secondary homothallism, because a single progeny spore gives rise to a self-fertile, but heterokaryotic, mycelium. Another 'unusual' example is provided by the diploid species of *Armillaria* for which there is evidence of haploid nuclei in tramal hyphae and dikaryotic subhymenial cells, indicating a haploidization process during fruit-body development before the normal meiotic cycle (Peabody, Motta & Therrien, 1978; Peabody & Peabody, 1986; Chiu, 1996).

Volvariella volvacea (Bull.:Fr.) Sing., the Chinese straw mushroom, may have been in cultivation longer than any other mushroom (Singer, 1961). In many markets it is the preferred fresh culinary mushroom owing to its unique flavour (Mau *et al*., 1997). Overall it is about the fifth most popular cultivated mushroom in the world (Chang, 1993; Royse, 1995), and has pharmaceutical value, including antitumour polysaccharides, immunosuppressive proteins and immuno-modulatory lectins (Kishida *et al*., 1992; Hsu *et al*., 1997; She, Ng & Liu, 1998).

Royse *et al*. (1987) demonstrated Mendelian segregation of five allozyme loci in *V. volvacea*, supporting the idea that breeding work could be undertaken between lines possessing desirable commercial traits. Possession of a meiotic process during fruit-body development may be redundant in true homothallics (Li, 1977; Chiu, 1993; Robertson, Bond & Read, 1998), but is not unusual. On the other hand, published evidence suggests that progenies resulting from selfing in *V. volvacea* are regularly phenotypically diverse; this *is* unusual. In this paper we report the first pulsed field gel electrophoresis (PFGE) study of the karyotype to examine the gross chromosomal stability and haploidy of *V. volvacea* and test whether DNA fingerprint polymorphisms segregate in progenies of successive selfed generations.

MATERIALS AND METHODS

Fungus, media and cultivation conditions

Volvariella volvacea strain V34 was used to initiate the experiments. This is a cultivated strain which is commonly used in Thailand. Cultures were maintained at 32 °C on potato dextrose agar (PDA) or mushroom complete medium (MCM; Raper & Miles, 1958), which consisted of ($g\ l^{-1}$): glucose, 20; magnesium sulphate, 0·5; potassium dihydrogen orthophosphate, 0·46; dipotassium hydrogen orthophosphate 1; peptone, 2; yeast extract 1 and agar 15. To obtain sufficient biomass for DNA extraction, mycelia were scraped from a plate culture and inoculated into 100 ml potato dextrose broth. The liquid cultures were then incubated at 32° on a rotary shaker operating at 100 rpm for 7 d unless otherwise specified.

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Fruiting and collection of sexual progenies

A 7-d-old plate culture was blended in 20 ml sterilized water using a Waring blender and 0·5 ml of the homogenate was pipetted as inoculum into 15 × 8 cm plastic bags of fermented compost and incubated at 32° for 20 d. The compost was prepared using either cotton waste or paddy straw and consisted of (w/w): 38·8% cotton waste/paddy straw, 0·6% lime, 0·6% wheat bran and 60% tap water and fermented for 2 d at room temperature before use. Colonized cultures were transferred to a humidified room (r.h. = 85%+) at 32° and the plastic bags were cut open. Fruit bodies appeared within 2–3 d and were harvested at the following developmental stages: *pinheads* (diam. about 2 mm); *egg* (universal veil still intact enclosing the well-differentiated stem and cap, basidia immature and had not started meiosis); *elongation*, with the lengthened stem rupturing the universal veil, leaving behind the genus-specific basal volva; and *mature* fruit bodies, with cap margin fully extended, the gill colour changing from white to reddish brown.

To collect sexual progeny, a sterilized filter paper was placed below a fully matured fruit body to get the spore print. Then a strip of the filter paper with spores was cut out aseptically and added to 2 ml of sterilized water. The concentration of the spore suspension was determined with a haemacytometer and about 500 spores were spread onto one plate of MCM which was incubated at 32° for 3 d. Well-isolated colonies were picked up and transferred onto MCM for culture maintenance.

DAPI-fluorescence staining of nuclei

For germination, basidiospores were spread onto a sterilized glass slide which had been coated with a thin layer of MCM medium and kept in a sterilized moist Petri dish. The whole setup was then incubated at 32° for 2 d. Nuclei in squashed gills, vegetative mycelia or slide cultures of germlings were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Chiu, 1993) and examined by fluorescence microscopy. DAPI was dissolved (final concentration 225 μg ml^{-1}) in 0·2 M Na/K phosphate buffer (pH 6·9) and the specimen was mounted in 50% (v/v) aqueous glycerol to retard fading during observation with a Nikon Microphot epifluorescence microscope.

Protoplast preparation and regeneration

Protoplasts were prepared from 4-d-old stationary broth cultures or gills from the egg stage of the fruit body. About 100 mg (f. wt) of fruit body tissue, or mycelium blotted dry using sterilized filter paper, was suspended in 2 ml of filter-sterilized Lywallzyme (Guangdong Institute of Microbiology, China) (10 mg ml^{-1} in 0·6 M mannitol). After 2 h digestion at 30° and 100 rpm, undigested hyphal debris was removed by filtration through a 5 mm tall column of cotton wool packed in a 5 ml syringe. The filtrate (approx. 10^7 protoplasts ml^{-1}) was centrifuged at 1150 ***g*** for 10 min. The protoplast pellet was resuspended in 0·6 M mannitol to a final concentration of 10^8–10^9 protoplasts ml^{-1}.

For regeneration, 0·1 ml of protoplast suspension (10^3–10^4 protoplasts ml^{-1}) was plated onto MCM containing 0·6 M mannitol as osmotic stabilizer. In parallel, samples were plated on MCM (without osmotic stabilizer) to estimate regeneration directly from cell debris rather than from protoplasts. Visible colonies were picked from the plates with mannitol after incubation at 30° for 3 d in darkness and transferred onto potato dextrose agar for culture maintenance.

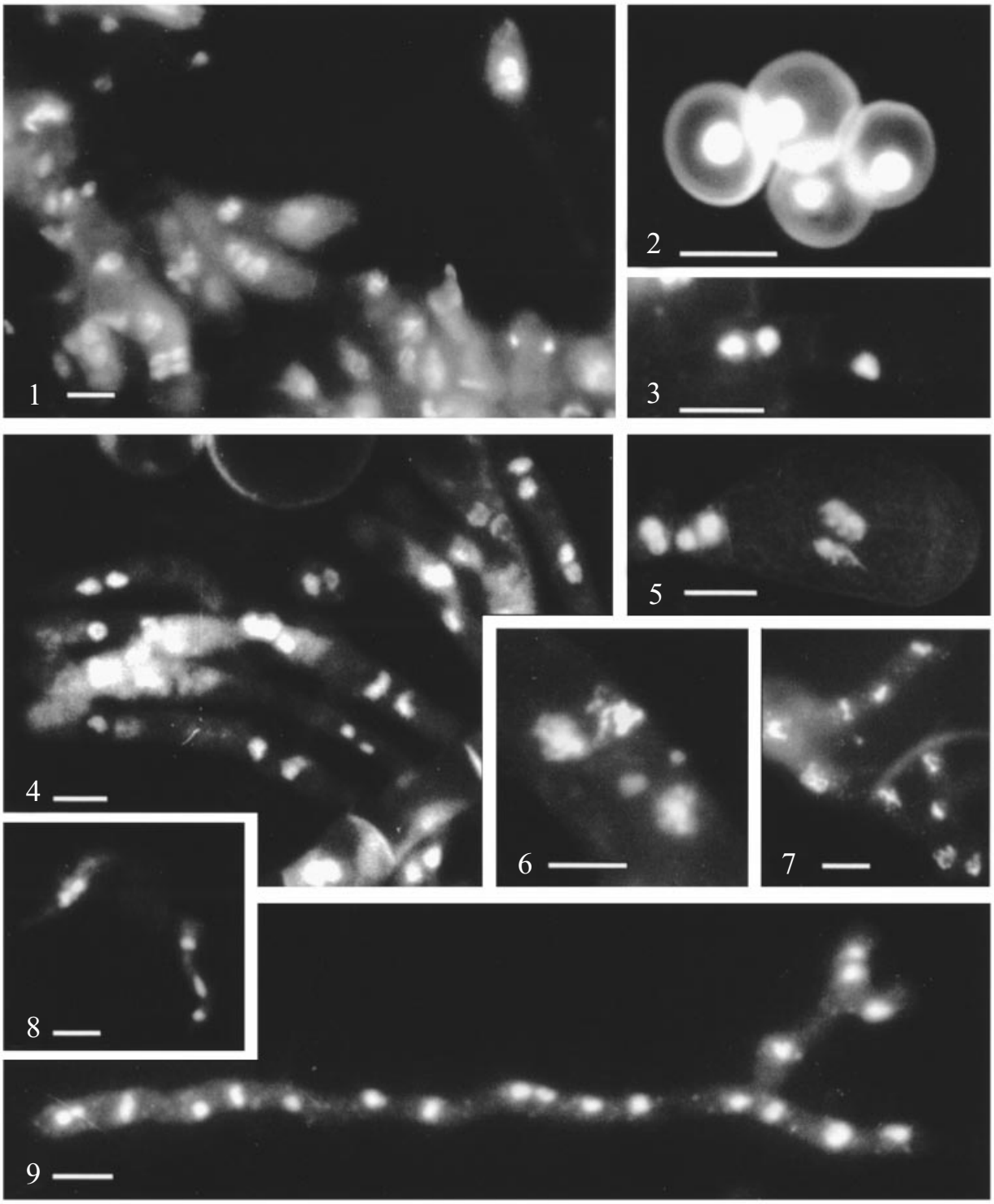
Preparation of chromosomal DNAs for pulsed field gel electrophoresis

Protoplasts were mixed with an equal volume of 1·5% melted low melting temperature agarose containing 0·125 M EDTA and 0·6 M mannitol (pH 7·5) using a pipette tip cut to 2 mm diam. Then the mixture was pipetted into Bio-Rad mould chambers and allowed to set at 4° for 1 h. The agarose plugs were incubated in a capped tube containing NDS buffer (0·01 M Tris–HCl (pH 7·5), 0·5 M EDTA (pH 8·0), 1% lauryl-sarcosine, 1 mg ml^{-1} proteinase K) at 50° for 48 h with gentle shaking to lyse the protoplasts *in situ* (Carle & Olson, 1985). The agarose plugs were washed with 0·05 M EDTA (pH 8·0) at 50° three times before storage at 4°.

PFGE was performed with a CHEF^{DR}II system (Bio-Rad) set at 50 V at 12 ° using 0·5 × TBE (0·45 M Tris-borate, 1 mM EDTA) as the electrophoresis buffer. Unless otherwise specified, the agarose plugs were inserted into the sample wells of a 1% SeaKem LE agarose (FMC) gel in 0·5 × TBE. Three conditions were used; (i) the pulse time was ramped from 3000 to 1200 s for 194 h; (ii) the same condition as in (i) but for 168 h with a 0·8% FastLane agarose gel (FMC); (iii) a ramped pulse from 1800 to 600 s for 90 h was followed by another ramped pulse from 600 to 90 s for 48 h. The following chromosomal DNA size markers were used: *Saccharomyces cerevisiae* Hansen for the range 225 kb to 2·2 Mb (Kwan *et al.*, 1991) and *Schizosaccharomyces pombe* Lindner for the range 3·5–5·7 Mb (Bio-Rad). The gel was stained with 0·5 g ml^{-1} ethidium bromide for 1 h and destained in water for 20 min before being photographed under uv illumination or the image was digitized using the Gel Documentation image analysis system (Bio-Rad model 670). The experiment was repeated once. As a control to the overall procedure, we also performed a similar analysis of the karyogram of *Coprinus cinereus* and obtained results similar to those reported in the literature (data not shown).

Arbitrarily-primed polymerase chain reaction

Use of PCR with arbitrary primers is a sensitive method for typing strains including siblings in heterothallic species (Chiu, Kwan & Cheng, 1993; Chiu *et al.*, 1996, 1998). Genomic DNA was extracted from tissue frozen in liquid nitrogen, and was purified following a protocol of White *et al.* (1990) with the addition of RNAse treatment and caesium chloride further to purify the DNA (Yoon & Glawe, 1993; Yoon, Glawe & Shaw, 1991). The concentration and purity of a DNA sample were measured by the spectrophotometric absorbance and the ratio of OD_{260}:OD_{280} (Sambrook, Fritsch & Maniatis, 1989). A sample having a ratio greater than 1·8 was considered suitable



Figs 1–9. Use of DAPI fluorecence-staining to reveal nuclear behaviour in *Volvariella volvacea*. **Fig. 1**. Meiosis in basidia. **Fig. 2**. A tetrad of uninucleate basidiospores. **Fig. 3**, Uni- and binucleate hymenial cells. **Fig. 4**. Multinucleate hyphal compartments of the gill trama giving rise to binucleate subhymenial cells. **Fig. 5**. Mitosis in a cystidium. **Fig. 6**. Mitoses in a hyphal compartment of the gill trama. **Fig. 7**. Asynchronous mitoses in a vegetative mycelial hypha. **Fig. 8**. First and second mitotic nuclear divisions in the hyphae emerging from two germinating basidiospores. **Fig. 9**. Establishment of a multinucleate hyphal system by repeated mitoses and branching. Scale bars = 10 μm.

for use, and its concentration and purity were further checked by agarose gel electrophoresis using ethidium bromide staining.

Five arbitrary primers were used (Chiu *et al.*, 1993; Chiu, Chen & Chang 1995). These were:

(i) M13FS (5′- CGCCAGGGTTTTCCCAGTCACGAC-3′);
(ii) M13RS (5′-AGCGGATAACAATTTCACACAGGA-3′);
(iii) *Eco*RI ext (5′-TAGGCGTATCACGAGGCCCT-3′);
(iv) DelC23 (5′-GTAAAAGCAGCCCCAGTGCCAAG-3′);
(v) GalK-54 (5′-TACGGTCCGGGAGCGCAGCA-3′).

The reaction mixture contained: 1 × PCR buffer, 3·5 mM $MgCl_2$, 200 μM each of dNTPs (Perkin–Elmer Cetus), 1 μM of the primer and 2·5 U *Taq* DNA polymerase (Boehringer–Mannheim) and 25–50 ng of genomic DNA. The thermal programme was: 2 low stringency cycles of 94° for 2 min, 35° for 1 min and 72° for 2 min, followed by 39 cycles of high stringency: 94° for 1 min, 55° for 1 min and 72° for 2 min and with the last extension time lengthened to 10 min. Experiments were repeated to verify consistency, and controls without DNA were amplified in parallel to check for contamination or carry-over. The DNA fingerprints were resolved on LE agarose gels (FMC) together with a molecular size marker consisting of a 100 bp ladder (Pharmacia; the product has a bright band at 800 bp). The amplification reaction was repeated once.

RESULTS AND DISCUSSION

Nuclear divisions

Unlike most basidiomycetes, the mycelium produced by a uninucleate basidiospore of *V. volvacea* can complete the life-cycle (Figs 1–9), producing a fruit body in which meiosis occurs (Fig. 1). Direct microspectrophotometric measurements of DNA quantities in nuclei by Chiu (1993) established that vegetative mycelium, fruit body tissues and (single) basidiospore nuclei are haploid. In arbitrary units, typical fluorescence values measured were: vegetative hyphal nucleus, 8; hymenium nucleus, 8; basidiospore nucleus, 9; basidiospore germling nucleus, 8; and the meiotic prophase diploid, 22. Indirect genetic evidence of gene segregations (Chang & Yau, 1971; Royse *et al.*, 1987; Chang & Li, 1991) and occurrence of recessive mutants (Chang & Li, 1991) also contribute to the conclusion that different isolates can mate and go through a conventional haploid–diploid–haploid meiotic cycle. There are no barriers to cross-fertility and no evidence for mating type factors. Yet mycelium from a single (uninucleate) basidiospore can produce fruit bodies, i.e. the organism exhibits a primary homothallic life-cycle. Since basidiospores are haploid and uninucleate, only one haploid set of chromosomes can occur in such a selfed (homothallic) fruit body and its progeny are expected to be identical to each other and to the parent.

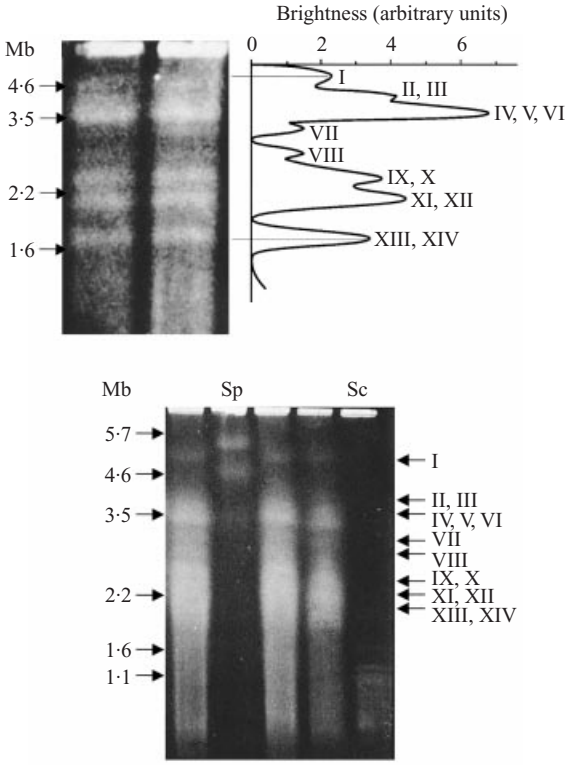


Fig. 10. Partial electrophoretic karyotype of *Volvariella volvacea*. Top panel shows chromosomal DNAs released from protoplasts of four-d-old mycelium (left) and fruit-body gills (right) resolved under electrophoretic condition (i) (see Materials and Methods) together with the densitometer trace. Bottom panel shows chromosomal DNAs resolved under electrophoretic condition (ii). Roman numerals indicate the *V. volvacea* chromosomal DNA band assignments. Chromosomal DNA size markers: Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*.

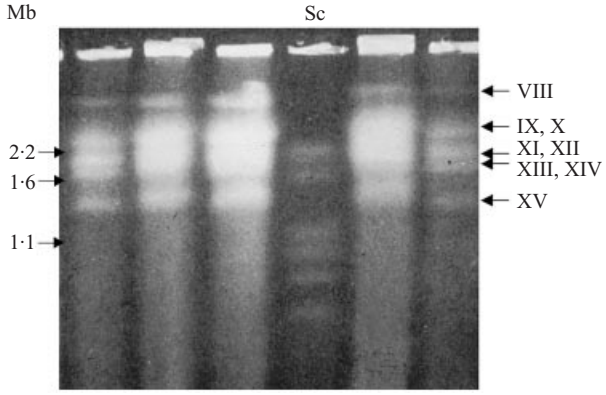


Fig. 11. Partial electrophoretic karyotype of *Volvariella volvacea*. Chromosomal DNAs released from protoplasts resolved under electrophoretic condition (iii). Roman numerals indicate the *V. volvacea* chromosomal DNA band assignments. Chromosomal DNA size marker: Sc, *Saccharomyces cerevisiae*.

The electrophoretic karyotype of Volvariella volvacea *Strain V34*

PFGE resolves more chromosomes than were reported from cytological study of *V. volvacea* (Li, 1977). The three sets of PFGE conditions resolved the chromosomal DNAs over different size ranges and combining the data revealed nine chromosomal DNA bands, five of them having higher staining intensity with ethidium bromide (Fig. 10), indicative of co-migration of different chromosomal DNAs (Sagawa & Nagata, 1992; Ásgeirsdóttir, Schuren & Wessels, 1994).

Sizes of chromosomes I–X were estimated using the *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* chromosomal DNAs as markers for type (ii) PFGE conditions (Fig. 10), and chromosomes XI–XV using *S. cerevisiae* DNAs for type (iii) conditions (Fig. 11). The chromosomal DNA bands of *V. volvacea* are estimated to have the following sizes: 5·0, 3·7 (doublet), 3·6 (triplet), 3·3, 3·1, 2·9 (doublet), 2·2 (doublet) 1·7 (doublet) and 1·4 Mb, respectively (Figs 10, 11). The genome of *V. volvacea* strain V34 is estimated to be 45 Mb by summation of these chromosome sizes and to comprise 15

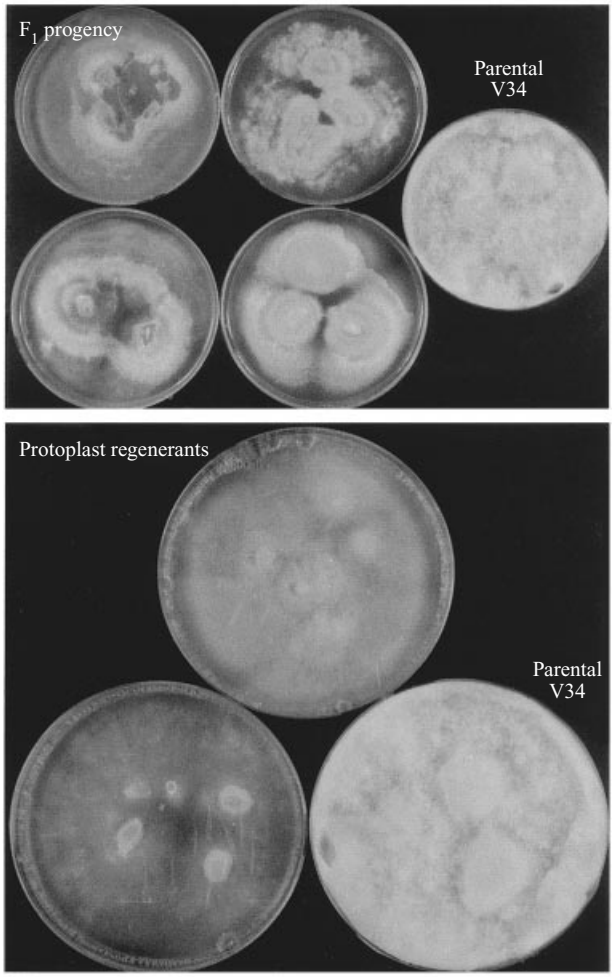


Fig. 12. Variant colony morphologies observed in meiotic progeny (top) and protoplast regenerants (bottom) of *V. volvacea* strain V34.

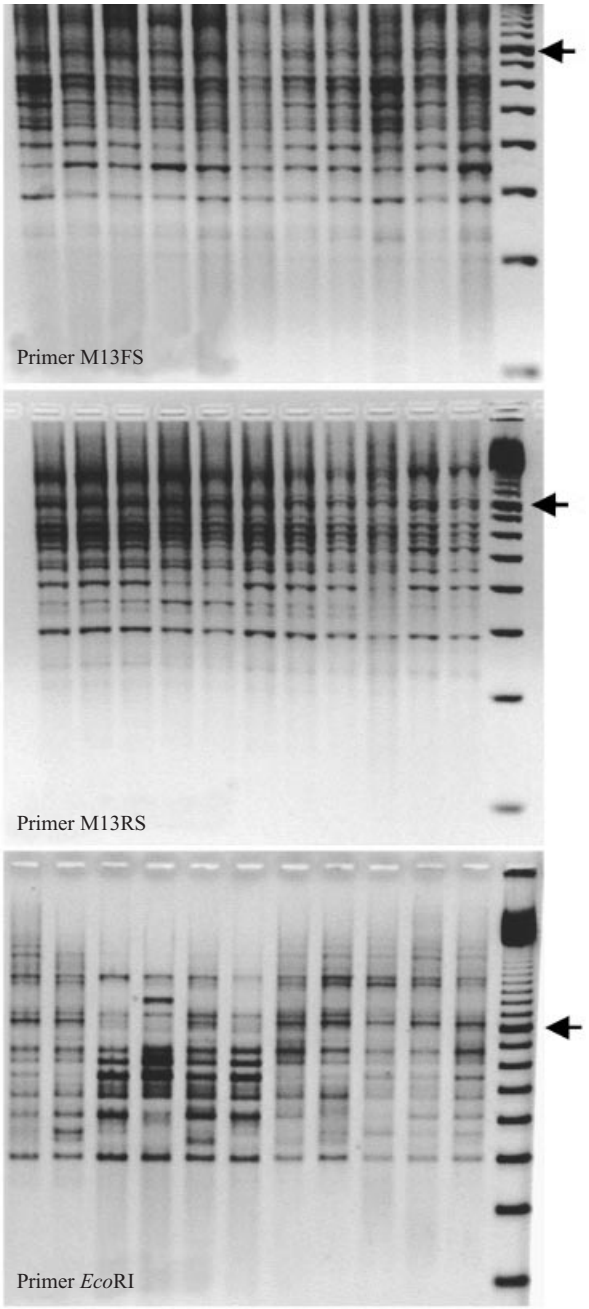


Fig. 13. AP-PCR fingerprints of the siblings from F_1 progeny A of *V. volvacea* strain V34. Top: M13FS as arbitrary primer. Middle: M13RS as arbitrary primer. Bottom: *Eco*RI as arbitrary primer. The lane at the right hand edge of each gel was loaded with a 100 bp ladder, the arrow indicates the 800 bp DNA band.

chromosomes. The odd number of chromosomes indicates that it could not be a diploid and supports the view derived from microspectrophotometric observations (Chiu, 1993) that the mushroom strain used here contains haploid nuclei.

Chromosome polymorphism in terms of addition or loss of supernumerary chromosomes, chromosome length polymorphism and chromosome number variation, has often been encountered in fungi (Chiu, 1996). The electrophoretic karyotypes of vegetative and fruiting stages (using protoplasts released from vegetative mycelia and gills) examined in *V. volvacea* strain V34 (Fig. 10) were the same in terms of chromosome size, number and banding intensity throughout vegetative growth and fruit–body development.

Genotype analyses

Protoplast regeneration frequency was about 3% in these experiments. Protoplast regenerants from four-day-old vegetative mycelium of strain V34 and meiotic F_1 and F_2 progenies expressed varied colony morphologies (Fig. 12). Although this confirms earlier observation of phenotypic variation in sexual progenies of *V. volvacea* (Chang & Li, 1991; Chang, Miles & Wai, 1981; Chang & Yau, 1971; Li & Chang, 1978; see discussion below), Zhao (1994) did not detect any difference in colony morphology among protoplast regenerants from strain V34. Nevertheless, we found that two of the 20 regenerants showed slow growth and thin colony morphology in contrast to the fluffy and fast-growing parent (Fig. 12).

Ten isolates analysed by AP-PCR were found to have identical DNA fingerprints with arbitrary primers M13FS and M13RS (Fig. 13) but other arbitrary primers, such as *Eco*RI, revealed phenotypic variation among the siblings (Fig. 13). To confirm this observation, more detailed analyses were carried out with another batch of meiotic progenies.

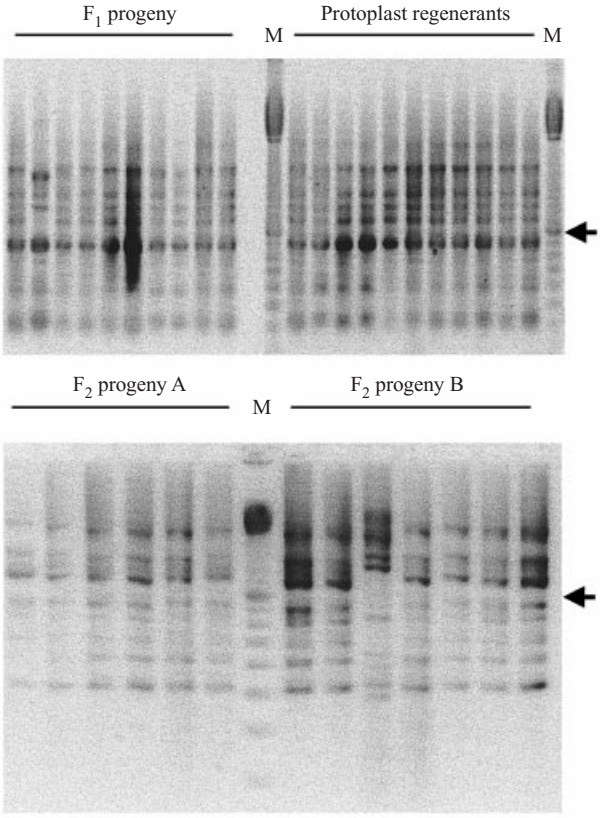


Fig. 14. AP-PCR fingerprints of F_1 B progeny, protoplast regenerants from vegetative mycelium, and two independent F_2 progenies (A and B) of *V. volvacea* strain V34, obtained using the arbitrary primer DelC23. Lanes labelled M were loaded with a 100 bp ladder, the arrow indicates the 800 bp DNA band.

Single spore progenies of the heterokaryotic strain V34

All five primers were used to examine the different life phases of *V. volvacea* strain V34. The results obtained with arbitrary primer DelC23 are shown in Fig. 14. Protoplasting permits a fine dissection of the bulk genotype by offering the possibility of obtaining regenerants from protoplasts which carried a single nucleus from the tissue of origin. AP-PCR fingerprints of protoplast regenerants of *V. volvacea* strain V34 mycelium revealed DNA polymorphisms using primers *Eco*RI ext (detected one variant only) and GalK54 (Fig. 15) in contrast to the uniformity observed with the other three primers used (Table 1). Only one of the two protoplast regenerants with non-parental colony morphology as illustrated above (Fig. 12) had a DNA fingerprint which differed from the parent. Thus, more than two genotypes were detected. This indicates that strain V34 is not a dikaryon but is a heterokaryon composed of haploid nuclei.

'Fingerprints' of bulk preparations of DNA of different physiological stages of *V. volvacea* (5-day-old mycelia, pinhead, egg, elongation stage, mature stem, mature cap) were identical with all the primers tested (Fig. 16; Table 1). Thus, the fungus shows stability in genetic makeup during morphogenesis, i.e. there is no selection for or against a particular genotype in the heterokaryon.

The second batch of F_1 progenies also revealed DNA polymorphisms (Figs 14, 17) except primer M13FS (Table 1). These two F_1 progenies comprised single spore isolates derived from spore prints from two separate fruit bodies arising on strain V34 and they showed different degrees of variation (Figs 13, 14 and 17; Table 1).

Single spore progenies of selfed isolates

Two self-fertile, monosporous F_1 isolates were chosen and fruited (not all F_1 progenies were self-fertile, cf. Table 2). F_2 progenies of single spore isolates with different colony morphologies (= non-random sampling) were prepared from spore prints and were analysed with AP-PCR. Despite originating from *selfed* haploids, the DNA fingerprints of the F_2 progenies were also as variable as were the F_1 progenies (Figs 14, 17; Table 1). Thus, in some way phenotypic variation persists in the *selfed* progeny of *V. volvacea*.

Before discussing the data presented here we wish to review critically that which is already in the literature. *Volvariella volvacea* is found in tropical and subtropical grasslands. The vegetative mycelium grows optimally at 32–35° but cannot tolerate temperatures below 10° (Li *et al.*, 1992). Vegetative hyphal compartments and the hyphae of fruit-body tissues are multinucleate and their septa lack clamp

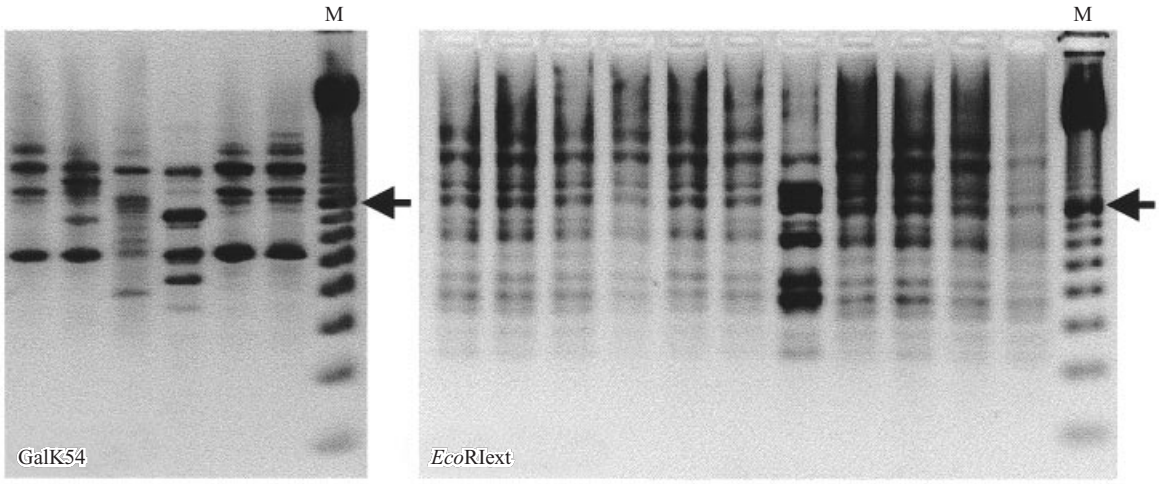


Fig. 15. AP-PCR fingerprints in protoplast regenerants of *V. volvacea* strain V34 obtained using arbitrary primers GalK54 and *Eco*RI ext. Lanes labelled M were loaded with a100 bp ladder, the arrow indicates the 800 bp DNA band.

Table 1. Incidence of DNA fingerprint variants in selfed progenies of *Volvariella volvacea*

Primer	Developmental stages	Protoplast regenerants	F_1 progeny A	F_1 progeny B	F_2 progeny A*	F_2 progeny B*
DelC23	0	0	1	1	0	1
*Eco*R I ext	0	1	5 (4)	1	0	0
GalK54	0	3 (4)	1	1	0	1
M13FS	0	0	0	0	0	1
M13RS	0	0	0	5 (2)	3 (2)	2 (3)
Sample size	6	10	10	10	5	5

* Non-random sampling; isolates show non-parental colony morphologies. Numbers in brackets show the number of different amplification patterns.

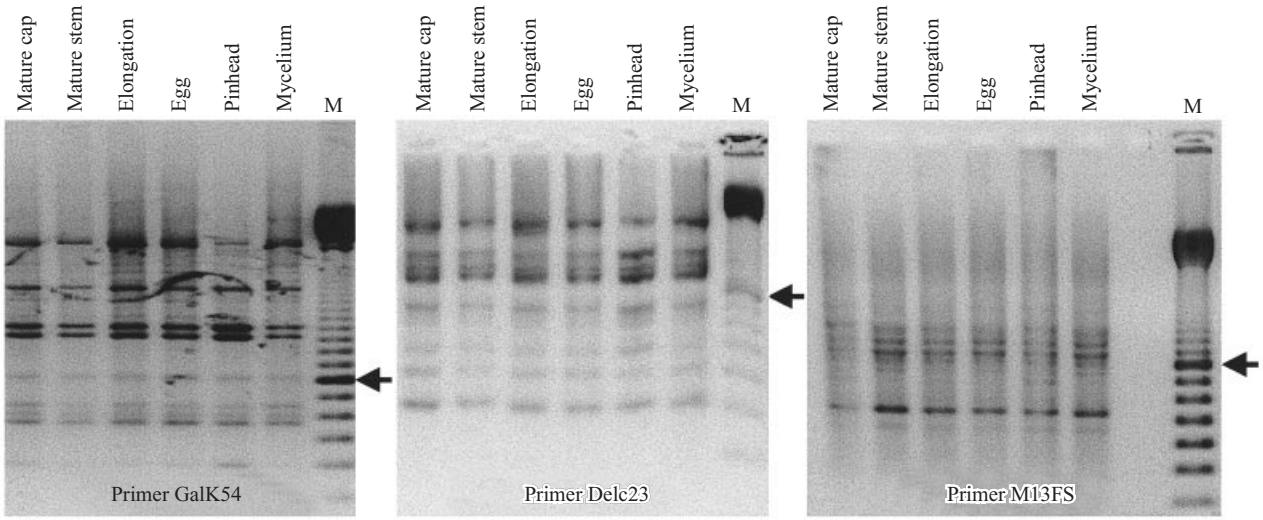


Fig. 16. AP-PCR fingerprints in different physiological stages of *V. volvacea* strain V34 obtained using the arbitrary primers GalK54, Delc23 and M13FS. Lanes labelled M were loaded with a 100 bp ladder, the arrow indicates the 800 bp DNA band.

connections (Figs 4, 7, 9) (Chang & Yau, 1971; Li, 1977; Chiu, 1993). *V. volvacea* has a haploid–diploid–haploid life-cycle with meiotic DNA replication taking place in basidia at prophase I of meiosis (Chiu, 1993). Most of the basidiospores (over 85%; Li, 1991; Chiu, 1993) are uninucleate (Fig. 2) but many of these uninucleate basidiospores germinate to form mycelia which are self-fertile (Chang & Yau, 1971; Li & Chang, 1978; Chang *et al.*, 1981; Li, 1991 and the present study).

On the other hand there seems to be no barrier to cross-fertility; with no evidence for mating type genes but clear demonstrations that different isolates can mate and go through conventional Mendelian segregations of markers (Chang & Yau, 1971; Chang, 1978; Royse *et al.*, 1987). These observations have depended on segregations of phenotypic variation in fertility, growth rate and colony morphology, although Chang & Li (1991) used aspartate-deficient and malachite green resistant mutants, and Royse *et al.* (1987) used isozymes. Cultivated strains from different countries showed genetic differences (Chiu *et al.*, 1995; Royse *et al.*, 1987), indicating that recombination occurs naturally in the wild as well as in laboratory cultures (Burt *et al.*, 1996; Kohli & Kohn, 1998).

Tests for self fertility (Li & Chang, 1978; Chang *et al.*, 1981; Li, 1991) in many cases showed a 1:1 ratio of self-fertile: self-sterile among progeny, but the authors claimed that this changed to a 3:1 ratio in the next generation. However, most of the sample sets were fairly small and when they are brought together it is clear that few differ significantly from a 1:1 ratio (Table 2), nor did the ratio obtained in the present study (self-fertile: self-sterile = 6:4 and 5:5 for F_1A and F_1B progenies, respectively). Though there appeared to be no statistical relationship between colony morphology and fertility, slow-growing mycelia were mostly self-sterile (Li & Chang, 1978; Li, 1991). In some fungi binucleate basidiospores are formed and can germinate to form self-fertile mycelia; self-sterility being associated with uninucleate spores in the progeny population (Elliott, 1972). This is secondary homothallism and cannot explain the situation in *V. volvacea*. *V. volvacea* forms four basidiospores into which the four meiotic nuclei migrate individually. The basidiospores remain uninucleate as there is no post-meiotic mitosis until germination (Chang, 1971; Li, 1977; Chiu, 1993; Figs 2, 8). Furthermore, Li (1991) measured nuclear DNA contents and showed that self-fertile and self-sterile isolates did not differ. Thus, neither variation in nuclear ploidy nor variation in nuclear number in basidiospores can explain segregation of genetic variation in selfed *V. volvacea*.

The data collected in Table 2 are remarkable in two respects. First, the progeny from crosses listed here (G12 × G52, H4 × H32, etc.) are from crosses between two single spore isolates, both of which were self-sterile, i.e. it was uniformly observed that self-sterile × self-sterile crosses gave progeny which were 50% self-fertile and 50% self-sterile. Secondly, the monosporous strains (with the code number V42–18 in Li, 1991 and HK-1 in Li & Chang, 1978) and other single spore isolates used for cultivation in mainland China

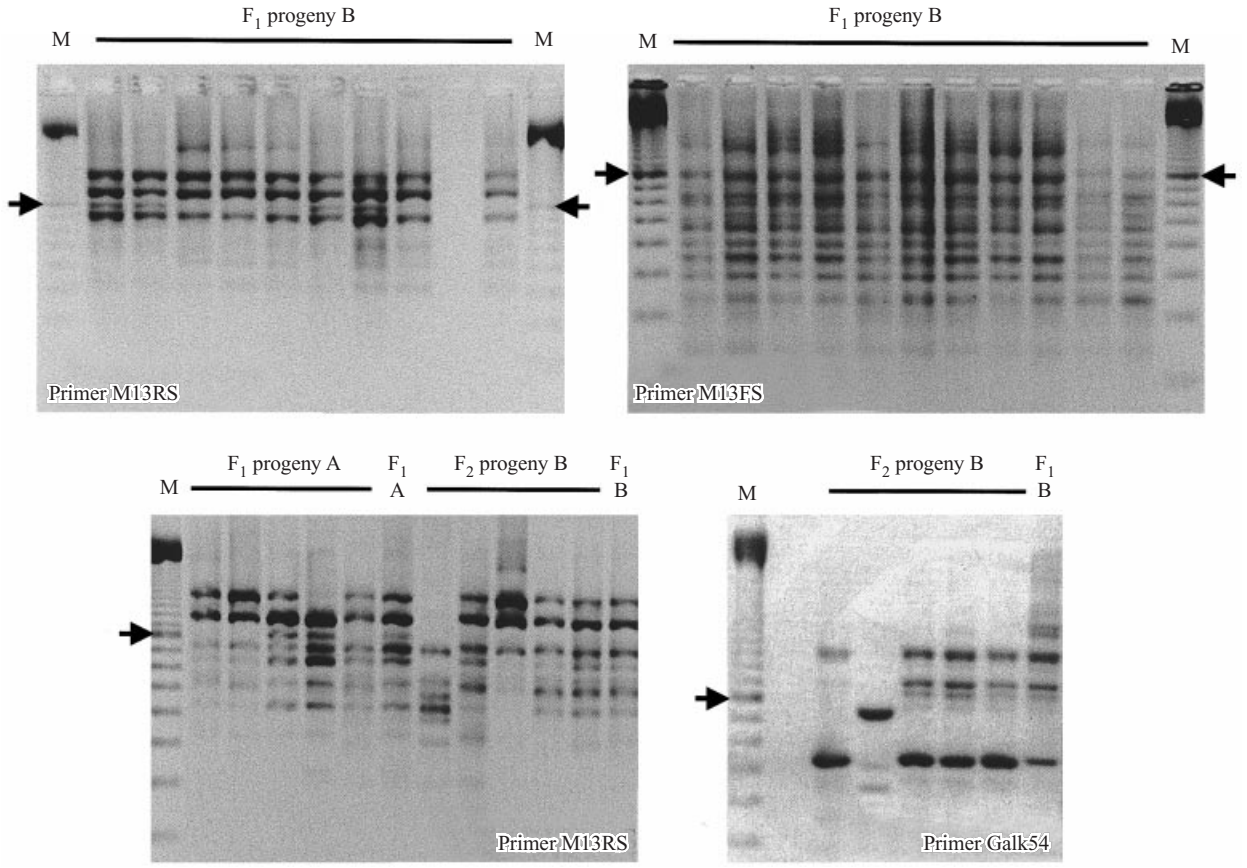


Fig. 17. AP-PCR fingerprints in sexual progenies of selfed haploid progeny of *V. volvacea* strain V34. Gels in the upper half of the figure show fingerprints of F_1 progeny group B obtained with the primers M13FS and M13RS. The lower panels show DNA fingerprints of F_2 progenies obtained from two self-fertile F_1 progeny of strain V34 (called F_1A and F_1B). Arbitrary primer GalK54 detects genetic differences in F_2B progeny. Arbitrary primer M13RS detects genetic differences in both F_2A and F_2B progenies. Lanes labelled Mwere loaded with a 100 bp ladder, the arrow indicates the 800 bp DNA band.

Table 2. Segregation of fertility in selfed and out-crossed sexual progenies of *Volvariella volvacea*

Culture	Self-fertile	Self-sterile	χ^2value for 1:1 ratio	Reference
Fruit body H	23	7	8·53*	1
Fruit body K	15	5	5·00*	1
HK-1	24	19	0·58	2
THAI-1	17	19	0·11	2
PJ	20	30	2	2
TEN	19	23	0·38	2
SAR	11	11	0	2
G11 × G12	5	10	1·73	2
G11 × G93	6	4	0·5	2
G12 × G52	6	9	0·67	2
G52 × G93	5	10	1·73	2
T5 × T14	7	7	0·07	2
T5 × T19	4	11	3·33	2
H4 × H32	9	7	0·31	2
H4 × H80	11	5	2·31	2
H32 × H89	12	3	5·47*	2
H80 × H89	3	11	4·64*	2
V42 × 18	30	12	7·60*	3
V14	19	12	1·58	3
V35	14	16	0·4	3
V7	11	15	0·62	3
Totals	271	246	1·21	$P =$ 0·25 for good fit

* With 1 degree of freedom, χ^2 for $P = 0{\cdot}05$ is 3·84; the asterisked values deviate significantly from a 1:1 ratio.
† Yates' correction applied when $n = 10$ or fewer.
References: 1, Chang & Yau (1971), 2, Li & Chang (1978), 3, Li (1991).

(personal communication from Professor Y. J. Pan, Director of the Shanghai Institute of Edible Fungi) have the phenotype 'self-fertile'. Yet their selfed fruit bodies yield progenies which are 50% self-fertile and 50% self-sterile (Table 2). Clearly, a selfed haploid should be fully homozygous so such a segregation among its progeny is completely unexpected. Change from self-fertility to self-sterility has been accounted for by mating type switching in *Ceratocystis caerulescens* (Harrington & McNew, 1997). Yet no regularity in pattern of pairings of self-sterile isolates was observed which might imply the presence of mating factor control in *V. volvacea* (Chang & Yau, 1971; Li, 1977; own data, not shown).

Subsequently, Chang *et al*. (1981) showed that segregation persisted over three successive generations of monosporous selfing. In this case the colony morphology was used as the test phenotype. The first generation of (92) spores was isolated from a culture obtained from Thailand and segregated a range of colony phenotypes. A single spore isolate from this set with 'normal' colony morphology was then fruited and the 132 single-spore progeny isolated in this generation segregated the same range of phenotypes as the first (parental) generation. For the third generation a single spore isolate from the second generation with 'normal' colony morphology was fruited and 118 single spore isolates segregated the same range of phenotypes as before. Finally, a single spore isolate from the third generation with 'normal' colony morphology was fruited and once more segregated the same range of phenotypes (85 progeny spores tested). This study demonstrated quite categorically that the ability to segregate variants survived, apparently undiminished, through three successive generations of selfing of haploid progeny.

Our results support these earlier reports but we can now extend the description with our observations that variation at the DNA level persists through the selfing of a haploid strain of *V. volvacea* but does not involve whole-chromosome aberrations. The present study employed arbitrary primers to detect the DNA fingerprints of parents and offspring and reveals change in DNA profiles which reflects genetic variation from parent to progeny in *V. volvacea*.

The mechanisms for generating genetic variation which come most readily to mind are: gene or chromosomal mutation, transposons, parasexual mitotic processes including somatic recombination, meiotic recombination, and, possibly, repeat-induced point mutations (RIPs) and deficiency in DNA repair (Magni & von Borstel, 1962; Dillon & Stadler, 1994; Montelone & Koelliker, 1995; Watters & Stadler, 1995). Production of aneuploid progenies with deviating DNA content per nucleus with that of the parental strain, however, are ruled out by previous fluorescence microspectrophotometric studies (Li, 1992; Chiu, 1993). Also, any one of these mechanisms cannot account alone for the high level of variation observed in *V. volvacea*.

In this study, variation in sexual progenies was quite high in the data of Table 1 (though the F_2 was non-randomly sampled and both progenies are small in size); using fertility as a criterion, the occurrence of non-parental phenotype in other studies was 21–80% (Table 2). Any mechanism which is postulated to account for the observations must produce these frequencies of variant progeny, *and* provide for continued segregation of variants despite a period of regular selfing.

The problem posed by the observations on *V. volvacea* published over the past 25 years is to explain how self-fertile monosporous isolates can continue to produce genotypically diverse progeny over several generations of selfing. This may not be the most helpful way of looking at the phenomenon. If we accept the phenomenon as an adaptive one (rather than as a defect or disease) it may be possible to identify the circumstances and adaptive criteria which have directed its development. We suppose that *V. volvacea* should be viewed as an outbreeding species which has evolved mechanisms to generate and/or preserve genetic diversity on those occasions when it encounters reproductive isolation and becomes a 'facultative homothallic'. In that case we would postulate that the organism requires a means to preserve heterozygosity which it enjoys in generation zero through successive selfings into generations 1, 2, 3, etc., so that the population remains 'healthy' in evolutionary terms until an unrelated mate is encountered. The value of the mechanism will be greatly diminished if based on a randomized mutational process.

The segregating variation is clearly located at the DNA level. We cannot yet distinguish between nuclear and organelle genomes, though the self-fertile: self-sterile segregation ratio of 1:1 argues against dependence on segregation of an organelle. Since genomes appear to be stable through the entire life cycle, in terms of electrophoretic karyotype and DNA fingerprint, and microspectrofluorometric measurement of the DNA content per nucleus shows that the F_1 progenies do not change in ploidy level in *V. volvacea* (Li, 1991; Chiu, 1993), we presume that more variation is generated through the meiotic cycle than through a mitotic (parasexual) process.

Similarly, stability of vegetative genomes (at least in comparison with the high level of variation detectable in basidiospore progeny) would argue against any mutational process (whether conventional or transpositional) being responsible, though the multinucleate condition of *V. volvacea* mycelium may have masked vegetative variation. A remarkably high mutation rate would have to be assumed to produce up to 50% variation in meiotic progeny, and this would be quite out of keeping with the presumed balance which preserves a constant rate of spontaneous mutation in microorganisms (Drake, 1991). Such a rate of mutation would be too disruptive unless directed away from crucial housekeeping sequences by unrealistic protective mechanisms. Furthermore, it may need to be limited to the meiotic cycle. Transposable elements have been identified in a number of filamentous fungi (Oliver, 1992; Kempken & Kuck, 1998). Transposition in the rice blast fungus, *Magnaporthe grisea*, generates DNA polymorphisms at frequencies up to 18% (Wu & Magill, 1995; Xia & Correll, 1995; Shull & Hamer, 1996), occurs during vegetative growth and can also generate karyotype variation (Talbot *et al*., 1993). It may be significant that some *V. volvacea* strains are vegetatively unstable (Zhang & Chang, 1992), and that 50% of progeny are self-sterile (Table 2). Both phenotypes might be due to transpositional inactivation, but *random* transposition on the scale required to create the variation observed is likely to be as disruptive as random mutation. Thus, for transposition to be a plausible

explanation of self-fertile variability we would have to assume specificity of targeting.

In *V. volvacea* strain V34 repetitive sequences totalled 44% of the total DNA (highly repetitive: 8%; moderately repetitive: 36%) as detected by the hybridization of homologous probes cloned in pUC18 with digested genomic DNA (Chen, 1994; Chiu *et al*., 1995). This value lies between that obtained with diploid *Phytophthora infestans* (60%; Goodwin, Drenth & Fry, 1992) and haploid *Agaricus bisporus* (16%; Arthur *et al*., 1983) and *Lentinula edodes* (10%; Kulkarni, 1991). RIP is well known in *Neurospora crassa* and is specific to the sexual cycle (Watters & Stadler, 1995). A RIP-like mechanism, which depends on identifying a sequence peculiarity (repetition, methylation, etc.) and then modifying that sequence could not operate through successive generations of selfing, because once modified in F_1 the sequence would not be recognizable in F_2. We therefore exclude this as a possible explanation. Whether the high percentage of repetitive sequences is related to self-fertile variability in *V. volvacea* is uncertain but remains a possible means of generating variable progeny from self-fertilized fruit bodies. In brief, it is difficult to see how transpositional mutagenesis could produce the observed 1:1 segregations of self-fertile:self-sterile progeny.

An additional plausible mechanism, which could produce 1:1 ratios from initially heterozygous loci, might be to regulate meiotic recombination mechanisms such that: (i) occurrence of recombination is enhanced (and this might be fine-tuned by making recombinational hot-spots or cold-spots); but (ii) repair of base mismatches in hybrid DNA is reduced or eliminated (this could also be fine-tuned, particularly by extending the length of hybrid regions). The result would be that (*a*) in every meiosis at least a proportion (which might be upregulated to be a high proportion) of the heterozygosity of the diploid nucleus would be preserved in the haploid daughter nuclei in the form of base mismatches in hybrid DNA; (*b*) at the first postmeiotic mitosis (in this organism this occurs at germination, see Fig. 8) these mismatches would be segregated into daughter nuclei and the *V. volvacea* spore germling would immediately become dikaryotic, even though the basidiospore had but one haploid nucleus; (*c*) assuming that recombination and mismatch-repair patterns differed between meioses then different spores would produce different dikaryons; (*d*) if half the base-mismatches at an initially heterozygous hypothetical fertility locus were repaired prior to the post-meiotic mitosis then a 1:1 ratio could result of self-fertile (= still heterozygous in the sporeling dikaryon): self-sterile (= repaired to homozygosity). In the past few years there have been a number of reports of continued genetic variation in selfed or mitotic progenies (Bagagli *et al*., 1995; Guo & Ko, 1995; Marra & Milgroom, 1996). As far as we can make out, the possibility of uncorrected hybrid DNA being a source of such variation has not been suggested previously.

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REFERENCES

Arthur, R., Herr, F., Straus, N., Anderson, J. B. & Horgen, P. A. (1983). Characterization of the genome of the cultivated mushroom, *Agaricus brunnescens*. *Experimental Mycology* **7**, 127–132.

Ásgeirsdóttir, S. A., Schuren, F. H. J. & Wessels, J. G. H. (1994). Assignment of genes to pulse-field separated chromosomes of *Schizophyllum commune*. *Mycological Research* **98**, 689- 693.

Bagagli, E., Furlaneto, M. C., Pizziranikleiner, A. & Alzevedo, J. L. (1995). Genetic recombinants in *Trichoderma pseudokoningii* (Rifai) without typical parasexuality. *Canadian Journal of Microbiology* **41**, 1132–1134.

Burt, A., Carter, D. A., Koenig, G. L., White, T. J. & Taylor, J. W. (1996). Molecular markers reveal cryptic sex in human pathogen *Coccidioides immitis*. *Proceedings of the National Academy of Sciences, U.S.A.* **93**, 770–773.

Carle, G. F. & Olson, M. V. (1985). An electrophoretic karyotype for yeast. *Proceedings of the National Academy of Sciences, U.S.A.* **82**, 3756–3760.

Chang, S. T. (1978). *Volvariella volvacea*. In *The Biology and Cultivation of Edible Mushrooms* (ed. S. T. Chang & W. A. Hayes), pp. 573–605. Academic Press: New York.

Chang, S. T. (1993). Biology and cultivation technology of *Volvariella volvacea*. In *Mushroom Biology and Mushroom Products* (ed. S. T. Chang, J. A. Buswell & S. W. Chiu), pp. 73–83. The Chinese University Press: Hong Kong.

Chang, S. T. & Li, S. (1991). Genetical studies on the sexuality pattern of *Volvariella volvacea*. In *Science and Cultivation of Edible Fungi* (ed. M. J. Maher), pp. 119–122. Balkema: Rotterdam and Brookfield, VT, U. S. A.

Chang, S. T. & Yau, C. K. (1971). *Volvariella volvacea* and its life history. *American Journal of Botany* **58**, 552–561.

Chang, S. T., Miles, P. G. & Wai, C. C. (1981). A study of monosporous isolates of *Volvariella volvacea*. *Mushroom Science* **11**, 603–619.

Chen, M.-J. (1994). *Molecular studies on the Chinese Straw Mushroom, Volvariella volvacea*. M.Phil. Thesis. The Chinese University of Hong Kong: Hong Kong.

Chiu, S. W. (1993). Evidence for a haploid life-cycle in *Volvariella volvacea* from microspectrophotometric measurements and observations of nuclear behaviour. *Mycological Research* **97**, 1481–1485.

Chiu, S. W. (1996). Nuclear changes during fungal development. In *Patterns in Fungal Development* (ed. S. W. Chiu & D. Moore), pp. 113–125. Cambridge University Press: Cambridge, U.K.

Chiu, S. W., Chen, M.-J. & Chang, S. T. (1995). Differentiating homothallic *Volvariella* mushrooms by RFLPs and AP-PCR. *Mycological Research* **99**, 333–336.

Chiu, S. W., Kwan, H. S. & Cheng, S. C. (1993). Application of arbitrarily-primed polymerase chain reaction in molecular studies of mushroom species with emphasis on *Lentinula edodes*. In *Culture Collection and Breeding of Edible Fungi* (ed. S. T. Chang, J. A. Buswell & P. G. Miles), pp. 265–284. Gordon and Breach Publishers Inc.: Philadelphia.

Chiu, S. W., Ma, A.-M., Lin, F.-C. & Moore, D. (1996). Genetic homogeneity of cultivated strains of shiitake (*Lentinula edodes*) used in China as revealed by the polymerase chain reaction. *Mycological Research* **100**, 1393–1399.

Chiu, S. W., Wang, Z. W., Yip, M. L., Leung, T. M., Lin, F. C. & Moore, D. (1998). Genetic diversity in a natural population of shiitake (*Lentinula edodes*) in China. In *Abstracts, 6th International Mycological Congress, 23–28 Aug., 1998,* p. 34. IMC-6: Jerusalem, Israel,

Dillon, D. & Stadler, D. R. (1994). Spontaneous mutation in the *mtr* locus in *Neurospora*: the molecular spectrum in wild type and mutator strains. *Genetics* **138**, 61–74.

Drake, J. W. (1991). A constant rate of spontaneous mutation in DNA-based microbes. *Proceedings of the National Academy of Sciences, U.S.A.* **88**, 7160–7164.

Elliott, T. J. (1972). Sex and the single spore. *Mushroom Science* **8**, 11–18.

Goodwin, S. B., Drenth, A. & Fry, W. F. (1992). Cloning and genetic analysis of two highly polymorphic moderately repetitive nuclear DNAs from *Phytophthora infestans*. *Current Genetics* **22**, 107–115.

Guo, L. Y. & Ko, W. H (1995). Continuing variation in successive asexual generations of *Pythium splendens* following sexual reproduction. *Mycological Research* **99**, 1339–1344.

Harrington, T. C. & McNew, D. L. (1997). Self-fertility and uni-directional mating type switching in *Ceratocystis caerulescens*. *Current Genetics* **32**, 52–59.

Hsu, H. C., Hsu, C. I., Lin, R. H., Kao, C. L. & Lin, J. Y. (1997). Fip-vv, a new fungal immunomodulatory protein isolated from *Volvariella volvacea*. *Biochemical Journal* **323**, 557–565.

Kempken, F. & Kuck, U. (1998). Transposons in filamentous fungi – facts and perspectives. *BioEssays* **20**, 652–659.

Kishida, E., Kinoshita, C., Sone, Y. & Misaki, A. (1992). Structures and antitumor activities of polysaccharides isolated from mycelium of *Volvariella volvacea*. *Bioscience Biotechnology and Biochemistry* **56**, 1308–1309.

Kohli, Y. & Koln, L. M. (1998). Random association among alleles in clonal populations of *Sclerotinia sclerotiorum*. *Fungal Genetics and Biology* **23**, 139–149.

Kulkarni, R. K. (1991). DNA polymorphisms in *Lentinula edodes*, the shiitake mushroom. *Applied and Environmental Microbioloy* **57**, 1735–1739.

Kwan, H. S., Li, C. C., Chiu, S. W. & Cheng, S. C. (1991). A simple method to prepare intact yeast chromosomal DNA for pulsed field gel electrophoresis. *Nucleic Acids Research* **19**, 1347.

Li, S. F. (1977). *Cytological and Genetical Analysis on the Life Cycle of Volvariella volvacea*. M.Phil. Thesis. The Chinese University of Hong Kong: Hong Kong.

Li, S. F. (1991). *Genetical and Cytological Studies on Variations of Volvariella volvacea*. Ph.D. Thesis. The Chinese University of Hong Kong: Hong Kong.

Li, S. F. & Chang, S. T. (1978). Variation in the homothallic basidiomycete, *Volvariella volvacea*. *Mushroom Science* **10**, 171–184.

Li, Y., Cho, K. Y., Wu, Y. Z. & Nair, N. G. (1992). The effect of lipids and temperature on the physiology and growth of *Volvariella volvacea*. *World Journal of Microbiology & Biotechnology* **18**, 621–626.

Magni, G. E. & von Borstel, R. C. (1962). Different rates of spontaneous mutation during mitosis and meiosis in yeast. *Genetics* **47**, 1097–1108.

Marra, R. E. & Millgroom, M. G. (1996). Selfing and heterothallism in *Cryphonectria parasitica*. Abstracts, American Phytopathological Society & Mycological Society of America joint annual meeting, Indianapolis, Indiana, U. S. A., July 27–31 1996, abstract 112M, p. 107.

Mau, J. L., Chyau, C. C., Li, J. Y. & Tseng, Y. H. (1997). Flavor compounds in straw mushroom *Volvariella volvacea* harvested at different stages of maturity. *Journal of Agricultural and Food Chemistry* **45**, 4726–4729.

Montelone, B. A. & Koelliker, K. J. (1995). Interactions among mutations affecting spontaneous mutation, mitotic recombination and DNA repair in yeast. *Current Genetics* **27**, 102–109.

Oliver, R. (1992). Transposons in filamentous fungi. In *Molecular Biology of Filamentous Fungi* (ed. U. Stahl & P. Tudzynski), pp. 3–11. VCH: Weinheim, New York, Basel & Cambridge.

Peabody, D. C., Motta, J. J. & Therrien, C. D. (1978). Cytophotometric evidence for heteroploidy in the life cycle of *Armillaria mellea*. *Mycologia* **70**, 487–498.

Peabody, D. C. & Peabody, R. B. (1986). Nuclear volumes and DNA content of three stages in the life cycle of *Armillaria bulbosa*. *Mycologia* **78**, 967–968.

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Raper, J. R. & Miles, P. G. (1958). The genetics of *Schizophyllum commune*. *Genetics* **438**, 530–546.

Robertson, S. J., Bond, D. J. & Read, N. D. (1998). Homothallism and heterothallism in *Sordaria brevicollis*. *Mycological Research* **102**, 1215–1223.

Royse, D. J. (1995). Speciality mushrooms. *Mushroom News* **43**, 4–19.

Royse, D. J., Jodon, M. H., Antonio, G. G. & May, B. P. (1987). Confirmation of intraspecific crossing and single and joint segregation of biochemical loci of *Volvariella volvacea*. *Experimental Mycology* **11**, 11–18.

Sagawa, I. & Nagata, Y. (1992). Analysis of chromosomal DNA of mushrooms in genus *Pleurotus* by pulsed-field gel electrophoresis. *Journal of General and Applied Microbiology* **38**, 47–52.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, vols. 1–3. 2nd edn. Cold Spring Harbor Laboratory Press: New York.

She, Q. B., Ng, T. B. & Liu, W. K. (1998). A novel lectin with potent immunomodulatory activity isolated from fruiting bodies and cultivated mycelia of the edible mushroom *Volvariella volvacea*. *Biochemical and Biophysical Research Communications* **247**, 106–111.

Shull, V. & Hamer, J. E. (1996). Genetic differentiation in the rice blast fungus revealed by distribution of the Fosbury retrotransposon. *Fungal Genetics and Biology* **20**, 59–69.

Singer, R. (1961). *Mushrooms and Truffles: Botany, Cultivation and Utilization*. Leonard Hill (Books) Ltd: London.

Talbot, N. M., Salch, Y. P., Ma, M. & Hamer, J. E. (1993). Karyotypic variation within clonal lineages of the rice blast fungus, *Magnaporthe grisea*. *Applied and Environmental Microbiology* **59**, 585–593.

Watters, M. K. & Stadler, D. R. (1995). Spontaneous mutation during the sexual cycle of *Neurospora crassa*. *Genetics* **139**, 137–145.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications* (ed. M. A. Inns, D. H. Gelfand, J. J. Sninsky & T. J. White), pp. 315–322. Academic Press: San Diego.

Wu, B. C. & Magill, C. W. (1995). Spontaneous mutations at fingerprint loci in clonal lineages of the rice blast fungus. *Experimental Mycology* **19**, 86–90.

Xia, J. & Correll, J. C. (1995). Examination of mitotic stability and hybridization potential between two genetically distinct haplotypes of *Magnaporthe grisea*. *Experimental Mycology* **19**, 171–177.

Yoon, C. S. & Glawe, D. A. (1993). Association of random amplified polymorphic DNA markers with stromatal type in *Hypoxylon truncatum sensu* Miller. *Mycologia* **85**, 369–380.

Yoon, C. S., Glawe, D. A. & Shaw, P. D. (1991). A method for rapid small-scale preparation of fungal DNA. *Mycologia* **83**, 835–838.

Zhang, J. & Chang, S. T. (1992). Study on productivity and quality of *Volvariella volvacea* (Bull. ex Fr.) Sing. stock culture after storage. *Edible Fungi of China* **11** (4), 3–9 [in Chinese with English summary].

Zhao, J. (1994). *Isolation, Identification and Application of Protoplast Fusion Products in Edible Mushrooms*. Ph.D. Thesis, The Chinese University of Hong Kong: Hong Kong.