Genetic Structure within Septobasidium Colonies Suggests Outcrossing and Frequent Non Self-Fusion

Daniel A Henk* and Rytas Vilgalys

Department of Biology and Biochemistry, University of Bath, Bath, UK

Abstract

Fungi in the genus Septobasidium form colonies that envelop and infect multiple scale insects. Infection of each insect is thought to occur only via meiotic basidiospores. In this study, we investigated the genetic status of colonies by genotyping different phases of the Septobasidium life cycle including single spore isolates, mature colony tissue isolates, and infected insects. Meiotic analyses showed segregation of two alleles at multiple loci among the progeny from a single fungal colony. Genotyping of multiple tissue isolates demonstrated that individual Septobasidium colonies were composed of a single heterozygous dikaryotic mycelium. Fungi from insects fused to a colony of *S. ramorum* had identical genotypes to each other, but insects not yet fused to a colony sometimes had unique genotypes. Based on these studies, Septobasidium species are suggested to be predominantly outcrossing, with colonies maintaining their genetic identity despite frequent non self-fusions.

Keywords: Coevolution; Phylogenetic; Host specificity; Symbiosis

Introduction

For fungi and many other colonial organisms, defining the units of natural selection is not always straight-forward [1,2]. The concept of the "individual" has been contentious in fungal biology [3] because filamentous fungi form interconnected, multinucleate networks often composed of genetically heterogeneous elements. Historically, two ideas have dominated most discussion of genetic individualism for fungi. An early view of the fungal individual known as the "unit mycelium" was that genetically distinct mycelia undergo frequent somatic fusion and anastomosis to form genetically heterogeneous networks as the individuals [4].

In this view of the mycelial individual, different genotypes cooperate to acquire nutrition, but not all genotypes contribute equally toward producing progeny. This idea was strongly contested by observations that showed successful somatic fusions occurred only between hyphae that were genetically identical or nearly so (the "individualistic mycelium", Rayner and Todd [3], Rayner [5]. Under this view, genetic identity of the fungal colony is maintained by somatic incompatibility responses among unrelated mycelia [3,6,7]. The emergence of the "individualistic mycelium" concept resulted in the dismissal of somatic fusion as a major means of generating genetic variation, and rather, sexual recombination is generally thought to be the dominant mechanism generating genetic variation in fungi [8].

Mating systems in fungi may be loosely divided into several types, including obligate outcrossing (heterothallism) and self-fertile (homothallism). Homothallic strains can produce basidiospores that germinate into mycelia that may fuse freely and are self- fertile [9]. In contrast, outcrossing occurs when two genetically distinct, mating compatible strains undergo hyphal fusion to produce a genetically stable dikaryon. In outcrossing species, mating compatibility initially overrides somatic incompatibility among monokaryotic strains [10]. However, somatic incompatibility still acts to maintain identity of the newly formed dikaryotic "secondary mycelium" which is dominant life phase in basidiomycete fungi [6]. In heterothallic basidiomycetes, the successfully mated mycelium produces meiotically recombinant haploid basidospores, which must fuse with appropriate mating compatible strains in order to regenerate the dikaryotic phase.

Fungi in the genus Septobasidium all share an unusual symbiotic life history, which offers unique opportunities for exploring mating biology and fungal individuality. Unlike other Urediniomycetes (which include rusts and their relatives), Septobasidium spp. live symbiotically with scale insects growing parasitically on woody plants, and individual colonies vary in size from a few mm in diameter to nearly 2 m. The lifecycle of Septobasidium was first elucidated by Couch [11-13], who interpreted its life cycle as follows based on the "unit mycelium" concept (Figure 1): 1) Septobasidium basidiospores infect first instar insects that settle on the plant surface; 2) The fungi grow out of infected insects and fuse with fungi from adjacent infected insects to form a mat of hyphae that covers both infected and uninfected insects; 3) Mature colonies produce basidiospores that can infect the offspring of the uninfected insects associated with the colony. Because the growth of the Septobasidium colony depends on fusion between multiple fungal mycelia (each derived from independent basidiospore infections), colonies may be composed of multiple genetically distinct nuclei. Couch [11-13] initially interpreted his observations in line with a genetic mosaic concept with many strains making up each colony.

However, a single genetic unit concept may also be consistent with Couch's observations. If infective spores do not actually represent different genotypes then the genetic identity of a unified mycelium would be maintained. This could result from homothallism or cryptic mitotic production of basidiospores. Alternatively, apparent fusion of genetically distinct strains may cause any of several antagonistic somatic incompatibility reactions resulting in either a) distinct genotypic patches within the larger colony or b) replacement of nuclei throughout the entire colony by a dominant pair of nuclei.

*Corresponding author: Daniel A Henk, Department of Biology and Biochemistry, University of Bath, Bath, UK, Tel: +4456798465; E-mail: d.a.henk@bath.ac.uk
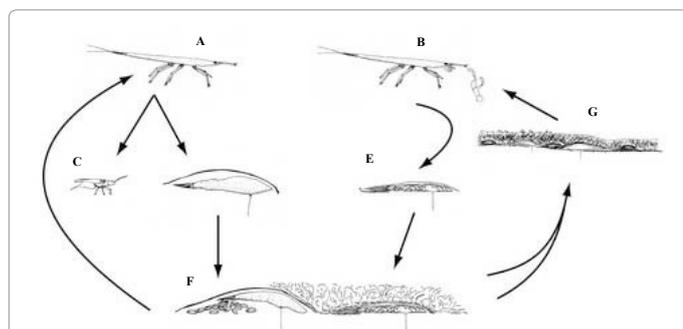
Figure 1: The lifecycle of the Septobasidium **symbiosis**. First instar scale insects newly emerged from eggs (A) may settle without encountering fungal spores and develop into healthy male (C) or female (D) scale insects. Insects that encounter budding basidiospores (B) become infected as the fungus invades the body cavity eventually filling it with haustoria (E). When the fungi have fully invaded the insect body hyphae begin to grow out of the insect and form a mat of mycelium that may cover adjacent insects (F). Fungi from infected insects fuse to form healthy mature fungal colonies composed of uninfected and infected insects (G).

Determining the mating system and genetic structure of colonies in Septobasidium is a necessary first step toward developing a model of the co-evolutionary dynamics in this potentially altruistic and mutualistic interaction. This study addresses genetic structure of Septobasidium colonies using DNA sequence data from multiple life history phases in consort with microscopic observation of nuclear status in hyphae. Two specific questions we addressed are 1) whether scale insects are infected by different fungal genotypes and 2) how genotypes are distributed within an adult fungal colony. Using microscopy and sequence data from multiple single spore isolates, we determined the minimum ploidy of spores and whether the spores represent recombinant genotypes. Using sequence data from multiple locations within a colony and from isolates of fungi within infected insects already fused to a colony we can detect intra-colony genetic variation. From infected insects that have not yet fused to the main fungal colony we can determine what fungal genotype or genotypes are the infective units. By comparing the results from each part of the fungal infection we can detect possible genetic mosaicism within the colony, and determine whether the mating system is likely to be homothallic or heterothallic. We demonstrate this approach to a single focal species, *S. ramorum* and also for nine other species.

Materials and Methods

Collection, culture, and DNA extraction from fungal material Septobasidium colonies were collected from Quercus, Nyssa, Acer, Cornus, Fraxinus and Liquidambar trees across the southeastern U.S. (Table 1). Infected branches were removed and stored at room temperature in paper bags for up to two weeks. Three small spatially separated sections (approximately 5mm in diameter) were sliced from the upper surface of each colony and floated on sterile water. Each rehydrated colony was then suspended, upper surface facing downwards, from the lid of a Petri dish containing Malt Yeast Agar (MYA - 15% g/L malt extract, 5% g/L yeast extract, and 15% g/L Agar). Spores that landed on the agar surface were allowed to germinate and produce colonies of fewer than 100 cells.

This usually occurred after 3-4 days. At least six yeast colonies, each derived from a single spore, were transferred to new plates and maintained as single spore isolates (SSI). From each colony that was collected, five tissue samples (TI) approximately 2 mm in diameter were removed from various locations within each colony and placed into

elution buffer for DNA extraction using the Forensic DNA extraction kit (OmegaBioTek). Insects that had settled on the fungal mat of *S. ramorum* colonies but were not connected to it were individually removed and placed into elution buffer for DNA extraction using the Forensic DNA extraction kit (OmegaBioTek).

Insects that were embedded and fused within *S. ramorum* colonies were removed, slightly crushed with a needle and pulled across the surface of MYA plates. The smeared contents of the scale insects were then observed microscopically to detect growth from the liberated haustoria. Mycelia from the haustoria were then transferred to fresh MYA plates. Aerial hyphae were placed into CTAB buffer for DNA extraction (1% CTAB, 50 mM Tris pH 8, 10 mM Na2EDTA, 0.7 M NaCl). Voucher collections of each collection sample, as well as representative's monokaryotic and dikaryotic cultures for each species are deposited at DUKE.

DNA was extracted following the Forensic DNA extraction kit (Omega Biotek) using instructions for whole blood preparation modified as follows. Tissue was disrupted by grinding with a pestle in 250 μl elution buffer in a microcentrifuge tube. Following disruption, 250 μl buffer BL and 25 μl of 100 μM Proteinase K solution were added before incubation at 70°C as described in the kit manual. DNA was collected using the spin coulombs and eluted in 50 μl of elution buffer.

DNA from SSI and aerial hyphae was extracted following the procedure of [14] with few modifications. Using a sterile loop, yeast cells were scraped from the surface of the MEA plates and placed into tubes with 400 μl CTAB and approximately 0.3 g sterile sand. Tubes were subjected to two minutes of high-speed vortexing to disrupt the yeast cells before addition of 400 μl of 24:1 isoamyl alcohol to chloroform. After brief mixing and high-speed centrifugation (10000 G) for 15 min, the aqueous layer was removed to a clean microcentrifuge tube. Then 0.6 volume of isopropanol was added and stored overnight at -4°C to precipitate DNA. After centrifugation and an ethanol wash, the DNA was in eluted in 100 μl sterile water. DNA from aerial hyphae was similarly extracted but rather than vortexing with sterile sand, the tissue was ground with a plastic pestle in a tube without sand.

PCR based identification of genotypes

We used polymerase chain reaction (PCR) to amplify at least two loci from each collection (Table 1). Primer sets were designed to amplify Septobasidium species (Table 2). Additional markers were developed in the focal species *S. ramorum*, by screening a clone library of genomic DNA fragments essentially as described in Morehouse et al. [15]. Genomic DNA from an SSI of *S. ramorum* (collection DAH045A) was partially digested using Sau3A. Fragments between 1000 bp and 500 bp were separated on an agarose gel and ligated into the p Zero plasmid vector (Invitrogen). The plasmid was used to transform TOP10 E. coli (Invitrogen) cells. After propagation, the plasmid was prepared with the QIAprep Spin Miniprep Kit (Qiagen) for sequencing with forward and reverse M13 primers. Clones from the resultant library were sequenced and those that had strong similarity to genes from other organisms were selected for primer design and use as genetic markers. Additionally, the marker STE20 was generated by an initial round of PCR with degenerate primers designed from GenBank comparisons [16] followed by cloning and primer redesign.

PCR amplification used Red Hot Polymerase (ABgene) and reactions conditions as follows: 4 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at annealing temperature (50°C for ITS and ASP25, 48°C for EF1α, β-tubulin and STK26, 46°C for STE20) and 45 s at 72°C then a final extension step for 10 min at 72°C. PCR products

Collection ID	Locality	Species	Locus-GenBank#s	Genotype IDs
DAH091	Durham, NC	*S. apiculatum*	ITS-DQ241405 EF1α-DQ648057	I017-I018 E015-E016
			βTUB-DQ241423	B011-β012
DAH130	Durham, NC	*S. burtii*	ITS-DQ241406 EF1α-DQ648058	I005-I006 E005-E006
DAH194D	St. Johns, FL	*S. burtii*	ITS-DQ241407	I003-I004
DAH157	Berkeley, SC	*S. castaneum*	EF1α-DQ648059 ITS-DQ241408	E003-E004 I007-I008
			EF1α-DQ648060	E007-E008
DAH176C*	Colleton, SC	*S. castaneum*	ITS-DQ241409	I091-I092
DAH232A*	Colleton, SC	*S. fumigatum*	EF1α-DQ648061 ITS-DQ241410	E035-E036 I009-I010
			βTUB-DQ241424	β003-β004
DAH011	Hoke, NC	*S. fumigatum*	ITS-DQ241411 βTUB-DQ241425	I011-I012 β005-β006
DAH147*	Berkeley, SC	*S. grandisporum*	ITS-DQ241412	I022
			EF1α-DQ648062	E023
DAH283B*	Adams, MS	*S. mariani*	βTUB-DQ241426 ITS-DQ241413	β014 I013
			EF1α-DQ648063	E009-E010
DAH092	Greene, AL	*S. mariani*	βTUB-DQ241427 ITS-DQ241414	β007-β008 I014
			EF1α-DQ648064	E011-E012
DAH191A	St Johns, FL	*S. pseudopedicellatum*	βTUB-DQ241428 ITS-DQ241415	β009-β010 I015-I016
			EF1 α-DQ241429	E013-E014
DAH045A	Durham, NC	*S. ramorum*	ITS-DQ241416	I001-I002
			EF1α-DQ648065 βTUB-DQ241430	E001-E002 β001-β002
			ASP25-DQ241433	A001-A002
			STE20-DQ241440 STK26-DQ241443	S001-S002 K001-K002
DAH045B	Durham, NC	*S. ramorum*	ITS-DQ241417	I002-I019
			EF1α-DQ648066 βTUB-DQ241431	E017-E018 β001-β002
			ASP25-DQ241434	A002-A003
			STE20-DQ241439	S002-S003
DAH078A	Durham, NC	*S. ramorum*	STK26-DQ241442 ITS-DQ241418	K003-K004 I002-I020
			EF1α-DQ648067	E019-E020
DAH078B	Durham, NC	*S. ramorum*	ASP25-DQ241435 ITS-DQ241419	A004-A005 I001-I021
			EF1α-DQ648068	E021-E022
			ASP25-DQ241436	A006-A007
DAH301A*	Durham, NC	*S. ramorum*	ITS-DQ241420 ASP25-DQ241437	I001-I021 A001-A008
			STK26-DQ241441	K005-K006
DAH042F	Wake, NC	*S. ramorum*	ITS-DQ241421 ASP25-DQ241438	I001-I020 A001-A009
DAH132A	Carteret, NC	*S. sinuosum*	ITS-DQ241422	I023-I024
			EF1α-DQ648069	E024-E025

Table 1: Septobasidium collections sequenced and genotype for each allele.

Locus ID	Primer Sequences	Atmplicon Length	Average Number of Segregating Sites
ITS	TCCGTAGGTGAACCTGCGG-ITS1[1] TCCTCCGCTTATTGATATGC-ITS4[1] CTTTTCATCTTTCCCTCACGG-ITS4NA[3]	563-950 bp	5.31[4] (5.90)[5]
EF1α	AGTTCGAGAARGARGCIGC-Sef30f AGNCGIAYIGGCTTGTCGG-Sef1070r	975-1002 bp	9.58 (10.79)
βTUB	ACCTYCTCATCTCSAARATCCG-SepBt1f AAGGGGACCATGTTGACKGC-SepBt1r	463-512 bp	7.32 (8.78)
ASP25	GCGACGGAGAAGACTTACCA-Plas25f CTTCCAATGGCACCCTTTT-Plas25r	470 bp	8.8
STE20	TGTNATGGAGTACATGGAGG-S20f TACNACTTCAGGGGCCATCCAGTA-S20r	339-343 bp	18.72
STK26	TCTCACGACATCATCCATCG-Plass26f2 CTTGGCGTCGTACTTTTCGT-Plas26r2	415-477 bp	12.96

Note: [1]White et al. [31]
[2]Gardes and Bruns [14]
[3]Walker and Parrent, 2004
[4]The Average number of segregating sites calculated as the sum of Watterson's expected segregating within each species/number of species sampled
[5]Values in parentheses calculated ignoring *S. grandisoporum*

Table 2: Primers, range of amplicon length and nucleotide variation for loci sequenced across the genus Septobasidium.

were purified using PCR purification kits (Qiagen) and used directly for sequencing reactions with Big Dye V.2 or V.3. Sequencing was conducted on an ABI 3700 DNA analyzer (Applied Biosystems/ Hitachi). Sequence chromatograms were viewed and edited using Sequencher 4.2 (Gene Codes Corp Ann Arbor MI). We determined genotypes using the presence of strong "double peaks" to indicate the presence of polymorphic sites. Restriction digests were designed using Sequencher 4.2 to select appropriate enzymes, and digested PCR products from TI were run on 3% agarose gels and visualized using EtBr staining.

Microscopic determination of nuclear state

Material from cultures and fresh collections were prepared for fluorescence microscopy using DAPI and EtBr staining (Table 1). Sample sections approximately 5 mm in diameter were removed from fresh collections and then placed on slides with 30 μl of 10 μg/ ml DAPI or 10 μg/ml EtBr and stored at room temperature in the dark for 15 min before being viewed. The samples prepared with EtBr did not require the 15 min waiting period. The material was usually squashed, but some preparations were made as transverse sections to identify each part of the colony. Nuclear status was then determined as binucleate, uninucleate or multinucleate. Hyphae and yeasts from cultures were similarly prepared and scored for the number of nuclei present between septa and within cells.

Results

Marker development

DNA sequences from six variable loci, ITS, EF1α, β-tubulin ASP25, STK26, and STE20, were used as genetic markers in this study (Table 2). Primers for amplification of ASP25 and STK26 were developed from a *S. ramorum* genomic library. The 470 bp long locus - 8 designated ASP 25 was most similar to part of aspartic proteinases (e ≤ 10), and the -550 bp long locus called STK 26 was most similar to serine-threonine kinases (e ≤ 10⁷). We found that STK 26 could also be amplified from *S. taxodii* and *S. burtii* without further primer modification. The STE20 locus did not contain any introns and was only 339 bp long,

but in the four specimens sampled, 20 polymorphic sites were detected. The ITS, β-tubulin and EF1α loci each contained introns. The ITS sequences from *S. burtii*, *S. castaneum*, *S. fumigatum*, *S. mariani* and *S. pseudopedicellatum* had a group I intron at position 1506 (relative to *Saccharomyces*) in the SSU portion of the amplicon. β-tubulin genes all contained a single splicesosomal intron varying in size from 85-92 bp. The EF1α genes contained two introns varying in size from 88-135 bp.

Nuclear status, recombination and spatial genetic structure detected using SSI and TI

Colonies from every species except *S. grandisporum* showed a similar pattern of polymorphism among SSI and TI samples (Table 3). SSI from each colony showed a maximum of two alleles at a locus (detected as double-peaks on chromatograms) and showed recombination among loci (Table 4). In all species except *S. mariani* SSI were found to possess a single allele, while TI samples possessed multiple alleles except in the case of *S. grandisporum* (Figure 2 and Table 5).

Single spore isolates of *S. mariani* sometimes possessed multiple alleles, and TI of the single *S. grandisporum* colony sampled possessed a single allele. Fluorescence microscopy showed that SSI growing as yeasts were predominantly uninucleate (Figure 3), though due to their small size and considerable autofluorescence this distinction was not always obvious. *S. fumigatum*, *S. grandisporum* and *S. sinuosum* SSI that had assumed a filamentous growth form showed a range of nuclear states, sometimes uninucleate but often multinucleate (Figure 3). Determination of the nuclear status of fresh collections was difficult due to autofluorescence and apparent lack of penetration by the stains. Where discernable, *S. apiculatum*, *S. ramorum* and *S. sinuosum* each showed only binucleate cells.

S. ramorum genotypes infecting individual insects

DNA from free-living insects not yet fused to colonies was successfully amplified from 10 of 40 extracts. Due to the microscopic size of the infected scale insects, each DNA extract was sufficient for only one to three PCRs. For one collection, DAH042F, three fungal

Species	#Spores/Basidium	SSI Ploidy	Recombination observed	TI Genotypes
S. apiculatum	3	1N	Yes	Two alleles all isolates
S. burtii	4	1N	Yes	Two alleles all isolates
S castaneum	4	1N	Yes	Two alleles all isolates
S. fumigatum	4	1N	Yes	Two alleles all isolates
S. grandisporum	1	1N	No	One alleles all isolates
S. mariani	4	2N	Yes	Two alleles all isolates
S. psuedopedicellatum	4	1N	Yes	Two alleles all isolates
S. ramorum	4	1N	Yes	Two alleles all isolates
S. sinuosum	2	1N	Yes	Two alleles all isolates
S. taxodii	4	1N	Yes	Two alleles all isolates

Table 3: Summary of data of ten Septobasidium species with different numbers of spores per basidium, including putative ploidy of single spore isolates (SSI) and number of alleles detected in tissue isolates (TI).

Locus	Single spore isolate genotypes					
	SSI#1	SSI#2	SSI#3	SSI#4	SSI#5	SSI#6
ITS	I0011	I001	I001	I001	I001	I002
EF1α	E001	E001	E001	E002	E001	E002
βTUB	β002	β001	β002	β002	β001	β001
ASP25	A001	A001	A001	A002	A002	A001
STE20	S001	S001	S001	S001	S002	S001
STK26	K002	K002	K001	K001	K001	K001

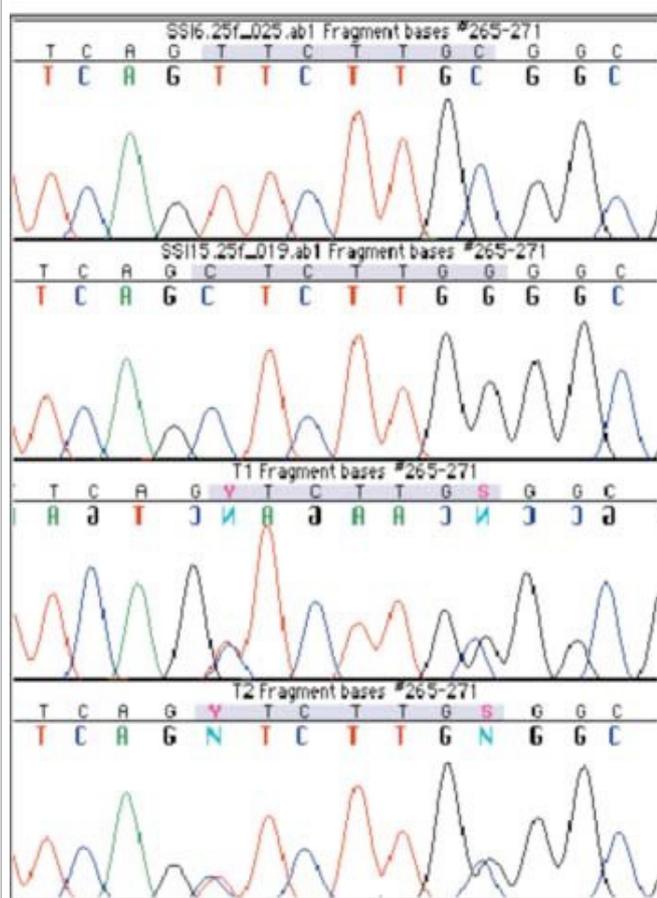**Table 4:** Segregation of alleles recovered from SSI of *S. ramorum* colony 045A.

Figure 2: Polymorphism segregating in SSI from a fungal colony and contained in TI from that colony. PCR-RFLP shows two alleles detected from each TI with each allele segregating among SSI (A). Chromatograms showing the segregating sites in SSI and characteristic "double peaks" of heterogeneous TI. The highlighted text is the consensus, while the chromatogram labels reflect the orientation of the read (B).

genotypes from infected insects were the same as the TI and three were monomorphic for the single sequenced locus. For collection DAH078B three insects were successfully amplified and each had the same genotype as the TI. However, an insect from an adjacent colony DAH078A possessed a recombinant genotype between TI from DAH078A and TI from DAH078B. All ten successfully isolated fungi from insects fused to colony DAH301 shared identical 3 locus

genotypes with a TI from DAH301. Microscopy revealed that fungi isolated from infected insects of DAH301 were binucleate (Figure 3).

Discussion

Our data indicate that colonies of most Septobasidium species consist of single outcrossed dikaryotic individuals, and that basidiospores represent haploid recombinant progeny. In *S. ramorum* genotyping revealed that although colonies arecomposed of a single dikaryotic individual, fungi from young infected insects not yet fused to the rest of the colony represent recombinant or homozygous genotypes. Based on Couch's observations of infection and our own results, basidiospores appear to be the most likely infective life stage in *S. ramorum*. These results are consistent with the modern view of genetic individualism as found in many other basidiomycetes.

Our results may also support Couch's observations that a colony is generated via multiple basidiospores. First, the evidence from *S. grandisporum* suggests that this fungus produces basidiospores that have identical genotypes. Second, the data from the other species, especially *S. ramorum*, show that single dikaryotic genotypes persist despite frequent fusions with other genotypes. Our results do not support spatial structuring of genotypes within a fused colony or multiple genotypes sharing the entire colony freely. For *S. grandisporum*, our data suggest that this fungus is homothallic and produces basidiospores with identical genotypes. In other species, especially *S. ramorum*, single dikaryotic genotypes may sometimes persist despite frequent fusions with other genotypes. Our results do not support spatial structuring of genotypes within a fused colony or multiple genotypes sharing a common colony (genetic mosaicism).

Previous microscopic work reported by Couch [13] for *S. grandisporum* suggested that cells were mostly dikaryotic but sometimes uninucleate. This is consistent with our microscopic results, though we also detected multinucleate cells. It is notable that the spores from this species do not primarily grow as yeasts but instead form one or a few generations of bud cells followed by a filamentous growth habit typical of dikaryotic mycelium. Although, our results cannot provide direct evidence for mitotic production of basidiospores or self-fertilization in *S. grandisporum*, the lack of any detectable allelic variation within a colony suggests this species is homothallic.

The results from *S. mariani* also contrast with the other species by sometimes having diploid spores. This may be a result of either incomplete meiosis resulting in heterokaryotic multinucleate spores or the production of diploid spores. Cryptococcus neoformans is known to produce diploid strains from cross serotype hybridization [17] and *Helicobasidium mompa*, a fungus in the same sub-class as Septobasidium, was also demonstrated to have diploid nuclei with unusual segregation [18]. Our microscopy did not reveal obvious consistently binucleate yeast cells in cultures of *S. mariani*, but it is possible that these nuclei are cryptic because of their close spatial proximity in the small yeast cells. This issue could be resolved for *S. mariani* using transmission electron microscopy or light microscopic observations of the complete cell cycle. Interestingly, Olive [19] observed that *S. apiculatum* typically produced some binucleate basidiospores. Although we examined *S. apiculatum*, we did not detect recombinant binucleate spores in that species. Our *S. mariani* results serve to highlight the potential complexity of the fungal lifecycle.

The mechanisms generating and maintaining genetic uniformity of Septobasidium colonies are not entirely clear. Although inbreeding and restricted segregating variation have the potential to generate multiple origins of shared somatic compatibility groups [20], these are unlikely

Locus	Single spore isolate genotypes					
	SSI#1	SSI#2	SSI#3	SSI#4	SSI#5	SSI#6
ITS	I014[1]	I014	I014	I014	I014	1014
EF1α	E011-E012	E011	E012	E011	E011-E012	E011
βTUB	β009-β010[2]	β009-β010	β009-β010	β009	β009-β010	β009-β010

Note: [1]ITS locus was scored as a single allele because the single polymorphic site may be due to inter-array heterogeneity than true heterozygosity
[2]β010 genotype was inferred rather than detected directly

Table 5: Alleles recovered from putatively diploid SSI of *S. mariani* colony 092.
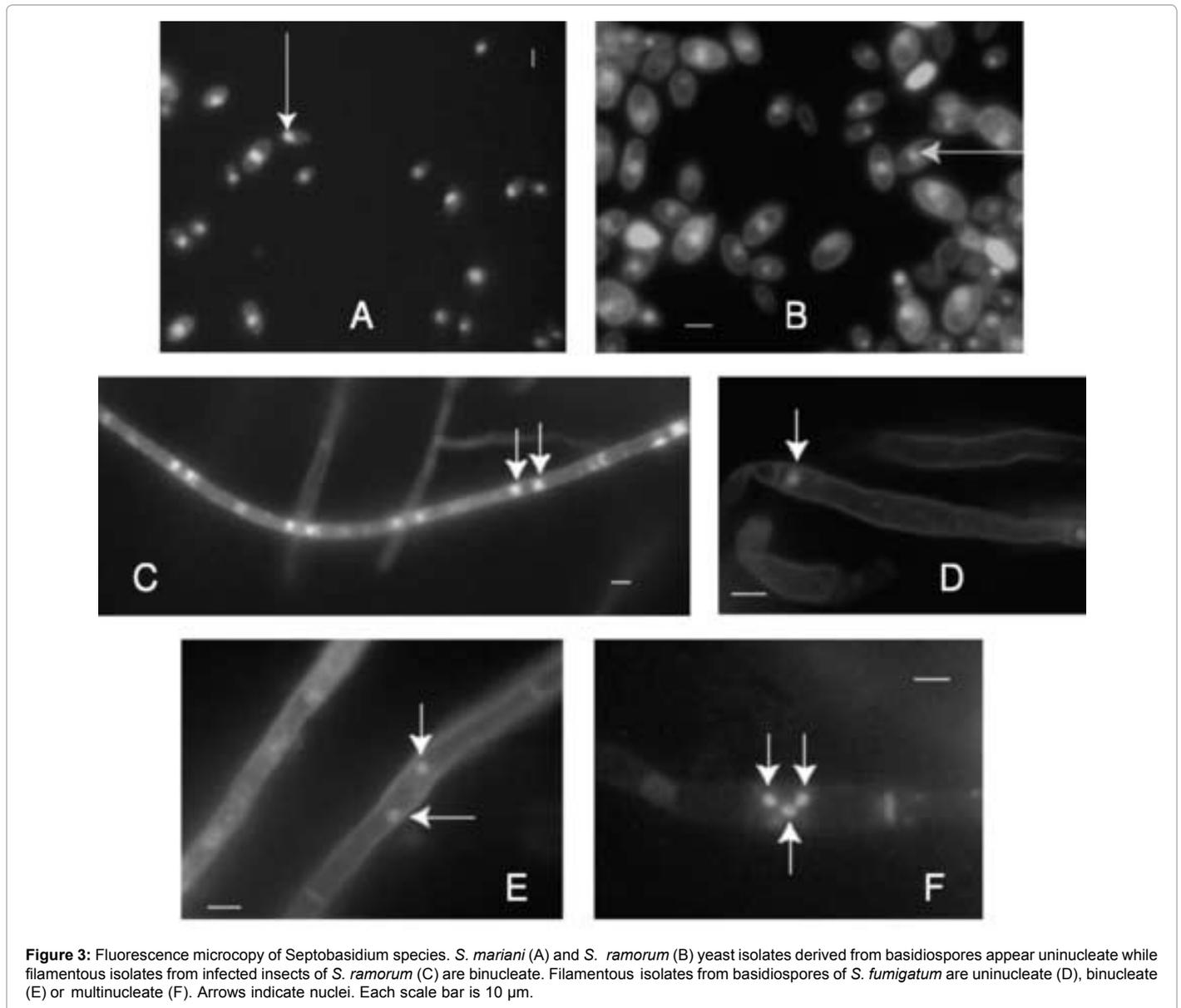
Figure 3: Fluorescence microcopy of Septobasidium species. *S. mariani* (A) and *S. ramorum* (B) yeast isolates derived from basidiospores appear uninucleate while filamentous isolates from infected insects of *S. ramorum* (C) are binucleate. Filamentous isolates from basidiospores of *S. fumigatum* are uninucleate (D), binucleate (E) or multinucleate (F). Arrows indicate nuclei. Each scale bar is 10 μm.

to explain fusions among progeny and parents in Septobasidium, given the high heterozygosity detected at other markers. The het loci responsible for somatic incompatibility in other filamentous fungi [21] may have modified function in Septobasidium or may have a different genetic architecture or selective regime leading to an extreme reduction in genetic variation relative to other loci.

Although our results conform to the idea that basidiomycete individuals are defined by genotype, some questions remain. Each Septobasidium colony represents a single genetic individual, but its stability may be at risk. Frequent non-self-fusions may make an individual vulnerable to invasion by other nuclei and cytoplasmic elements, disrupting the dominant dikaryon. Also, almost all of the resources available to a genetic individual must be acquired through non-self-fusions, making the genetic individual entirely dependent on other genotypes for survival. In some sense, this is a community of individuals providing benefit to a single dominant individual for at least one season of reproduction. From this perspective, Septobasidium

conforms closely to a "unit mycelium" in which a community of genotypes function as an individual [22-27].

The genetic life-history of Septobasidium may disconnect aspects of the fungus - insect interaction. Each insect is infected by a unique genotype, but it is the dominant dikaryon alone that draws most nutrition from living infected insects and provides any potential protection to the scale insect colony. The origin of the dominant genotype is unclear, and it may originate from a single infected insect or arises from nuclear exchange among different fusing fungal genotypes later in colony formation.

Interactions between scale insects and Septobasidium affect each other's reproductive fitness depending on whether the fungus can or cannot infect its insect host. Since only uninfected insects are fertile and able to contribute insect progeny to the next generation, a fungus that successfully infects (and sterilizes) an insect has five potential fates: 1) The fungus may not reproduce because it is unable to gain sufficient resources to fruit or is not an infection caused by a strain or strains capable of sexual reproduction; 2) The fungus may establish a new colony and fruit successfully without encountering another fungal genotype; 3) The fungus may be met and overrun by another genotype; 4) The fungus may invade other fungi and fruit successfully; 5) The fungus may partially invade another fungus by donating only one of its nuclei or its cytoplasmic components to a new dominant dikaryon. From these potentialities, it is clear that in order for an allele to propagate through the fungal population it must be able to infect insects, but the most infective genotypes are not necessarily successful at propagating through the population because the fungal competition component occurs after infection. The effect of the insects on fungal fitness at this stage is complex.

Another category of fungus/insect interaction is it that of fused insects with existing dikaryons rather than the dispersing fungal propagules. A dominant dikaryon does not affect the fitness of the fused scale insects directly because they are already sterilized. However, the dominant dikaryon may have a strong impact the fitness of the fused scale insects indirectly by modifying the fitness of uninfected scale insects associated with the fungal colony. Fungi can modify that fitness component through multiple avenues, including protection from predators, desiccation and the funguses own infective spore production. A colony of fused infected scale insects can affect the existing dikaryon's fitness by modifying the production of spores or the dikaryon's competitive ability in non-self-fusions.

The Septobasidium symbiosis is potentially mutualistic or cooperative at multiple levels of genetic organization. That individual insects may be sacrificed for the greater good of the nearby insect colony is nearly mirrored by the infective fungi associated with them. The nuclei of these fungi, derived from meiotically produced basidiospores, may be sacrificed for the benefit of the fused fungal colony. The individuals sacrificed are likely to be very closely related to the individuals receiving benefit. Coevolution may have a prominent role in the loss of "individualistic mycelium" behavior because of the Septobasidium's dependence on infecting individual scale insects [28-31].

References

1. Buss L (1982) Somatic cell parasitism and the evolution of somatic tissue compatibility. Proc Nat Acad USA 79: 5337-5341.

2. Pringle A, Taylor JW (2002) The fitness of filamentous fungi. Tren Microbiol 10: 474-481.

3. Rayner ADM, Todd NK (1982) Population and community structure and dynamics of fungi in decaying wood. In: Frankland JC, Hedger JN, Swift MJ (eds.), Decomposer Basidiomycetes: their biology and ecology. Cambridge University Press, Cambridge 129-142.

4. Buller AHR (1933) Researches on fungi. Longmans, Green and co., London.

5. Rayner ADM (1991) The challenge of the individualistic mycelium. Mycologia 83: 48-71.

6. Worrall JJ (1997) Somatic incompatibility in basidiomycetes. Mycologia 89:24-36.

7. Jacobson KM, Miller OK, Turner BJ (1993) Randomly amplifies polymorphic DNA markers are superior to somatic incompatibility tests for discriminating genotypes in natural populations of the ectomycorrhizal fungus *Suillus granulatus*. Proc Nat Acad USA 90: 9159-9163.

8. Carlile MJ (1987) Genetic exchange and gene flow: their promotion and prevention. In: Evolutionary biology of the fungi. Rayner, ADM, Braiser CM, Moore D (eds.), Cambridge University Press, Cambridge 203-214.

9. Kistler HC, Miao VPW (1992) New modes of genetic change in filamentous fungi. Annu Rev Phytopathol 30: 131-152.

10. Rayner ADM, Coates D, Ainsworth AM, Adams TJH, Williams END (1984) The biological consequences of the individualistic mycelium. In: Jennings DH, Rayner ADM (eds.), The ecology and physiology of the fungal mycelium. Cambridge University Press, Cambridge 509-540.

11. Couch JN (1931) The biological relationship between Septobasidium *retiforme* (B. & C.) Pat. and *Aspidiotus osborni* New. and Ckll. Quart J Microscop Sci 74:383-437.

12. Couch JN (1935) Septobasidium in the United States. J Elisha Mitchell Sci Soc 51: 1-77.

13. Couch JN (1938) The genus Septobasidium. University of North Carolina Press, Chapel Hill, NC.

14. Gardes M, Bruns T (1993) ITS primers with enhanced specificity of basidiomycetes: application to the identification of mycorrhizae and rusts. Mol Ecol 2: 113-118.

15. Morehouse EA, James TY, Ganley ARD, Vilgalys R, Berger L (2003) Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. Mol Ecol 12: 395-403.

16. James TY (2003) Evolution of mating type genes in mushroom fungi (Homobasidiomycetes). Ph. D. Dissertation. Duke University, Durham, North Carolina.

17. Lengeler KB, Cox GM, Heitman J (2001) Serotype AD strains of *Cryptococcus neoformans* are diploid or aneuploid and are heterozygous at the mating-type locus. Infect Immun 69: 115-122.

18. Aimi T, Iwasaki Y, Kano S, Yotsutani Y, Morinaga T (2003) Heterologous diploid nuclei in the violet root rot fungus *Helicobasidium mompa*. Mycol Res 107: 1060-1068.

19. Olive LS (1943) Cytology of various basidial types in the genus Septobasidium. Mycologia 35: 557-572.

20. Kohn LM (1995) The clonal dynamic in wild and agricultural plant-pathogen populations. Can J Bot 73: S1231-S1240.

21. Glass LN, Kaneko I (2003) Fatal attraction: Non-self recognition and heterokaryon incompatibility in filamentous fungi. Euk Cell 2: 1-8.

22. Johannesson H, Stenlid J (2004) Nuclear re-assortment between vegetative mycelia in natural populations of the basidiomycete *Heterobasidion annosum*. Fung Genet Biol 41: 563-570.

23. Kay E, Vilgalys R (1992) Spatial distributions and genetic relationships among individuals in a natural population of the oyster mushroom *Pleurotus ostreatus*. Mycologia 84: 173-182.

24. Lively CM, Dybdahl MF, Jokela J, Osnas EE, Delph LF (2004) Host sex and local adaptation by parasites in a snail-trematode interaction. Am Nat 164: S6-S18.

25. May RM, Anderson RM (1990) Parasite-host coevolution. Parasitol 100: 89-101.

26. Michod RE (1999) Individuality, immortality and sex. In: Levels of selection in evolution. Keller L (eds.), Princeton University Press, Princeton 53-74.

27. Nuismer SL, Otto SP (2004) Host–parasite interactions and the evolution of gene expression. Proc Nat Acad USA 101: 11036-11039.

28. Aimi T, Yotsutani Y, Morinaga T (2002) Cytological analysis of anastomoses and vegetative incompatibility reactions in *Helicobasidium monpa*. Curr Microbiol 44:148-152.

29. Thompson JN (1994) The coevolutionary process, University of Chicago Press, Chicago.

30. Uetake Y, Arakawa M, Nakamura H, Akahira T, Sayama A, et al. (2002) Genetic relationships among violet root rot fungi as revealed by hyphal anastomosis and sequencing of the rDNA ITS regions. Mycological Research 106:156-163.

31. White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications. Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) Academic Press, Inc., New York 315-322.