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Ontogeny and phylogeny of a *Scutellospora heterogama* mutant, with implications for morphological recognition of species in Glomeromycota

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ABSTRACT

A putative mutant of *Scutellospora heterogama* has been maintained for 19 pot culture generations over 15 y. The mutant differed phenotypically from the wild-type parental lineage in characteristics of the spore wall: albino *versus* pigmented red-brown color, high plasticity in acidic mutants *versus* rigid and finely laminate, deep red-purple reaction *versus* no reaction in Melzer's reagent, respectively. This variation was equivalent to or greater than that between any two species in *Scutellospora* or any other genus in Glomeromycota. Comparison of spore ontogenesis revealed that the terminal (mature) state of the albino mutant was similar to a transient intermediate stage in the wild-type. The albino phenotype, therefore, did not result from emergence of a unique morphological innovation. Rather, it arose from a mutation that led to premature termination of spore ontogenesis so that a unique transient juvenile stage became permanent in mature spores. Because this mutation was homogeneous in all progeny populations, it is hypothesized to be a recessive trait expressed only after the allele was distributed in all nuclei of the fungal thallus. Sampling of the genomes of the putative mutant and wild-type isolates by microsatellite-primed PCR suggested a local mutation. The profile of the mutant was identical to that of the wild-type parent and was 60–97 % similar to those of four other *S. heterogama* isolates. Phylogenetic analysis of the D1–D2 domains of the 25S *rRNA* gene and a *β-tubulin* gene with and without three variable introns placed the albino mutant solidly within the *S. heterogama* clade. These results suggest that stability of morphological traits is not a suitable criterion by itself to recognize species. The albino phenotype was a discrete and heritable mutation that became fixed in a population and was stable through time and space. In the absence of negative selection, this mutation could persist, disperse and then be misinterpreted as a new species in nature. Genetic markers expose this mutation as a population-level variant and therefore of no macroevolutionary significance. Assessment of genetic divergence amongst multiple isolates is important in ascertaining the contribution of morphological characters toward recognition of species in glomeromycotan clades.

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Introduction

Scutellospora heterogama is a widely distributed species in Gigasporaceae, Glomeromycota (Schüßler *et al.* 2001). Oehl *et al.*, 2008 partitioned Gigasporaceae into five genera of four families, with *S. heterogama* renamed *Fuscutata heterogama*. We reject this new classification for reasons that are addressed elsewhere (Morton & Msiska 2010). In an international collection of fungi in this phylum at West Virginia University (INVAM; Morton *et al.* 1993), 24 accessions of *S. heterogama* have been deposited from North America, South America, and Asia. In 1994, one of these accessions, WV858, produced a mixture of two mature spore phenotypes. One phenotype yielded brownish-red spores typical of the species and the other produced white to pale orange spores. Almeida & Schenck, 1990 recovered a similar divergent albino phenotype from two different cultured accessions in INVAM when the collection was housed in Florida (isolates #139 and #964). They were unable to establish this putative mutant alone in culture and hypothesized that loss of pigment was linked to loss of infectivity and ability to form a mycorrhizal association. However, we were able to establish the mutant from accession WV858 in monospecific culture from albino spores as starter inoculum. Because arbuscular mycorrhizal fungi (AMF) are multinucleate and it is unclear if these nuclei are homokaryotic or heterokaryotic (Bever & Wang 2005; Pawlowska & Taylor 2004), a minimum of 200 spores was used as inoculum. The albino phenotype remained true to type in all progeny of 19 pot culture generations over 15 y, with no mosaic patterns of expression or reversion to wild-type.

The phenotype of the albino mutant is distinctive from that of the wild-type. Pigmentation is absent from both layers of the spore wall when spores are newly formed and healthy. The inner layer of a spore wall is highly plastic (henceforth termed "amorphous") with a deep reddish-purple staining reaction in Melzer's reagent. The amorphous trait and Melzer's reaction are linked properties, and so they actually represent only one character state change (Morton *et al.* 1995). In combination, this morphological variation is equivalent or greater than that between any *Scutellospora* species described to date and therefore would be perceived as a new species if spores were recovered only from the field. Since this mutant arose as a variant in pot culture, the observed transformations were hypothesized to more likely represent a unique and localized mutation within the *S. heterogama* genome.

With both wild-type and mutant phenotypes in hand, a three-pronged approach was taken to determine the extent of the linkage between morphological and genotypic variation. First, ontogenetic criteria were used to characterize the origin and nature of the mutant phenotype. Arbuscular mycorrhizal fungi, as whole organisms, share many similarities with indeterminant plant species in modular growth and development (Morton 1990; Mishler 1988). Ontogeny is simplified when the focus narrows to structural transformations in discrete structures, such as spores, within which subcellular diversity is much higher than that found in other fungal groups (Morton 1990). Ontogenetic comparisons in representative genera (Morton *et al.* 1995) established a universal pattern within Glomeromycota. A spore wall is synthesized first from the subtending hyphal wall, and it can consist of one to several layers. For some genera, such as *Gigaspora* and *Glomus*, no further differentiation occurs. For other genera, such as *Scutellospora*, additional structures are formed within the cytoplasm of the spore. First, one or more discrete bilayered colorless flexible "inner walls" are synthesized, followed by formation of a disc-shaped structure termed a germination shield on the innermost of these walls. These internal structures are termed "germinal walls" because the last one formed always is involved in germination shield formation and any others present are considered functionally equivalent historical relicts preserved because of sequential formation in a linear trajectory during ontogenesis (Morton *et al.* 1995). *S. heterogama* was one of the first species to be characterized (Franke & Morton 1994), and this analysis established that all of the stages in spore ontogenesis were discrete and comparable across taxa. Thus, it was feasible to compare *S. heterogama* mutant and wild-type phenotypes and ascertain at which stage in the ontogenetic sequence one phenotype diverged from the other and the extent to which that change impacted on other developmental stages.

Second, microsatellite-primed PCR was used to randomly sample regions of the genome assess and obtain a general measure of variation between the albino mutant, its wild-type parent, and other typical members of *S. heterogama*. Longato & Bonfante, 1997 used this method to measure similarity among several isolates of *Glomus mosseae*.

Third, molecular markers were used to determine genetic and phylogenetic distances between the albino mutant, its parent, and a sampling of geographically distant isolates sharing the typical *S. heterogama* phenotype. The large subunit 25S *rRNA (LSU)* gene was selected because the D1 and D2 regions are variable enough to be discriminatory in AMF (Van Tuinen *et al.* 1998). In metazoan groups, these domains are sufficiently discriminatory at the species-level to complement the mitochondrial cytochrome oxidase I gene in bar-coding projects (Sonnenberg *et al.* 2007). The *β-tubulin* (*TUB2*) gene was selected as an independent test of evolution because only one copy was found in *Gigaspora* and *Scutellospora* lineages (Msiska & Morton 2009a). With 45 species evaluated to date, exon sequences yield phylogenetic trees whose terminal clades strongly correspond with known glomeromycotan species (Msiska & Morton 2009b). The *TUB2* gene also contains three introns of variable sequence and length (Msiska & Morton 2009a). These introns are alignable within isolates of any given species, and so they could be used to evaluate intraspecific variation in *S. heterogama* and provide additional information on the relative position of albino mutant clones. The necessity for utilizing a combination of morphological and molecular data to define and rank glomeromycotan species is discussed.

Materials and methods

Fungal germplasm

Scutellospora heterogama WV858 was isolated in Jul. 1991 from the rhizosphere of *Andropogon virginicus* growing in isolation next to an acid mine drainage pond near Manown, West

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