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GENETIC VARIATION OF MORPHOLOGICAL CHARACTERS WITHIN A SINGLE ISOLATE OF THE ENDOMYCORRHIZAL FUNGUS *GLOMUS CLARUM* (GLOMACEAE)[1]

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The nature of variation in morphological characters in spores of arbuscular endomycorrhizal fungi (Order Glomales, Class Zygomycetes) has received little attention, despite the importance of these characters in modern taxonomy of the order. We tested the hypothesis that genetic variation exists in spore size and color (presumably important taxonomic characters) within a single isolate of the glomalean fungus *Glomus clarum*. Phenotypic variation in size and color of spores was determined from a pot culture population (designated P). A 10% selection pressure was imposed on replicate pot cultures of the first progeny culture generation (G1) by selecting the smallest, largest, yellowest, and whitest spores from the P generation and inoculating *Sorghum bicolor* plants. The experiment was repeated for another generation (G2), but with a 5% selection pressure. In both the G1 and G2 generations, significant differences in spore size and color were observed among the various treatments, indicating substantial genetic variation in these characters. Despite efforts to keep the physical environment constant across generations, we observed variation in the overall means of spore size and color among the generations (regardless of treatment), indicating a strong nongenetic influence on character expression. This study provides empirical evidence that will help delimit species boundaries among isolates of *Glomus clarum* and similar morphospecies. It also demonstrates a promising method to help elucidate the nature of character diversity in obligately asexual fungi.

Key words: arbuscular mycorrhizal fungi; asexual fungi; directional selection pressure; Glomales; quantitative genetics.

Arbuscular mycorrhizal (AM) fungi (Order Glomales, Class Zygomycetes) are an important group of ubiquitous soil-borne fungi that assist plants in nutrient acquisition. The fungi colonize the roots of most higher plant species (Trappe, 1987) and effectively increase the volume of soil that can be explored by the plant. The fungi absorb inorganic soil nutrients, most notably phosphorus, which are translocated to the plant host in exchange for photosynthetically fixed carbon. These fungi are often important in plant growth and reproduction, and may influence plant competition, especially in nutrient-limiting soils (Allen, 1991). The impact of the fungi on plants and plant community processes also likely depends upon the identity of the fungi associated with the plants, since different fungal species may vary in their effectiveness at plant growth promotion (Powell, Clarke, and Verberne, 1982; Ollivier et al., 1983; Wilson, 1988; Ravnskov and Jakobsen, 1995), and in their ability to bind soil particles into microaggregates (Miller and Jastrow, 1994). Our ability to investigate these issues depends heavily upon our developing knowledge of the taxonomy of these fungi.

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Morphological characters of spores, such as size, color, and wall structure are of taxonomic importance in AM fungi. A clear understanding of the nature of character variability is essential to evaluate current species concepts and taxonomic schemes. Progress has been made in understanding the nature of characters with ontogenetic techniques (Franke and Morton, 1994; Bentivenga and Morton, 1995; Morton, 1995; Stürmer and Morton, 1997), but the extent of genetic control on character variation in these fungi still remains poorly understood. Traditional genetic analyses of AM fungi have been difficult because they reproduce asexually and are obligate symbionts (culturable only in association with host plant roots).

Different populations of a single species of an AM fungus can vary in ecotypic traits such as drought tolerance (Stahl and Christensen, 1991) and tolerance to heavy metals (Weissenhorn, Leyval, and Berthelin, 1993). Furthermore, individual isolates within a species can also differ in the relative benefit conferred to a host plant (Stahl and Smith, 1984; Louis and Lim, 1988; Boerner, 1990; Sylvia et al., 1993). At more basic levels, isolates within a single species of AM fungus have been reported to vary in isozyme banding patterns (Hepper et al., 1988) and random amplified polymorphic DNA (RAPD) markers (Wyss and Bonfante, 1993). While variation in these characters is indicative of genetic variation, their heritability has not been examined in AM fungi.

Recently, J. D. Bever and J. B. Morton (unpublished data) examined the variability and inheritance of spore shape within a single isolate of *Scutellospora pellucida*. Replicate single-spore cultures, started each from either

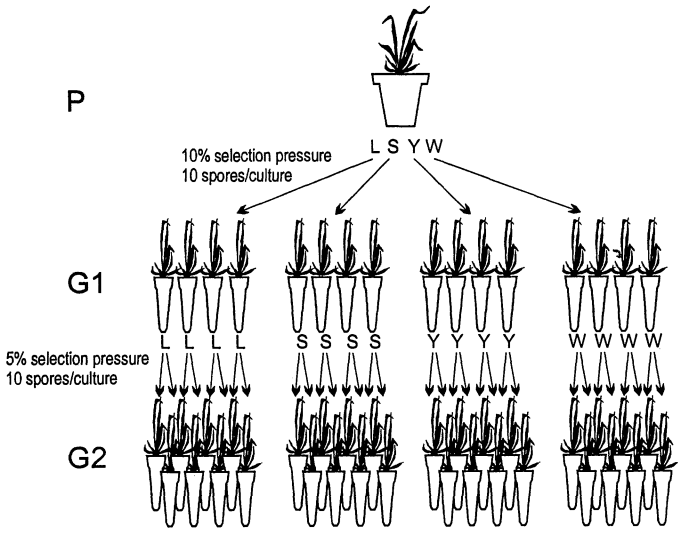

Fig. 1. Graphical representation of the experimental design. L = largest spores; Y = yellowest spores; W = whitest spores; S = smallest spores. See text for details.

subglobose or elliptical spores, produced progeny populations that were significantly more globose or elliptical, respectively. This was the first study to show that morphological variation among individuals in a population is heritable and can respond to selection pressure.

In the present study, we employed quantitative genetic methods to test the hypothesis that genetic variation exists in spore size and color (presumably important taxonomic characters) within a single isolate of the glomalean fungus, *Glomus clarum* Nicolson & Schenck. We further examined how these characters respond to imposed directional selection pressure in an effort to understand how genetic and environmental components interact to influence morphological character expression. This study represents a first step toward separating these components relative to taxonomic characters in glomalean fungi, an essential task for delineation of species boundaries.

MATERIALS AND METHODS

Glomus clarum isolate FL979 from the International Culture Collection of Arbuscular and VA Mycorrhizal Fungi (INVAM, West Virginia University) was grown in pot culture on *Sorghum bicolor* (L.) Moench. This and all subsequent cultures were grown under controlled light (175 μmol photons·m^{-2}·s^{-1} photosynthetically active radiation, measured at the soil line; 16 h daylength) and temperature (26° ± 3°C) conditions. The culture medium consisted of two parts (v/v) of a number 3 quartzite sand (mean particle size 0.9 mm) and one part soil (Lily series, 0.9% organic matter, pH 5.5, 8.0 μg/g bicarbonate-extractable phosphorus). Prior to use, the mixture was steam-pasteurized at 100°C for two 1-h periods separated by a 24-h cooling period. The pH of the pasteurized growth medium was adjusted to 6.2 by adding $CaCO_3$ and allowing 2 wk for equilibration.

Spores were extracted after 4 mo using wet sieving and sucrose gradient centrifugation (Daniels and Skipper, 1982). Spore preparations were further cleaned by individually pipetting spores into watch glasses containing distilled/deionized water. Two hundred freshly extracted spores of the parental culture generation (P) were examined to determine phenotypic variation in size (measured with an ocular micrometer) and color. Color was quantified by comparison with a printed color chart

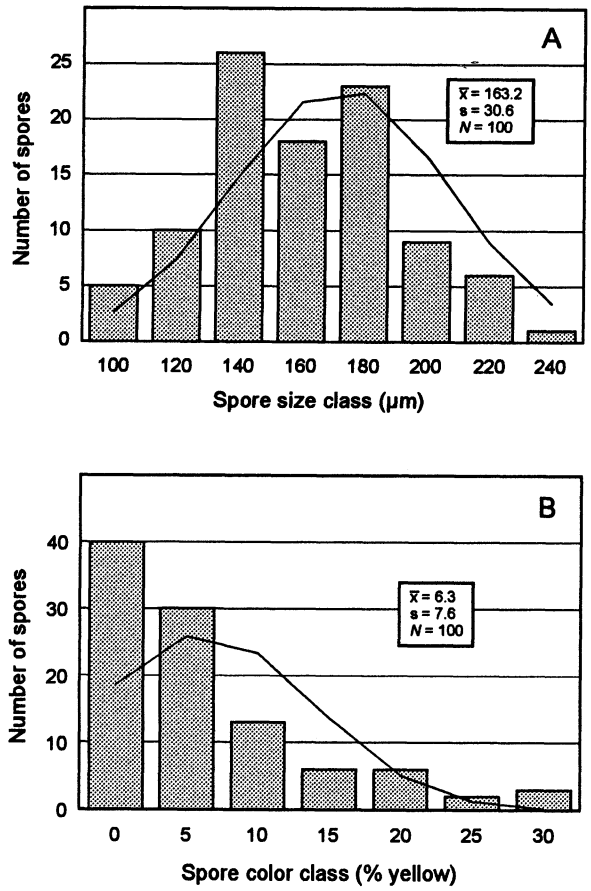

Fig. 2. Size (A) and color (B) distributions of *Glomus clarum* FL979, parental culture generation (P). Solid line indicates the predicted normal distribution, based on the mean and standard deviation.

(available from the authors) and estimation of the percentage of each of the component colors: cyan-magenta-yellow-black (C-M-Y-K). Both the spores and the chart were simultaneously illuminated with a bifurcating fiber optic light source (color temperature 3400 K).

To determine the effect of selection pressure on size and color distribution, a 10% selection pressure (10/100 spores) was imposed. Four replicate cultures on *S. bicolor* were started each from ten spores from the size extremes (largest and smallest spores, respectively) and color extremes (darkest and lightest spores, respectively), for a total of 16 cultures (Fig. 1). In the case of the lightest-colored spores, a 10% selection pressure could not be realized, since more than 10% of the population lacked color altogether (0% yellow, Fig. 2). The selection pressure actually applied was ~40%.

Spores were pipetted directly onto the roots of 10-d-old *S. bicolor* seedlings that were planted in 4 × 21-cm Conetainers™ (Stuewe and Sons, Corvallis, OR), containing ~150 g of the sand-soil mixture, and arranged in a completely randomized design. Cultures were allowed to grow for 4 mo, under the same conditions detailed above. Size and color of 100 spores from each cone were measured as above in four randomized blocks.

The selection regime was repeated with a second pot culture generation (G2) by imposing a more intense 5% selection pressure (10/200 spores) using the same criteria outlined above (although the selection for the lightest color was again not realized). Cultures were sewn as above with *S. bicolor* as the host. Soil, temperature, and lighting conditions were as described previously and the cultures were arranged in a completely randomized design. The same selection pressure applied to a given culture in the G1 was applied to the progeny cultures in the

TABLE 1. Results of analysis of variance from size and color selection treatments in generation G1.

	Source	df	SS	*F*	*P*
Size:	Treatment[a]	3	48 030.18	8.87	0.0001
	Block[a]	3	20 214.98	3.73	0.05
	Treatment × Block	9	16 248.24	1.63	ns
	Error	1584	1 757 807.04		
Color:	Treatment[a]	3	2803.55	36.43	0.0001
	Block[a]	3	179.67	2.33	ns
	Treatment × Block	9	230.89	0.66	ns
	Error	1584	61 250.75		

[a] Tested over the treatment by block interaction.

G2. For example, G1 cultures that were started from the smallest spores were used to start the smallest-spored cultures in the G2 (Fig. 1). Thus, selection pressure regimes were not mixed within a specific selection lineage. From each G1 culture, two replicate G2 cultures were started, for a total of 32 cultures (Fig. 1). The impact of the imposed selection pressure on genotypic variation was assessed by comparing size and color (as outlined above) in the various generations.

Statistical analysis—We employed two approaches to test for the existence of genetic variation in size and color. The size and color of spores within a generation were analyzed using an analysis of variance with the general linear models procedure of SAS (1988). Within a generation, the existence of genetic variation was evaluated as the significance of the selection treatment tested over the treatment by block interaction. The existence of the genetic variation was also tested as the significance of the regression of the mean size and color of the spores in the G2 generation against the corresponding values in the G1 generation. We note that since selection was imposed between these generations, the slopes of these regressions are likely to be greater than the heritabilities. Estimates of broad-sense heritabilities (H^2) of the size and color for this isolate were obtained from the equation for the response of the population (R) to the selection differential (S) for selection in both directions ($H^2 = R/S$; eq. 11.2 in Falconer, 1989). In addition, for the G1 generation we calculated an overall estimate of heritability for size and color as the ratio of the sum of the responses to selection in the upward and downward direction over the sum of the selection differentials [$H^2 = (R_{up} + R_{down})/(S_{up} + S_{down})$]. This equation has the advantage of reducing to a form that is not dependent upon the average measurement from the parental population, which may vary because of slight environmental differences.

The extent of genetic correlation between size and color determines the degree of independence the two traits have in their evolutionary responses to selection. If the traits are genetically uncorrelated, then they will have completely independent responses to selection, while if they are completely correlated (i.e., $r_G = 1$), the evolution of the two traits will proceed in parallel. Two analogous approaches were used to test for the existence and degree of genetic correlation between the two traits. Within a generation, the existence of a genetic correlation was evaluated as the difference between unselected traits within a generation. We also tested for the existence of a genetic correlation by regressing the size or color in the G2 generation against the color or size, respectively, of the G1 generation. As noted above, these regressions do not equal the genetic correlation alone because selection was imposed between these generations. To test whether a genetic correlation was complete within a generation, we evaluated the significance of the interaction of the selection treatment with the multivariate profile contrast tested over the profile by treatment by replicate interaction (J. D. Bever, unpublished data). In these cases, if the slope of the profile varies with treatment, then the genetic correlation is not perfect and there is some independence of the two characters. An estimate of the broad-sense genetic correlation of size and color (r_G) for this fungal isolate was obtained as the ratio of the product of the correlated responses to selection (CR) for the two characters over the product of the direct responses to selection for the two characters [$r_G = (CR_S \times CR_C)/(R_S \times R_C)$]; eq. 19.7 in Falconer, 1989). As for heritabilities, estimates of the genetic correlation were obtained both for the two directions of selection and for a combined estimate, which is not dependent upon the measurements in the parental generation.

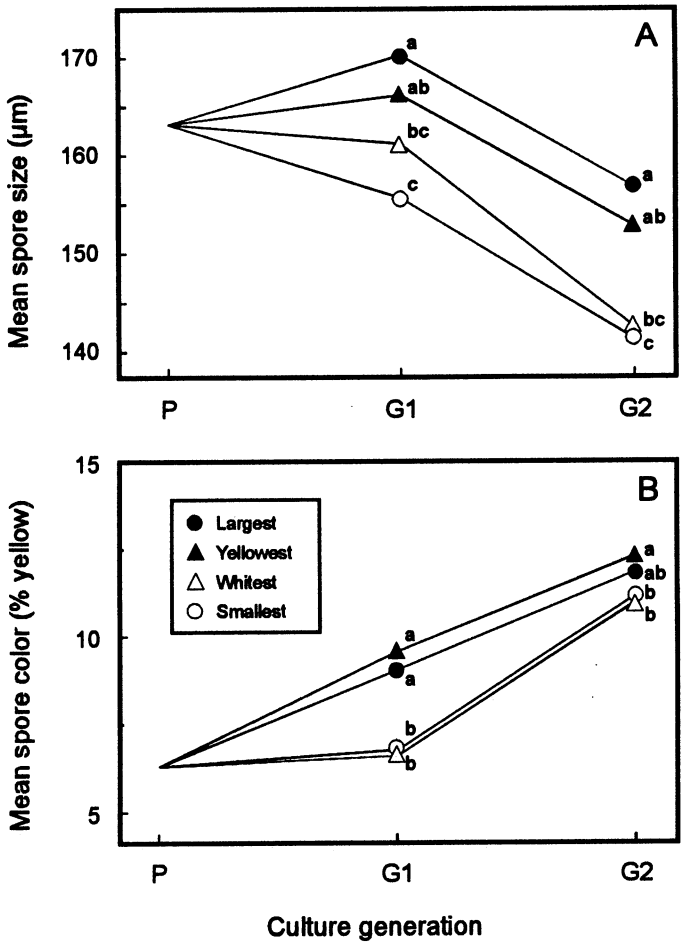

Fig. 3. Mean spore size (A) and color (B) of all treatments in each generation. P = parental culture generation; G1 = first experimental generation; G2 = second experimental generation. Within a generation, means with the same letter are not significantly different according to the least significant difference procedure ($P < 0.05$).

RESULTS

The P generation exhibited substantial phenotypic variation in size (100–240 µm) and color (0–30% yellow, Fig. 2). Color of spores varied only in the percentage of yellow; therefore only these data are presented. Size data were normally distributed (Fig. 2A), whereas color data were skewed toward light-colored spores (Fig. 2B). Spore size and color were phenotypically correlated in the parental generation (data for P shown in Fig. 5A, $r^2 = 0.31$).

The selection pressures exerted on the G1 generation resulted in progeny cultures that differed significantly in both size and color (Table 1). For example, G1 cultures that were started from larger spores produced progeny spores that were significantly larger than those produced from smaller parental spores (Fig. 3). Note that selection

for white spores, which was much weaker than the other traits being selected, did not result in spores that were whiter than the parental generation, possibly reflecting a paucity of heritable variation in this direction. From this response to the first generation of selection, the realized broad-sense heritability for size was estimated as 0.12, 0.15, and 0.134 for selection in the upward, downward, and overall, respectively. The corresponding estimates of realized broad-sense heritabilities for color were 0.184, 0.0, and 0.123. Note that the heritability estimate for color of zero for downward selection is likely to be an artifact of slight differences in the environment of the P and G1 generation.

Size and color appeared to covary in their response to the first generation of selection, such that yellow spores produced progeny that were relatively large and white spores produced progeny that were relatively small. This result reflects a positive genetic correlation between spore size and color, which was significantly greater than 0. This correlation, however, was also significantly less than 1 as tested with use of multivariate profile analysis (Wilk's λ for the interaction of profile with treatment: $F_{3,9} = 5.78$, $P < 0.0175$). In fact, this genetic correlation, r_G, was calculated to be 0.358, 0.265, and 0.263 from selection in the upward direction, downward direction, and overall, respectively.

The ANOVA results from the G2 generation were similar to those of the G1 generation. However, the spores produced in the G2 generation tended to be smaller and darker overall than previous generations (Fig. 3). The overall mean for spore size (irrespective of selection treatment) for the P, G1, and G2 was 163.2, 163.3, and 148.3 μm, respectively. Similarly, mean spore color (over all treatments) for the three generations was 6.3, 8.0, and 11.6% yellow, respectively. These shifts possibly result from an unmeasured change in the environments (however defined) between the generations, or may be due to some unknown internal mechanism. Because of the possibilities of genotypes varying in their response to an environmental shift, we did not use the G2 generation for calculations of actual values of heritabilities or genetic correlations. We therefore were unable to evaluate whether the magnitude of heritable variation was depleted as a result of the first generation of selection.

Nevertheless, comparisons between the G2 and G1 generation were in broad agreement with the results within the G1 generation. A positive regression of spore size in the different generations was observed (Fig. 4A, $r^2 = 0.33$). Larger spores from the G1 generation tended to produce progeny spores in the G2 that were larger. A significant, but weaker, regression was also found with color (Fig. 4B, $r^2 = 0.14$). Moreover, darker spores in the G1 generation produced G2 progeny spores that were significantly larger than those produced by light-colored spores (Fig. 5B, $r^2 = 0.42$), in agreement with our previous observation of a positive genotypic correlation between the size and color.

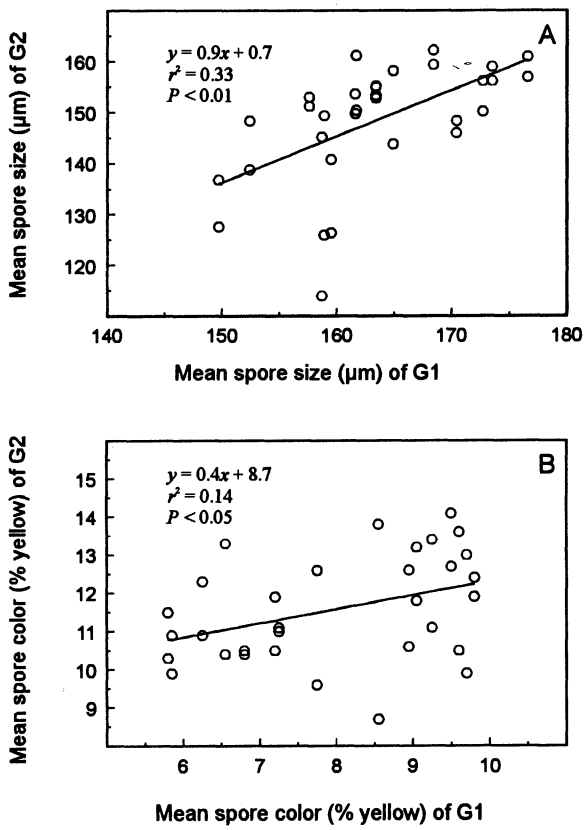

Fig. 4. Regression of spore size (A) and color (B) of the second experimental generation (G2) vs. the first experimental generation (G1). Each data point represents the mean of 50 spores.

DISCUSSION

The data presented here clearly demonstrate that genetic variation in size (Fig. 4A) and color (Fig. 4B) exists within a population of spores of *G. clarum*. While the heritabilities were not high (0.134 and 0.123 for size and color, respectively), they were sufficient to allow these characters to respond to selection (Fig. 3). Previous work on AM fungi found substantial genetic variation in spore size and shape within a single field population (J. D. Bever and J. B. Morton, unpublished data). However, in this study, we find measurable genetic variation within a single isolate maintained in a laboratory environment for over 3.5 yr. During this time, a relatively constant selective environment along with frequent bottlenecks during purification and subculturing and the solely asexual reproduction of this fungus would probably work to reduce variability. Nevertheless, other forces, such as mutation and the recombination of nuclei through hyphal division and fusion, may work to create genetic variation with the end result that a low level of heritable variation is maintained. While we cannot identify the mechanism(s) responsible for maintaining this variation in this study, we have demonstrated an approach to measuring heritable variation that may prove useful in efforts to elucidate of these mechanisms.

Size and color were phenotypically correlated (Fig. 5A) and a component of this correlation is due to a positive genetic correlation ($r_G = 0.263$). One plausible explanation for this phenotypic and genotypic correlation is that the pigment in *G. clarum* spores is associated with a laminated layer that thickens as the spore develops.

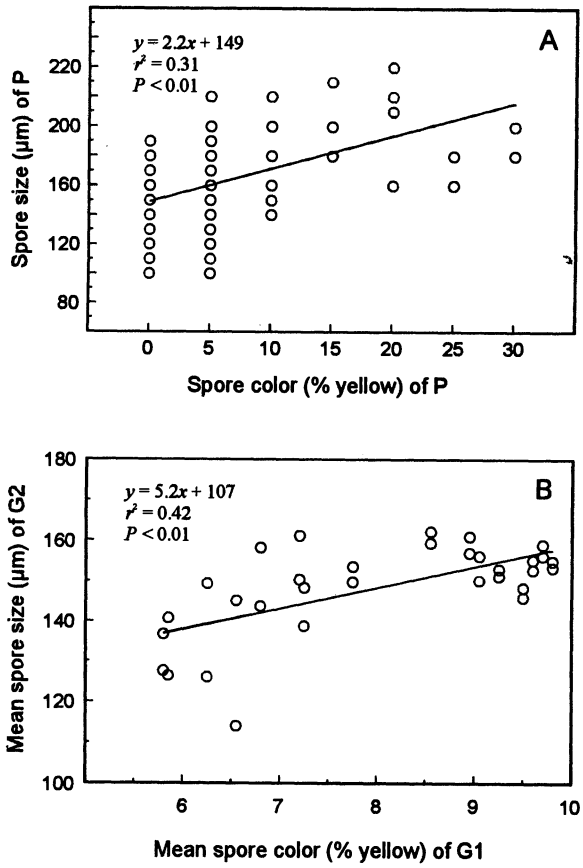

Fig. 5. (A) Phenotypic correlation of color and size in the P generation (N = 100 spores, some data points are obscured). (B) Evidence of genotypic correlation of color and size, as indicated by the regression of spore size of the second experimental generation (G2) against spore color of the first experimental generation (G1). Each data point represents the mean of 50 spores.

Larger spores tend to have thicker walls since they have had more time to develop. As the pigmented layer becomes thicker, spores become darker in appearance. Recent ontogenetic studies of *G. clarum* have confirmed this (Stürmer and Morton, 1997). Even still, this genotypic correlation noted here was only partial (r^2 = 0.42, Fig 5B) and was significantly less than one (Wilk's Lambda $F_{3,9}$ = 5.78, P < 0.0175), suggesting a degree of independence in these two characters. As a consequence of the independence of the two characters, it may be theoretically possible to select for a population of *G. clarum* with large, white spores or with small, yellow spores through selection techniques similar to those used here.

Even though a strong genetic component of size and color expression was noted, selection pressure did not eliminate phenotypic variation, thus indicating that environmental conditions also influence character expression. In fact, even in the relatively constant environments of the P and G1 generations, the genotypic differences only accounted for 13.4 and 12.3% of the phenotypic variation for size and color, respectively; therefore, the majority of the variation (86.6 and 87.7%, respectively) for both of these characters was due to environmental factors. We reached a similar conclusion from the regressions of the means of the G2 generation on the corresponding means of the G1 generation. These regressions were able to explain only 33 and 14% of the variation in spore size and color, respectively, of the variation in spore color of the G2. These observations suggest that spore size and color are strongly influenced by environmental conditions in addition to genetic control.

The large influence of unknown factors (e.g., environment) on the phenotypic variation of spore size and color is further illustrated by the observation that spores in the G2 tended to be smaller and yellower than previous generations, independent of the intense selection pressure (Fig. 3). The overall mean (irrespective of treatment) for spore size in generation G2 was ~15 µm smaller than that of either the P or G1 generations (9.1% shift; Fig. 3A). Similarly, the mean color of all spores in the G2 was several percentage points yellower than in previous generations (84% shift, Fig. 3B). The cause of these changes in size and color between generations is not clear. All cultures were grown under controlled conditions with the same host, similar soil conditions, and under similar temperature and lighting regimes. It appears that fungal expression of these characters is sensitive to some unknown parameter(s), either external or internal to the fungus.

The fact that this variation exists in important taxonomic characters (size and color) is of particular importance since it has implications in the delineation of species boundaries. For example, *G. clarum* was originally described as being hyaline to white but becoming yellow in age (Nicolson and Schenck, 1979). A morphologically similar species, *G. manihot* Howeler, Sieverding & Schenck, was reported to range from white to yellow to yellow brown (Schenck et al., 1984). Our data demonstrate that environmental factors can have a large impact on these characters. Moreover, microevolutionary forces, including directional selection, could result in a shift in the average size and color of spores in the population. Therefore, the boundaries between larger or darker populations and smaller or whiter populations are suspect because they may result from microevolution within a single population. However, we do not know from our data whether sufficient genetic variation exists within the population for a sustained response to multiple generations of selection. Indeed, an exhaustion of genetic variation within a population could constrain a microevolutionary shift and provide support of a species boundary based on certain characters. Nevertheless, the evidence reported here suggests that the boundary between *G. clarum* and *G. manihot* may be dubious.

This study provides empirical evidence on the nature of morphological character diversity in a glomalean fungus and demonstrates that a single isolate of *G. clarum* might evolve to have different spore characteristics. However, even with this genetic potential, expression of spore color and size is strongly influenced by local environmental conditions. Understanding the extent of this evolutionary potential will improve our ability to delineate *G. clarum* from putatively distinct species with similar morphologies. The methods employed here may also prove useful in genetic studies of other obligately asexual or clonally reproducing fungi.

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