Molec. Gen. Genetics 102, 187—195 (1968)

Segregation of Heterokaryons in the Asexual Cycle of *Phycomyces*

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Received June 4, 1968

Summary. Artificial heterokaryons between carotene mutants of *Phycomyces blakesleeanus* Bgff. have been prepared by squeezing cytoplasm out of two different mutant sporangiophores and allowing the fused droplets to regenerate.

These heterokaryons are used to study the distribution of nuclei at different stages of the asexual life cycle. It is proposed that the nuclear ratio is constant in all parts of the mycelium, sporangiophores and sporangia, and that random samples of nuclei are packaged into spores. This model permits quantitative predictions regarding the proportions of phenotypes in the asexual progeny and these predictions are corroborated by experiments. The nuclear ratio remains constant during repeated mycelial transfers.

Introduction

The genetics of *Phycomyces* has received little attention because of the long dormancy of the zygotes, the complication of their analysis and the scarcity of genetic markers. However *Phycomyces* is a good laboratory organism for the study of sensory perception mechanisms (reviews by Delbrück, 1963; Shropshire, 1963) and other complex biological problems. These developments encourage a renewed analysis of its genetic properties. In the heterogeneous collection of primitive fungi classed together as *Phycomycetes* few genetic studies have been made (Emerson, 1954).

This publication is concerned with the genetic properties of the asexual cycle of *Phycomyces blakesleeanus* Bgff. The multinucleate spores, upon germination form a mycelium, whose extensively branched hyphae do not contain septa or crosswalls and never fuse or anastomose, either with the same or with another mycelium. Long, unicellular sporangiophores grow out of the mycelium and form at their distal tips a globular sporangium containing a large number of spores. The sporangiophores exhibit responses to light, gravity, stretch, and other stimuli.

Some of the results to be discussed here were reported early in this century in a less quantitative or conclusive way. Burgeff (1912, 1914) discovered morphological variants in his *Phycomyces* stocks which probably arose from spontaneous mutations. These mutants occurred as heterokaryons with wild type and part of the spores of a heterokaryotic mycelium were heterokaryotic, in agreement with the cytological investigations of Swingle (1903) who showed that the spores incorporate groups of nuclei from the sporangium. Burgeff also developed an ingenious technique to produce heterokaryons from pure strains.

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The first stable mutants of *Phycomyces* were produced in 1966 by E. T. YOUNG (unpublished) and by S. GOODGAL and L. EDGAR (unpublished) after treatment with nitrosoguanidine. A wide variety of mutants are now available. We have used artificial heterokaryons between mutants blocked in the biosynthetic pathway for β-carotene to study quantitatively the distribution of nuclei during the normal vegetative cycle and during the transfer of small pieces of mycelium to new medium.

Materials and Methods

Strains. The strain 1555 (National Regional Research Laboratory) of *Phycomyces blakesleeanus* BGFF. is a sexually *minus* strain that has been used in many biophysical investigations. Several mutants blocked in the biosynthetic pathway for β-carotene were obtained after nitrosoguanidine mutagenesis as described below. To obtain zygotes these strains were plated together with the sexually *plus* strain 1554 NRRL.

Culture Methods. The minimal medium contains 30 g glucose; 2 g L-asparagine·H_2O; 0.5 g $MgSO_4 \cdot 7H_2O$; 1.5 g KH_2PO_4; 0.25 mg thiamine-HCl; 1000 ml distilled water. In the experiments to be reported here a complete medium was used, consisting of the above medium supplemented with 1 g yeast extract.

This medium was used in 1.2-ml shell vials, or, solidified with 10 g/l agar, in plastic plates and plastic trays (Model 96 SC, Lambda Sci. Corp.) containing 1.2-ml wells. The volume of medium was 30 ml in plates and 0.5 ml in vials and wells.

To insure germination the spores were heat-shocked at 48° for 15 minutes before inoculation of the nutrient medium (RUDOLPH, 1960). All cultures were incubated at 22° in an air-conditioned room. Spore stocks were prepared from plates with a dense crop of sporangiophores by washing the lid with about 5 ml distilled sterile water, centrifuging and resuspending twice and filtering the spore suspension through a cloth. To obtain the spores from a single sporangiophore the sporangium was picked up and squeezed with a tweezer carrying a drop of water between its tips and transferred to a tube with water. Spore suspensions may be kept for more than a year at 4°. To transfer mycelia, pieces of 1—2 mm diameter were cut out from the mycelium and laid on a new plate.

Mutagenesis. Vegetative spores at a concentration of 10^7 per ml were treated with 0.5 mg/ml of N-methyl-N′-nitro-N-nitrosoguanidine in 0.2 M acetate buffer, pH 5, for 80 minutes at 22°. The suspension was shaken occasionally. Survival is about 50%. The spores were washed several times by centrifugation and resuspension in water and seeded at a concentration of about 50 viable spores per agar plate. The first crop of sporangiophores was plucked and discarded, and the covers of the plates removed. The second crop of sporangiophores was screened for the presence of white or red mutants when they started to form sporangia, since the coloration is particularly conspicuous at this stage. The wild type, yellow sporangiophores around non-yellow sporangia were plucked, and the spores of the putative mutant were harvested the next day.

Heterokaryon Formation. The method described by WEIDE (1939) was found easier and faster than the original method of BURGEFF (1914) although for large scale experiments more reproducibility would be desirable. Two sporangiophores that have not yet formed sporangia are plucked from mycelia of different genetic type, placed side by side on an empty Petri plate and cut together in half. The bottom halves are removed; the top parts are squeezed out gently in such a way that their contents form a single droplet. Immediately a wet paper towel is placed in a small circle around them, the plate is covered and stored in the dark. After about 24 hours sporangiophores are formed by regenerating mixtures of two different carotene mutants. These sporangiophores are clearly yellow, indicating complementation in the production of β-carotene. In some cases the yellow color appears in the original mixture 4—6 hours after surgery. Some of the spores from the sporangiophores give rise to yellow mycelia, others to white or red mutant types. The mutant mycelia in subsequent generations produce only mutant mycelia of the same type, and are considered homokaryotic; the yellow mycelia have yellow and mutant progeny, and are considered heterokaryotic.

Viable Counting and Scoring. Spore titers are measured with the Petroff-Hauser chamber. Viable spores are counted by plating between 50 and 300 spores on agar plates and counting the mycelia 24—36 hours later.

For the determination of the proportions of spores producing different types of mycelia agar plate counts are not reliable because the mycelia spread widely with variable speeds. Instead the spore suspension is diluted and seeded into tubes or plastic trays in such a way as to obtain an average of about 0.2—2 viable spores per vial. The mycelia are scored when they start to form sporangia by determining the number of empty vials and the numbers of vials with mycelia of each color. The average number of viable spores per vial, m, is determined using Poisson's formula $e^{-m}=P(0)$, where $P(0)$ is the proportion of empty vials. The average number of each color is calculated applying the same relationship to the proportions of vials not containing that particular color. Thus, for example, the proportion of heterokaryotic spores coming from a heterokaryotic mycelium is determined by counting empty, yellow (including mixtures of white and yellow) and pure white vials, calculating $m=-\ln P$ *(empty)* and $m_y=-\ln P$ *(not yellow)*, and the proportion of heterokaryons or yellow is given by $h=m_y/m$.

Results

Carotene Mutants

Under the conditions described in Methods albino mutants of *Phycomyces* are found with a frequency of 1 to 2 per thousand survivors; red mutants are more scarce. Four mutants were used in this study, the albinos *alb 5*, *alb 10* and *alb 12* and the red *r 1*. The albino mutants form white mycelia, turning black with time; the young sporangia are white, becoming dark grey when mature. The red mutant *r 1* forms bright red mycelium, sporangiophores and young sporangia; many red lipid granules can be seen along its sporangiophores. The biochemical characteristics of these mutants have been investigated by MEISSNER and DELBRÜCK (in press) who found that, in relation to the β-carotene content of wild type, *alb 5* contains about 1% β-carotene, *alb 10* less than 0.1% β-carotene, *alb 12* about 1% lycopene and *r 1* about 320% lycopene. Except for their color these mutants resemble very much the wild type; however the sporangiophores of *alb 10* and *r 1* are thinner and not as long; those of *alb 12* are somewhat disturbed in their bending mechanics.

These mutants show abortive zygote formation with the sexually *plus* strain, except for the mutant *alb 5*, which still contains some β-carotene and is able to form a few viable zygotes. This finding supports the relationship of some of the sexual hormones of the *Zygomycetes* to the carotenoids (PLEMPEL, 1965; VAN DEN ENDE, 1967). At present only a small number of zygotes from a cross involving *alb 5* have been analyzed; the results indicate that the *alb 5* character is chromosomally inherited.

A Theory for Segregation

The observation that yellow heterokaryotic mycelia containing two types of carotene mutant nuclei produce some spores which give rise to yellow mycelia and some which form mycelia of the mutant types indicates that different types of nuclei may be included in the same spore. Our aim is the study of the distribution of nuclei in the different phases of the asexual life cycle. There are several events in the development of *Phycomyces* during which substantial alterations in the ratio of different types of nuclei may occur: the distribution of nuclei into germ tubes after germination of the spore; the frequent formation of new branches in the hyphae; the appearance of sporangiophores and sporangia; and the for-

mation of spores. Different assumptions on the behavior of the nuclei at these moments will lead to very different porportions of the different types of spores.

Our model for the behavior of the nuclei in the asexual cycle is based on the following assumptions:

1. The mycelium of *Phycomyces* is a wholly randomized coenocyte, in which all parts and branches contain the different types of nuclei in the same proportions.

2. The nuclei of each sporangiophore and its sporangium are derived from a large sample of mycelial nuclei, thus preserving the same nuclear proportions.

3. Nuclear division does not occur during or after spore formation.

4. The nuclei in the spores are random samples taken from the general population of nuclei.

Table 1. *Distribution of nuclei per spore in Phycomyces blakesleeanus strain NRRL 1555*

Number of nuclei n	Proportion of spores $f(n)$
1	0.003
2	0.090
3	0.420
4	0.410
5	0.074
6	0.003
$\geqq 7$	<0.001

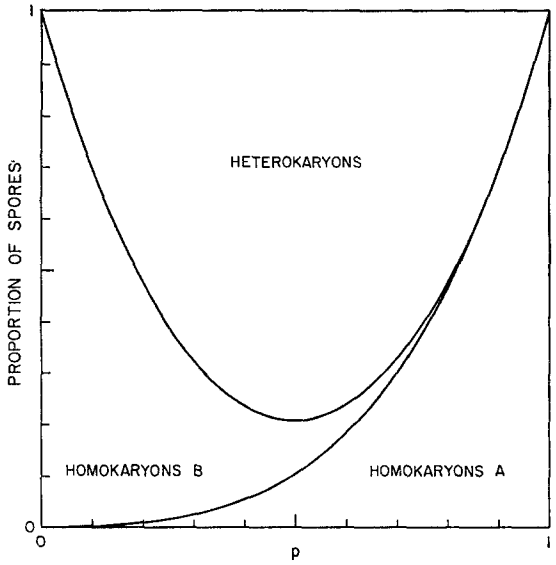

Fig. 1. Theoretical proportions of the types of spores formed by a heterokaryotic mycelium in which the number of nuclei per spore is distributed as in Table 1. Ordinates indicate the proportions of each type of spore as a function of the nuclear proportion p in the heterokaryon

This model permits specific quantitative predictions. If a mycelium has a proportion p of its nuclei of type A and $1-p$ of type B and forms spores in such a way that $f(n)$ is the proportion of spores with n nuclei, the proportion of spores containing only type A nuclei is $S_A = \sum_{n=1}^{\infty} p^n \cdot f(n)$, and type B, $S_B = \sum_{n=1}^{\infty} (1-p)^n \cdot f(n)$. The rest of the spores will contain both types of nuclei and will develop heterokaryotic mycelia. Fig. 1 gives the proportions of different types of spores as a function of p, using the actual distribution of nuclei per spore given in Table 1.

The values for S_A and S_B depend on a single parameter, p. Thus a relation exists between them that has to be fulfilled by every mycelium. This relation is plotted as the continuous curve of Fig. 2, and the values of the parameter p have been indicated on it.

Segregation Experiments

The predictions of the model depend on the distribution of nuclei per spore, $f(n)$. Measurements of this distribution have been done with several strains

(JOHANNES, 1950; HARM, unpublished, 1964). Our results, counting 2000 Feulgen-stained spores (ROBINSON, 1957) from several mutants and heterokaryons of strain 1555, all of which gave the same distribution, are summarized in Table 1.

To test our model we measured the proportions of spores of the homokaryotic types A and B formed by several different heterokaryotic mycelia; they are expected to follow the relation given in Fig. 2. The heterokaryon between *alb 5* and *r 1* was used because the two homokaryotic types, white and red, are clearly distinguishable even from those heterokaryons that have a very unbalanced nuclear ratio. This

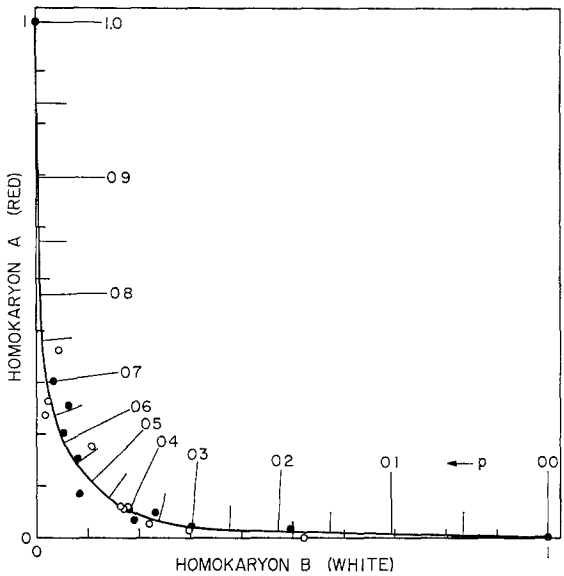

Fig. 2. Relation between the proportions of the two homokaryotic types of spores produced by heterokaryotic mycelia. The continuous curve represents the theoretical expectations from the model (see text); the values of the nuclear proportion p in the mycelium are indicated as a curve parameter along this line. The open and solid circles are the results of double determinations on ten different heterokaryotic mycelia containing *alb 5* and *r 1* nuclei

assumption was checked by collecting spores from mycelia scored as homokaryotic or heterokaryotic by their color and making sure that in fact the heterokaryons, but not the pure types, segregated in succeeding generations.

Spores of a heterokaryon between *alb 5* and *r 1* were plated on agar; 30 young mycelia were isolated by transfer to new plates and the spores of 10 of these mycelia having different shades of yellow were collected. The proportions of spores forming white, red and yellow mycelia were determined twice in the same stocks, and the values have been plotted in Fig. 2 by open and solid dots. The results are in agreement with the expected relationship between red and white types; and the errors of measurement seem to account for the observed dispersion around the theoretical curve, but minor deviations from it cannot be excluded.

The observations, then, support the model in general, and the "random packaging" method of spore formation in particular. Another feature of the model, the prediction that all regions of the mycelium have the same nuclear proportion p, was tested further by a different experiment. Since the nuclei, before being

distributed into spores, are grouped in isolated sporangiophores, any local fluctuation in the nuclear proportion would lead to different segregations in the spores coming from different sporangiophores.

It was observed that every sporangium of a heterokaryotic mycelium contains both heterokaryotic and homokaryotic spores. A more quantitative demonstration of the equivalence of different sporangia is reported in Table 2. We counted the proportion of heterokaryotic spores contained in each of five sporangia taken from different regions of a heterokaryotic mycelium containing

Table 2. *Proportion of heterokaryotic spores in five sporangia of the same heterokaryotic mycelium*

Each sporangium was scored on 768 agar wells of plastic trays, which may have some tendency to understimate the number of slow-growing albinos. The same mycelium was tested on glass tubes in experiment B, Fig. 3.

Yellow (with or without white)	All white	Empty	Proportion of heterokaryons
277	41	450	83.8
234	39	495	82.7
322	51	395	81.2
177	8	583	95.3
477	49	242	85.5

alb 10 and *alb 12* nuclei. The results show that different regions of the same heterokaryon contain the same nuclear proportions and provide direct support for the corresponding assumptions of our model.

The nuclear proportion p can be determined from the proportion of one type of homokaryotic spore (Fig. 1) or from the proportion of heterokaryotic spores, if one knows which is the more frequent homokaryotic type (Fig. 1) or from the relation of the two homokaryotic types (Fig. 2). Closely similar values were obtained by applying these methods to our scorings of mycelia used in the previous experiments.

Mycelial Transfer

In some experiments it is important to use a heterokaryon with a known nuclear proportion or to obtain large amounts of mycelia or spores from a certain heterokaryon. For this purpose we have studied the behavior of the nuclei during the transfer of pieces of mycelium to new medium, using heterokaryons with different proportions of *alb 10* and *alb 12* nuclei. In each transfer a piece of 1—2 mm diameter was laid on a new agar plate and allowed to grow. Spore stocks were prepared from the successive plates and the proportions of heterokaryons determined. The results (Fig. 3) indicate that the nuclear ratio of a heterokaryon is maintained for at least 8 mycelial transfers, which include more than 100 nuclear generations. However there are two important qualifications to be made. If the donor plate contains mature spores, the pieces of mycelium may become contaminated. Since the spores of *Phycomyces* are disseminated by contact, this contamination is probably avoided by not touching the sporangia. In experiment A, Fig. 3, a plate identical to the one scored as 8th transfer contained white streaks, which may have resulted from this form of contamination. On the other hand,

in experiment C only young mycelia, which had not yet formed spores, were transplanted by picking pieces of mycelium from their peripherial regions. Growth was normal after 7 transfers, but after the 8th transfer it was much less vigorous, and after the 9th it was very slow and completely sterile. This phenomenon may be similar to the "senescence" described in *Podospora anserina* (RIZET, MACOU and SCHECROUN, 1958).

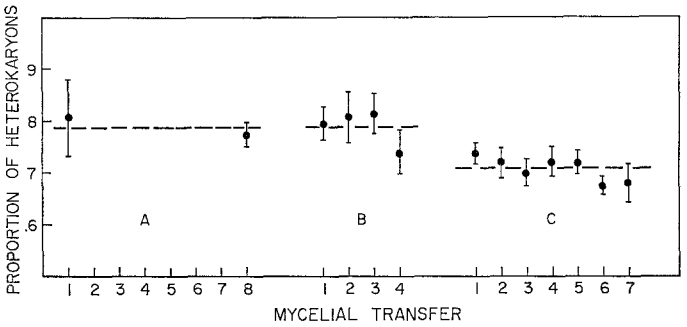

Fig. 3. Influence of mycelial transfer and continuous growth on the nuclear ratios of heterokaryotic mycelia of *alb 10* and *alb 12*. Pieces of mycelium about 1—2 mm in diameter were transferred to new plates. The donor mycelia in experiments A and B were 5 to 7 days old, well established and with abundant spore formation; in experiment *C* the donor mycelia were only 1 to 2 days old, with a diameter of about 4 cm and had not yet formed sporangiophores. In each plate the proportion of heterokaryotic spores produced by the mycelium was scored using 700 vials; the brackets represent estimates of the standard error

Discussion

Natural heterokaryosis in *Phycomyces* does not occur by fusion of vegetative hyphae, as in other genera of fungi. Methodologically this is very convenient, since it guarantees that each mycelium and its spores stem from a single spore. However heterokaryosis can arise sometimes by mutation or by lack of segregation in the sexual cycle (BLAKESLEE, 1906; BURGEFF, 1912, 1914, 1928; ORBAN, 1919; HARM, 1964, unpublished). In fact it was BURGEFF working with *Phycomyces*, who discovered this peculiarity of multinucleate cells.

Random packaging of groups of nuclei in heterokaryotic vegetative sporangia may lead to high proportions of heterokaryotic spores. In contrast the sporangiophores formed by germinating zygotes, which are very similar to the vegetative ones in appearance and behavior and which are necessarily heterokaryotic since they contain the meiotic products, are known to produce nearly always homokaryotic spores. A different process has to be postulated for spore formation in the zygotesporangia which avoids the production of heterokaryotic spores.

The artificial formation of heterokaryons in *Phycomyces* opens the way for the analysis of complementation, which will be particularly useful in the studies of sensory physiology. Heterokaryosis has permitted the crossing of carotene mutants which are sexually inactive, and the propagation of recessive mutants which do not form viable spores.

The method of artificial heterokaryon formation also permits mixing the cell contents of one sporangiophore with cytoplasmic fractions or subcellular particles of another (ZALOKAR, 1968, unpublished) or introducing substances for which the

cell is not permeable. This system may be fruitful in the study of membrane organization and regeneration.

Our model for segregation requires that the nuclear ratio in the sporangiophores be the same as in the mycelium. Each sporangiophore must receive from the mycelium a number of nuclei large enough for the statistical fluctuation in the nuclear ratio not to be detected. The inaccuracy of the measurements permits this number (about 50) to be small compared with the more than 3×10^5 nuclei included in the mature sporangiophore. It has been suggested that all the nuclei in the sporangiophores are formed in the mycelium: DAVID (1967, unpublished) found that efficient labeling of sporangiophores with (^{32}P)phosphate requires addition of the labeled compound before the onset of sporangiophore formation and WAGENMANN (1964, unpublished) did not detect nuclear divisions in the sporangiophores.

Heterokaryosis may play an interesting role in the adaptability and evolution of this species in nature. Under normal circumstances the segregation of a heterokaryon of the strain studied here produces from 45% ($p=1/6$ is the minimum in a spore with six nuclei) to 80% heterokaryotic spores. In the absence of selection, the proportion of heterokaryons will rapidly diminish; selection in favor of the heterokaryons may maintain a polymorphism, with a considerable proportion of the population being heterokaryotic and the continuous outflow of homokaryotic segregants permitting rapid selection of non-heterotic genes. The proportion of homokaryotic segregants and, consequently, the selective pressure needed to maintain heterokaryosis may be regulated by changing the distribution of nuclei per spore; different strains of *Phycomyces* are in fact known to contain different numbers of nuclei per spore.

Significant differences from randomness were found in the distribution of nuclei in *Neurospora* heterokaryons (PROUT, HUEBSCHMAN, LEVENE and RYAN, 1953; ATWOOD and MUKAI, 1955) and there occur also long term fluctuations of the nuclear ratio during mycelial growth (GRINDLE and PITTINGER, 1968). The problems of nuclear behavior in fungal heterokaryons and their implications have been reviewed by DAVIS (1966). In contrast to the other known systems, *Phycomyces* appears to follow closely the hypothesis of a completely random distribution of nuclei during mycelial growth, during mycelial transfer and during spore formation.

Acknowledgements. We thank Prof. MAX DELBRÜCK for valuable criticism and discussions and Miss PATRICIA REAU for excellent technical assistance. This work was supported by a grant from the U.S. National Science Foundation, No. GB 4642, and a fellowship from the Max-Planck-Gesellschaft, West Germany.

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