Genet. Res., Camb. (1977), **30**, *pp.* 211–219
With 1 text-figure
Printed in Great Britain

Continuous variation of genic dosage in *Phycomyces*

By JUAN RAMON MEDINA

Departamento de Genetica, Facultad de Ciencias,
Universidad de Sevilla, Spain

(*Received* 8 *December* 1976)

SUMMARY

A computer simulation of the genetic analysis of the asexual life cycle of *Phycomyces*' heterokaryons has been carried out. We have studied experimentally the relationship between the nuclear proportion in heterokaryotic mycelia containing prototrophic and auxotrophic nuclei and their growth rates. We discuss possible evolutionary implications of heterokaryosis in coenocytic fungi and the genetic applications of the quantitative complementation technique.

1. INTRODUCTION

The hyphae of the fungus *Phycomyces blakesleeanus* lack transverse walls and their nuclei are completely intermixed by strong cytoplasmic streaming (Bergman *et al.* 1969). The mycelium grows radially and emits aerial sporangiophores, each with its own apical sporangium full of nonmotile, asexual spores containing 1–6 nuclei. The frequencies, $f(1), f(2), \ldots, f(n)$, of spores with $1, 2, \ldots, n$, nuclei are known (Heisenberg & Cerda-Olmedo, 1968).

In *Phycomyces* it is possible to obtain heterokaryons between any two strains, A and B (Ootaki, 1973). The proportion, p, of A-type nuclei in the heterokaryotic mycelium can assume initially any value between 0 and 1. As the spores are produced by packing the nuclei of the sporangium at random, 3 types of spores occur in heterokaryotic sporangia: A-type homokaryotic spores, B-type homokaryotic spores, and heterokaryotic spores, with frequencies S_A, S_B and S_H respectively. The sporangium and the mycelium have similar nuclear proportions:

$$S_A = \sum_{n=1}^{n=6} p^n f(n); \quad S_B = \sum_{n=1}^{n=6} (1-p)^n f(n); \quad S_H = 1 - \sum_{n=1}^{n=6} (p^n + (1-p)^n) f(n). \quad (1)$$

These equations allow us to estimate the nuclear proportion in any heterokaryotic mycelium from the frequencies of heterokaryons and both types of homokaryons in its progeny. So, in *Phycomyces*, the phenotypic effect of a continuous variation of the genic dosage can be studied by using heterokaryons between two strains with quantifiable phenotypic differences.

This type of study has been applied, up till now, to heterokaryons between the wild-type and mutants with altered phototropism (Cerda-Olmedo & Medina,

1976) and to heterokaryons between mutants with abnormal β-carotene synthesis (De la Guardia *et al.* 1971).

In this work we have investigated the mycelial growth rate as a function of the nuclear proportion in a heterokaryon between an auxotrophic strain, S80, and a prototrophic strain, S18. Mutations affecting the synthesis of β-carotene (Meissner & Delbrück, 1968) were used as genetic markers. A nicotinamide-requiring mutant, S102, was isolated after mutagenesis of the wild type with nitrosoguanidine. Heterokaryons containing Nic^- and revertant nuclei were isolated after mutagenesis of S102 with nitrosoguanidine. The maximal proportion of auxotrophic nuclei compatible with normal growth on minimal medium was studied in these heterokaryons.

Since the methods used in this and other works for estimating the nuclear proportions have several sources of error, we have first investigated its reliability by computer simulation of the actual process.

2. MATERIALS AND METHODS

(i)

The heterokaryon S18 (*carA*57 *madC*202 (−))*S80 (*carR*21 *aux* (−)) was used. The S80 strain is unable to convert lycopene into β-carotene, due to the *carR* mutation, and, as a consequence, has a red mycelium; the mycelium of S18 is white because, due to the *carA* mutation, it synthesizes less than 1% of the normal amount of β-carotene; the heterokaryotic mycelia S18*S80 are reddish-yellow, as they accumulate, according to their nuclear proportions, different amounts of lycopene and β-carotene.

The auxotrophic strain does not grow on a minimal medium containing (per litre of distilled water), glucose, 30 g; L-asparagine, 2 g; $MgSO_4.7H_2O$, 0·5 g; H_2KPO_4, 1·5 g; thiamine-HCl, 0·25 mg. The strain S80 grows on a rich medium obtained by supplementing the minimal medium with yeast extract (1 or 10 g/l). All media were prepared after Bergman *et al.* (1969).

Single-spore mycelia, isolated after plating about 20 spores/plate on acid medium, pH = 3·4, where the growth is colonial, were replicated three times on both rich and minimal medium, and the surface covered by the mycelia in four days was measured.

For genetic analysis, spores from each mycelium were collected and dispersed in rich liquid medium. Each suspension was distributed, with a repetition syringe, into 800 2 ml vials. The average number of spores per vial was intended to be about 1. After 8 days of incubation, the numbers of vials containing: no mycelia (*a*), white mycelia (*b*), red mycelia (*c*), white and red mycelia (*d*), and yellow mycelia (*e*) mixed or not with homokaryotic mycelia, were counted. It is not easy to distinguish the pure red or white mycelia when they grow in the same vial with a reddish-yellow heterokaryotic mycelium; so we cannot subdivide with confidence group (*e*) into those vials with and without red mycelia and those with and without white mycelia. Assuming that the number of spores per vial follows

a Poisson distribution, the average number of viable spores per vial will be: $m = -\ln(a/(a+b+c+d+e))$. The average number of heterokaryotic spores per vial will be: $m_H = -\ln((a+b+c+d)/(a+b+c+d+e))$; the proportion of heterokaryotic spores, then, will be: $S_H = m_H/m$. For example, from mycelium D (Table 2) we obtain: $m = -\ln(427/800) = 0{\cdot}627$; $m_H = -\ln((427+18+52+1)/800) = 0{\cdot}474$; $S_H = 0{\cdot}474/0{\cdot}627 = 0{\cdot}756$. The empirical value of S_H enables us to estimate the nuclear proportion, p, by solving the equation:

$$1-S_H-\sum_{n=1}^{n=6}(p^n+(1-p)^n)f(n) = 1-S_H-\sum_{n=1}^{n=6}((0{\cdot}5+X)^n+(0{\cdot}5-X)^n)f(n) = 0;$$

where $X = p-0{\cdot}5$. The $S_H = F(X)$ curve is symmetrical around the $X = 0$ axis, since changing X for $-X$ does not change the value of S_H. Thus, for every solution, p_1, such that $0 < p_1 < 0{\cdot}5$, there is another solution, p_2, such that $0{\cdot}5 < p_2 < 1$ and $p_1+p_2 = 1$. Moreover, there is only one pair, p_1, p_2, of positive, real, symmetrical, solutions of the above equation between 0 and 1. Since

$$d(S_H-F(p))/dp = \sum_{n=1}^{n=6}((1-p)^{n-1}-p^{n-1})\,nf(n))$$

is positive when $0 < p < 0{\cdot}5$ and negative when $0{\cdot}5 < p < 1$ the $S_H = F(p)$ curve increases monotonically when $0 < p < 0{\cdot}5$ and decreases monotonically when $0{\cdot}5 < p < 1$ (having a maximum for $p = 0{\cdot}5$), and, therefore, there is only one value of p between 0 and 0·5 such that $F(p)-S_H = 0$.

In this work, the values of p_1 and p_2 were obtained by an interative method based on the fact that the expression $(F(p)-S_H)\,(F(p+\Delta p)-S_H)$ must be positive if neither p_1 nor p_2 or if both p_1 and p_2 are between p and $p+\Delta p$ and negative if only one solution is in that interval. A computer program obtains the successive values of the above expression, setting $\Delta p = 0{\cdot}01$ and $p_{i+1} = p_i+\Delta p$; $p_0 = 0$. When the first value of p, for which the expression is negative, is obtained, seting $\Delta p = 0{\cdot}001$ allows us to obtain an estimate of p differing from the true value by less than 10^{-3}. Evidently, by taking smaller Δp this difference may be reduced to any prefixed level of accuracy. There are simpler graphical methods that allow us to obtain an estimate of p directly from the value of S_H (Heisenberg & Cerda-Olmedo, 1968) but they are less accurate.

Once p_1 and p_2 have been estimated, we can decide which is the correct value of p by comparing the values of b and c. For example, the S_H values of mycelia A and F are 0·358 and 0·357. The corresponding values of p_1 and p_2 are, in both cases, 0·15 and 0·85. We decide that the correct p values are 0·85 for A and 0·15 for F since $101 > 1$ and $1 < 107$, respectively.

(ii) *Analysis of* S102 *revertants*

Spores of NRRL1555 (−) were collected and mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine according to Bergman *et al.* (1973). The mutagenized spores were plated on rich acid medium at about 20 survivors/plate. The survival rate was 3·3 %. After 5 days of growth on rich medium a total of 2603 survivors

were transplanted to minimal medium. An auxotrophic strain, designated S102, was isolated. Addition of several metabolites allowed us to determine that S102 grows on minimal medium supplemented with nicotinic acid or nicotinamide (1 μg/ml). S102 (*nicA 101* (−)), does not grow on minimal medium supplemented with kynurenine, formylkynurenine, 3-hydroxy-anthranilic acid or quinolinic acid. Either the biosynthetic pathway of nicotinamide is different in *Phycomyces* from *Neurospora* or the *nicA* gene affects a reaction subsequent to quinolinic acid synthesis.

Spores of S102 were collected, incubated on rich liquid medium for three hours and then washed three times, resuspended in citrate–phosphate buffer, pH = 7·5, and mutagenized as above. The mutagenized spores were plated on minimal acid medium at about 10^5 survivors/plate. After five days several revertants were isolated. The experiment was repeated, and in both cases no spontaneous revertants were isolated, indicating that the frequency of spontaneous reversion was less than $1/6 \times 10^6$ survivors. Spores from 20 normal-growing revertants were collected and plated on both rich and minimal acid medium at about 20 spores/plate. A total of 30 descendants of each original revertant were transplanted to minimal medium and the numbers of growing and non-growing transplants were counted.

(iii) *Simulation of genetic analysis*

The program simulating the genetic analysis has four parts. Each part uses as input data the output of the preceding part. The random processes were simulated with the RANDU subroutine, producing pseudorandom numbers uniformly distributed in the 0–1 interval. The input data of the first part are the empirical values of the frequency, $f(n)$, of spores containing n nuclei and a known nuclear proportion, p. This part obtains a sample of N spores drawn at random from an infinite population where the frequency of each type of spore is deterministically derived from the initial p value by means of equation (1). The second part of the program simulates the distribution of the N spores into M vials, $1/M$ being the *a priori* probability of each spore falling into a particular vial. This part also classifies the vials according to the types of spores received. The third part, by means of the Poisson distribution approximation described, calculates the average numbers of spores per vial and of heterokaryotic spores per vial, and estimates the value of p. The whole process was simulated 20 times for each set of values of N ($100 \leqslant N \leqslant 2000$, $\Delta N = 100$), M ($100 \leqslant M \leqslant 2000$, $\Delta M = 100$) and p ($0 < p < 1$, $\Delta p = 0{\cdot}1$).

3. RESULTS

Table 1 presents some of the results of 20 independent estimates of the nuclear proportion. The mean of the distribution of the estimates is, in all cases, very similar to the corresponding initial value. The distribution has, in all cases, a small standard deviation. When all results of the simulation are taken into account, it is possible to conclude that the estimation of the nuclear proportion is reliable

Table 1. *Computer simulation of the genetic analysis of* Phycomyces *heterokaryons*

N	$p = 0{\cdot}1$	$p = 0{\cdot}2$	$p = 0{\cdot}3$	$p = 0{\cdot}4$	$p = 0{\cdot}5$	
200	0·1100	0·1890	0·2915	0·3970	0·4995	
500	0·1004	0·1942	0·2915	0·3955	0·5010	
800	0·0985	0·1943	0·3004	0·3975	0·4985	Mean
1100	0·0993	0·1920	0·2985	0·3845	0·4872	
1400	0·0996	0·2005	0·3005	0·3857	0·4877	
200	0·0309	0·0416	0·0249	0·0348	0·0268	
500	0·0213	0·0236	0·0239	0·0233	0·0268	
800	0·0267	0·0288	0·0176	0·0170	0·0198	S.D.
1100	0·0326	0·0184	0·0209	0·0260	0·0222	
1400	0·0209	0·0182	0·0251	0·0332	0·0275	

Mean and standard deviation of the distribution of the 20 independent estimates of the initial nuclear proportion (p) for each value of the number of spores analysed (N). In all cases, $M = 800$.

Table 2. *Genetic analysis of the S18*S80 heterokaryon*

Mycelium	a	b	c	d	e	p
A	631	101	1	0	64	0·85
B	298	87	10	1	404	0·68
C	623	12	19	1	136	0·46
D	427	18	52	1	302	0·40
E	430	7	86	1	271	0·29
F	624	1	107	0	68	0·15

From each original heterokaryotic mycelium (A, B, ..., F) spores are collected, dispersed in rich liquid medium and the volume inoculated into 800 vials. After 8 days, the numbers of vials without mycelia (a), with S18 mycelia (b), with S80 mycelia (c), with S18 and S80 mycelia (d), and with heterokaryotic mycelia (e) are counted. From these numbers the proportion of S18 nuclei (p) in the original mycelium may be estimated as described in Materials and Methods.

if the total number of spores analysed is 200 or more, provided the average number of spores per vial is not greater than 1. So, if the number of vials tested is $M = 800$, the estimates are reliable if m (the average number of spores per vial) lies between 0·25 and 1.

Table 2 shows some of the results of the genetic analysis of the heterokaryon S18*S80. In Materials and Methods we describe how to get the value of the nuclear proportion from the numbers $a, b, \ldots, e$. The observed variations in the numbers of vials without mycelia (a) are due to differences in the viability of the spores, which is not related to the nuclear proportion. In Fig. 1, the growth rates of different mycelia of the S18*S80 heterokaryon are represented versus their respective proportions of prototrophic nuclei. On rich medium, the auxotrophic homokaryon, the prototrophic homokaryon and the heterokaryons grow at the same rate. On minimal medium, the auxotrophic homokaryon does not grow at all and those heterokaryons whose proportions of prototrophic nuclei are greater

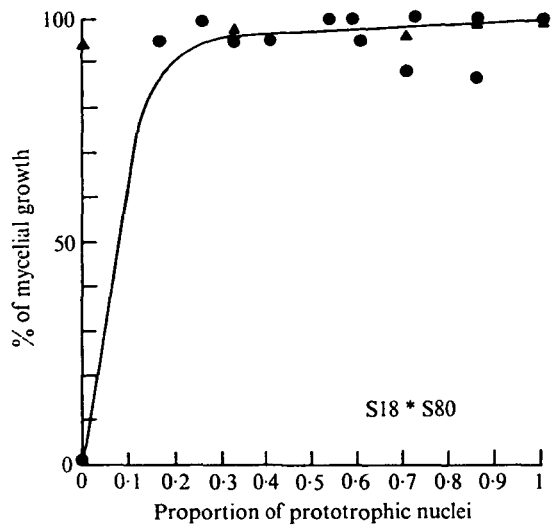

Fig. 1. Growth rates on minimal medium (●) and on rich medium (▲) of different mycelia of the S18*S80 heterokaryon as a function of their respective proportions of S18 nuclei. The growth rates are measured as the quotient in % between the growth area of the heterokaryotic mycelium in 4 days and the growth area of the S18 homokaryon in the same time and on the same medium.

than 0·15 grow at the same rate as the prototrophic homokaryon. Thus, in this test, the *aux* mutation is completely recessive.

For simplicity, a linear relationship between the growth rate and the nuclear proportion, for $0 < p < 0{\cdot}15$, has been arbitrarily assumed, but a sigmoidal or more complex relationship cannot be discarded. Since the maximum number of nuclei per spore is 6, the minimal value of the nuclear proportion in any heterokaryon arising in the asexual progeny of a mycelium is 0·166. More deviant heterokaryons would be expected in the progeny of mutagenized spores which have experienced at least 1 nuclear division prior to the mutagenic treatment. So the proportion of auxotrophs in the progeny of 20 revertants of S102, isolated after mutagenesis of spores grown for 3 hours in rich medium, varied between 0/30 and 28/30. A frequency of 28/30 auxotrophs in the progeny of a revertant would be expected when the proportion of auxotrophic non-reverted nuclei in the mycelium is about 0·9. Thus 10 % of the normal dosage of the *nicA* product allows normal growth.

4. DISCUSSION

The results of the simulation of the genetic analysis of *Phycomyces* heterokaryons show that the accuracy of the estimation of the nuclear proportion depends basically on two factors: the total number of spores analysed and the average number of spores per vial. As M increases the minimal value permissible and the optimal value of m decrease. For example, with $M = 1600$ the optimal value of m would be 0·5 and its minimal value 0·125. This is because as M increases and

m decreases the distribution of spores per vial will approach more closely the assumed Poisson distribution, while the reliability will not decrease since the total number of spores analysed would remain, on the average, constant. The value $M = 800$ used in this work represents a practical compromise between the amount of experimental work needed to get a p estimate and the size of the sample of spores analysed needed to restrict errors due to random fluctuations.

The proportion of heterokaryotic spores in the progeny of a mycelium decreases as its nuclear proportion approaches 0 or 1; therefore, the optimal value of m is greater for deviant heterokaryons. However, since the proportion of heterokaryotic spores is greater than 0·3 for all the possible heterokaryons, this dependence of m on the unknown parameter p may be ignored.

Though our simulation experiments delimit the conditions where the mathematical method of p estimation is reliable, they are not concerned with the validity of the underlying biological hypotheses. In *Phycomyces* their correctness seems to be experimentally verified (Cerda-Olmedo & Reau, 1970) but in *Neurospora* the proportion of homokaryotic spores is, frequently, greater than expected (Atwood & Mukai, 1955; Klein, 1958). Since the accuracy of the p estimate depends strongly on the $f(n)$ values, the distribution of nuclei in the spores has been carefully studied in several mutants and heterokaryons of *Phycomyces* and no variation of the wild type distribution has been observed (Johannes, 1950; Heisenberg & Cerda-Olmedo, 1968; Harm, unpublished results). In any case, these possible differences would not affect appreciably our conclusions since the growth rate is independent of the nuclear proportion in the studied range. To apply safely the quantitative complementation technique to any problem, the best solution would be to employ only unicleate spores. The isolation of these spores is already feasible in *Phycomyces* (Reau, 1972). Another critical point is the assumed equality between the nuclear proportion in the mycelium and in the spore-producing organ. When the mycelium is not a coenocyte and the number of nuclei per cell is small, it would be better to seed fragmented mycelium (Snider, 1963).

While in diploid cells only three values (0, 0·5 and 1) are possible, in coenocytic mycelia the proportion of each allele may assume any value between 0 and 1. Burgeff (1914) postulated that the phenotype of a heterokaryon may change according to its nuclear proportion; later, Pittenger & Atwood (1956) proved that, in *Neurospora*, the growth rate of a heterokaryon between the wild type and a pantothenate-requiring mutant is a function of its nuclear proportion. The heterokaryon was proved to grow normally for proportions of the prototrophic nuclei greater than 5 %. We have obtained similar results with *Phycomyces*. The S18*S80 heterokaryon grows normally for proportions of prototrophic nuclei greater than 15 %. A heterokaryon containing nicotinamide-requiring and prototrophic revertant nuclei grows normally though its proportion of prototrophic nuclei is as small as 10 %. In all three cases, for most of the nuclear proportion range the heterokaryons' growth is similar to the wild type growth. If this is the rule, allelic interactions in coenocytic fungi would protect transiently a part of genetic variability from the action of natural selection.

In S18*S80 we have not observed colour differences between young and old mycelia or between different mycelial sectors or between different replicates of each mycelium. So we conclude that the nuclear proportion of each mycelium is very stable. This implies that the rates of division of S18 and S80 nuclei are very similar and that cytoplasmic streaming is very effective in mixing nuclei. In the S18*S80 system, natural selection can only operate between mycelia with different initial nuclear proportions. The peculiarities of the asexual life cycle of *Phycomyces* would produce a total extinction of heterokaryons not favoured by natural selection. A pair of balanced lethal mutations would be an extreme case of such a selective advantage. Four of the twenty S102 revertants analysed do not segregate auxotrophs. They may be prototrophic homokaryons or heterokaryons with balanced lethal mutations. We do not know the extent of forced genetic variability in natural strains of *Phycomyces*.

Classically, heterokaryosis has been applied to qualitative complementation studies, aiming to elucidate whether two recessive mutations are or are not allelic. The quantitative study of allelic interaction is not restricted to recessive mutations and provides information about the type of gene mutated (structural, regulatory, etc.), and about the genic product behaviour (De la Guardia *et al.* 1971). For example, the *aux* and *nicA* gene products act probably through diffusible molecules that are not part of a molecular aggregate; otherwise the mutations would be more dominant. Since 15% of the normal gene dosage allows in both cases for a normal phenotype, the activities of the normal alleles of the two genes must be regulated or in excess in the wild type in relation to physiological needs.

The author wishes to acknowledge the contribution of E. Cerda-Olmedo to the present work and to thank him for allowing the experiments to be carried out in his laboratory.

REFERENCES

ATWOOD, K. C. & MIKAI, F. (1955). Nuclear distribution in conidia of *Neurospora* heterokaryons. *Genetics* **40**, 438–443.

BERGMAN, K., BURKE, P. V., CERDA-OLMEDO, E., DAVID, C. N., DELBRÜCK, M., FOSTER, K. W., GOODELL, E. W., HEISENBERG, M., MEISSNER, M., ZALOKAR, M., DENNISON, D. S. & SHROPSHIRE, J. R. (1969). *Phycomyces. Bacteriological Reviews* **33**, 99–157.

BURGEFF, H. (1914). Untersuchungen über Variabilitat, Sexualitat und Erblichkeit bei *Phycomyces nitents*: Kunze. *Flora (Jena)* **107**, 259–316.

CERDA-OLMEDO, E. & MEDINA, J. R. (1976). Quantitative measurement of genic interaction in *Phycomyces* phototropism. Submitted for publication.

CERDA-OLMEDO, E. & REAU, P. (1970). Genetic classification of the lethal effects of various agents on heterokaryotic spores of *Phycomyces*. *Mutation Research* **9**, 369–384.

DE LA GUARDIA, M. D., ARAGON, C. M. G., MURILLO, F. J. & CERDA-OLMEDO, E. (1971). A carotenogenic enzyme aggregate in *Phycomyces*: evidence from quantitative complementation. *Proceedings of the National Academy of Sciences* **68**, 2012–2015.

HEISENBERG, M. & CERDA-OLMEDO, E. (1968). Segregation of heterokaryons in the asexual cycle of *Phycomyces*. *Molecular and General Genetics* **102**, 187–195.

JOHANNES, H. (1950). Ein sekundäres Geschlechtsmerkmal des Igogamen *Phycomyces blakesleeanus. Bgff. Biologisches Zentralblatt* **69**, 463–468.

KLEIN, D. T. (1958). Randomness of nuclear distribution in conidia of *Neurospora* heterokaryons. *Zeitschrift für Vererbungslehre* **89**, 323–327.

MEISSNER, G. & DELBRÜCK, M. (1968). Carotenes and retinal in *Phycomyces* mutants. *Plant Physiology* **43**, 1279–1283.

OOTAKI, T. (1973). A new method for heterokaryon formation in *Phycomyces*. *Molecular and General Genetics* **121**, 49–56.

PITTENGER, T. H. & ATWOOD, K. C. (1956). Stability of nuclear proportions during growth of *Neurospora* heterokaryons. *Genetics* **41**, 227–241.

REAU, P. (1972). Uninucleate spores of *Phycomyces*. *Planta* **108**, 153–160.

SNIDER, P. J. (1963). Estimation of nuclear ratios directly from heterokaryotic mycelia in *Schizophyllum*. *American Journal of Botany* **30**, 255–262.