

Marker stability throughout 400 days of in vitro hyphal growth in the filamentous ascomycete, *Sclerotinia sclerotiorum*

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Abstract

The stability of routinely used, population genetic markers through approximately 1 year of continuous laboratory growth was investigated in the common, plant pathogenic ascomycete *Sclerotinia sclerotiorum*. Given reports of accelerated mutation rates at higher temperatures, both a permissive temperature, 22 °C, and a temperature at the high end of tolerance, 30 °C, were employed. Because mycelial growth rate was tracked among mitotic lineages established for each strain, a subsidiary objective was addressed, testing the stability of a 30 °C-competent phenotype. Twelve laboratory strains of *S. sclerotiorum*, including the genome sequence isolate, 1980, were propagated serially for up to 400 days at 22 °C. Five of these strains were also propagated at 30 °C. No mutations were observed in mycelial compatibility groupings (MCGs), DNA fingerprints, alleles at 7 microsatellite loci, or alleles at 56 AFLP loci. All of these markers show variation in field populations, which are likely much larger and influenced by different and more stochastic environmental processes. In *S. sclerotiorum*, population genetic markers were stable over time through serial transfer and growth of laboratory strains at both 22 °C and 30 °C. The strain isolated after extended drought and capable of infecting plants at 28 °C demonstrated the stability of its high temperature-competent phenotype, in addition to its stable growth rate at 22 °C. This observation has implications for modeling pathogen tolerance or adaptation under conditions of environmental stochasticity, including climate warming.

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1. Introduction

Population studies of fungi rely on the stability of genetic markers in strains propagated in the laboratory. Although the stability of genotypes is generally assumed, some markers, such as those based on simple DNA sequence repeats or the distribution of repetitive DNA elements in the genome may be subject to much higher mutation rates than those based on nucleotide substitutions. Variation within a marker system can vary significantly. For example, microsatellite loci are thought to have mutation rates ranging from about 10^{-3} to 10^{-6} per locus per gamete per generation (Primmer et al., 1996; Schug et al.,

1997; Weber and Wong, 1993). Mutation rates for dominant marker systems such as amplified fragment length polymorphism (AFLP) analysis have not been well studied.

High genetic diversity is often interpreted as a sign of genetic exchange and recombination but the mutation rate of markers is usually unknown and could be a confounding factor. The mutation rate per genome and per base pair (per replication) in *Saccharomyces cerevisiae* is 2.7×10^{-3} and 2.2×10^{-10} and in *Neurospora crassa*, 3.0×10^{-3} and 7.2×10^{-11} , respectively (Drake et al., 1998). The mutation rates of any given marker may diverge from this norm. Furthermore, environmental factors, such as elevated temperatures can raise the mutation rate and represent a stress to which pathogens may have to adapt under scenarios of climate change. Specifically, heat can be mutagenic in promoting base loss and base mutations (Drake et al., 1998).

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Sclerotinia sclerotiorum is a plurivorous, necrotrophic plant pathogen responsible for substantial economic losses, with an ample literature and research history in population genetics using the markers employed in the present study (Bolton et al., 2006; Malvárez et al., 2007). While not a model research system, it is a ubiquitous plant pathogen with an active genome project conducted in collaboration with those investigating the closely related and equally ubiquitous necrotrophic pathogen, *Botrytis cinerea*.

We have previously detected one mutation in our markers, the loss of a hybridizing band in the DNA fingerprint from a sector in routine serial culture of the laboratory standard strain, LMK 211. A sector is a wedge-shaped zone in a radially expanding fungal colony on solid growth medium. It originates with a mutation that then propagates from the source cell via mitosis and branching hyphal cell growth.

The purpose of this study was to test four commonly used marker systems in the filamentous ascomycete, *Sclerotinia sclerotiorum*: (i) mycelial compatibility groups (MCGs) (Kohn et al., 1990, 1991; Schafer and Kohn, 2006), (ii) fingerprints based on hybridization of a dispersed repetitive element with restriction-digested genomic DNA (Kohn et al., 1991), (iii) AFLPs (Vos et al., 1995), and (iv) microsatellites (Sirjusingh and Kohn, 2001), for stability over long term culture in the laboratory. If mutations were detected, a relative mutation rate could be estimated from mutations arising over a fixed time period in these populations of relatively fixed size. Given reports of accelerated mutation rates at higher temperatures, both a permissive temperature, 22 °C, and a temperature at the high end of tolerance, 30 °C, were employed. In the course of this experiment, mycelial growth rate was tracked among the experimental vegetative lineages established from each strain. As a subsidiary objective, the stability of a high temperature-competent phenotype was tested using these data.

2. Materials and methods

Twelve isolates, propagated as laboratory strains, were used in this experiment (Table 1). LMK 211 has been maintained in serial culture and used as a standard for scoring in all Southern hybridizations for DNA fingerprinting conducted in the Kohn laboratory since 1989. Strain 19-434 was selected for the present study in order to determine both the possible acceleration in the mutation rate caused by higher temperature and also the stability of its temperature tolerance phenotype which combines excellent growth at the usual temperatures for the species, e.g. 20–22 °C, with strong growth at a usually inhibitory temperature, 30 °C.

The 12 strains were propagated on Potato Dextrose Agar (PDA, Difco Laboratories, Detroit) in 30-cm glass race tubes (2.5 cm inside diameter). The race tubes were fabricated at the University of Toronto; for a description see Ryan et al. (1943). The serial growth in race tubes com-

menced on October 27, 2004 (transfer 1) and terminated on March 31, 2006 (transfer 44). The initial inoculation of the first race tube was with a 3 mm³ block of mycelial inoculum (also termed a mycelial plug) from the growing mycelial front of a Petri dish culture of PDA that had been incubated for 1 week at 22 °C. Transfer from a fully colonized race tube to a fresh tube was of a 2 mm wide strip cut from the entire growing mycelial front of the colonized tube. For each strain, two replicates were grown in the dark at room temperature (22 °C). Five of the 12 strains (LMK 211, 1980, 19-434, LMK 199, O-CEF-33) were also grown in the dark at 30 °C with two replicates. For each replicate line, the position of the mycelial front was marked on the race tubes every 3–5 days, or, in the case of strains growing at 30 °C, every one to 2 weeks. Strains were archived at each transfer to a new race tube. If a strain terminated growth or became contaminated, the most recently archived strain was used to inoculate a new race tube. During the experiment, strains were archived in several ways; plugs of young actively growing mycelium were stored in water at 4 °C or in 10% glycerol at –20 °C and –80 °C. Dry, mature sclerotia were also stored in 2-ml screw cap tubes at 4 °C.

At different points in the experiment all strains showed a sharp reduction in growth rate, deemed likely to result in death of the colony, which was repeatable when we went back to the most recently archived sample. Given that the objective was to monitor genetic marker stability, not strain physiology, one attempt was made to refresh each experimental lineage derived from a strain. Here, 5–6 carrot disks were placed in a glass jar with a lid in distilled water, so that half the carrot protruded from the water, and autoclaved for 20 min. One plug of mycelium was placed mycelium side down on each carrot disk and incubated at 22 °C until new sclerotia were formed. The mature sclerotia were then plated on PDA and the resulting mycelium was used to inoculate a new race tube. The duration of the refreshment procedure was approximately 4 weeks at 22 °C. Some experimental lineages terminated growth during the experiment (Fig. 1, as indicated by termination of the growth measurement line) despite an attempt to refresh the growth of the lineage.

How many nuclear generations are represented in these experiments? On average, the hyphal tips of the strains used here, as well as hyphae one cm behind the growing fronts have septa every 150 µm. This translates to more than 60 cellular compartments per centimeter. As several strains grew at a rate of about 3 cm per day, this means that more than 180 compartments are produced per day, a rate that can only be much higher than the average number of nuclear divisions. Since the hyphae of *S. sclerotiorum* are highly multinucleate, it is therefore not possible to estimate a nuclear generation time. A reasonable assumption, however, is that an actively growing strain experiences about 1 nuclear generation per hour, or 24 generations per day, about the maximum for any eukaryote (e.g. 62.5 min for the nuclear cycle of *Saprolegnia ferax*; Heath

Table 1

List of strains and their genotypes for 10 genetic markers that did not change throughout up to 400 days of vegetative growth, with pertinent terminology defined

Strain ^a	Clone ^b	MCG	RFLP fingerprint	Microsatellite alleles at seven loci ^c							AFLP Genotype	Provenance
				5-3	7-3	17-3	55-4	106-4	110-4	114-4		
1980	2	2	2	C	A	B	C	A	C	C	H	Bean, Nebraska 1980 ^d
LMK 211	2	2	2	C	A	B	C	A	C	C	H	Canola, Ontario 1989 ^e
LMK 199	1	1	1	B	B	B	C	C	C	B	G	Canola, Ontario 1989 ^e
O-CEF-33	1	1	1	B	B	B	C	C	C	B	G	Canola, Ontario 1999 ^f
19-434	1	1	1	B	B	B	C	C	C	B	G	Soybean, Ontario 2002 ^g
SS02-5	X	X	X	A	A	A	A	A	A	A	I	Pea, Washington 2002 ^h
SS02-12	X	X	X	A	A	A	B	A	A	A	I	
SS02-33	X	X	X	A	A	A	B	A	A	A	I	
SS02-41	X	X	X	A	A	A	A	A	A	A	I	
SS02-57	X	X	X	A	A	A	B	A	A	A	I	
SS02-87	Y	Y	Y	B	B	B	B	B	A	B	K	
SS02-122	Z	Z	Z	A	A	A	B	A	B	A	J	

^a A strain is a serially transferred, laboratory-maintained isolate. The term isolate is here used only for the first culture established from the host. In this experimental evolution study, each strain serially transferred in race tubes is termed a lineage in the text because it is a mitotic lineage established from the progenitor strain.

^b A clone of *S. sclerotiorum* is a genotype identified with two unlinked markers that is repeatedly recovered from a wide geographical area; all members of a clone of *S. sclerotiorum* are mycelially compatible, i.e. in the same mycelial compatibility group or MCG (Kohn et al., 1990; Schafer and Kohn, 2006), and share the same DNA fingerprint or a fingerprint differing by 5 or fewer hybridizing bands (Kohn et al., 1991; Hambleton et al., 2002).

^c Sirjusingh and Kohn, 2001.

^d 1980 is the *Sclerotinia* genome sequence strain (http://www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/Home.html).

^e Kohn et al. (1991).

^f Hambleton et al. (2002).

^g Isolated under drought conditions and demonstrated to maintain its laboratory growth rate at 30 °C and infect soybean under greenhouse conditions at 28 °C (Kohn, Fazio and Rajcan, unpublished).

^h Isolated in 2002, from pea in one field in Quincy, Washington. These Washington isolates represent a population distinct from that of the Ontario and Nebraska isolates, with no MCGs or DNA-RFLP fingerprints in common (Malvárez et al., 2007).

and Rethoret, 1981). Over 400 days, this would correspond to about 10,000 generations and the end points for each pair of replicate lines would be separated by about 20,000 generations from one another through divergent evolution from the common ancestor.

Marker assays were conducted regularly throughout the experiment. The distance of mycelial growth for each isolate at each marker-assay point is shown in Fig. 1. The termination of a line represents the final cessation of growth for that lineage in the experiment.

Mycelial compatibility assays, as described by Schafer and Kohn (2006), were performed for the progenitor of each isolate at the beginning of the experiment as well as at the first, second, and final assay points. For the initial MCG testing all 12 isolates were paired in all combinations including self-selfs as a control (always compatible). For assays 1, 2, and 4, each isolate was paired with itself, its own progenitor, a progenitor from the same initial MCG, and a progenitor representing any other of the MCGs.

RFLP fingerprints were assayed by the method of Kohn et al. (1991) at each assay point.

Amplified fragment length polymorphism (AFLP) analysis (Vos et al., 1995) was performed on genomic DNA using the AFLP Microbial Fingerprinting protocol (Applied Biosystems, Foster City, CA) with slight modifications as described previously (Grünwald and Hoheisel, 2006). DNA (20 ng) was digested and adaptors were ligated in a 11 µl reaction volume with EcoRI (5 U), MseI (1 U),

T4 DNA ligase (1 Weiss U), 1.0 µl MseI and EcoRI adaptor, 1.1 µl 0.5 M NaCl, 0.5 µl BSA (1.0 mg/ml), and 1.1 µl 10X T4 DNA ligase buffer (New England Biolabs, Beverly, MA; 50 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 25 µg/ml BSA) for 2 h at 37 °C. Restriction-ligation samples were diluted 1:4 with sterile deionized water. Preselective amplifications totaling a 10 µl reaction of 3.0 µl restriction-ligation sample with 0.25 µl EcoRI core primer, 0.25 µl MseI core primer and 3.25 µl AFLP amplification core mix (Applied Biosystems) were performed on a GeneAmp 9700 thermal cycler (Applied Biosystems) programmed as follows: 2 min at 72 °C; 20 cycles of 20 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C; and cooling to 4 °C. Amplicons (10 µl) were checked on 1.2% agarose gels (4V/cm for 3–4 h) in 1X TBE buffer and visualized with ethidium bromide and UV illumination. A ten microlitres pre-amplified sample was diluted 1:8 with sterile deionized water. Selective PCR was performed on 1.5 µl diluted, pre-amplified product in a 10 µl reaction volume with 0.5 µl MseI-AC primer at 5 µM, dye-labeled 0.5 µl EcoRI-AC primer at 1 µM and 3.75 µl AFLP core amplification mix (Applied Biosystems). Reactions were performed under the following conditions: 2 min at 94 °C; 10 cycles of 20 s at 94 °C, 30 s at 66 °C and 2 min at 72 °C (annealing temperature is lowered by 1 °C during each cycle); followed by 20 cycles of 20 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C; and a final extension for 30 min at 60 °C and cooling to 4 °C. Products (1.0 µl)

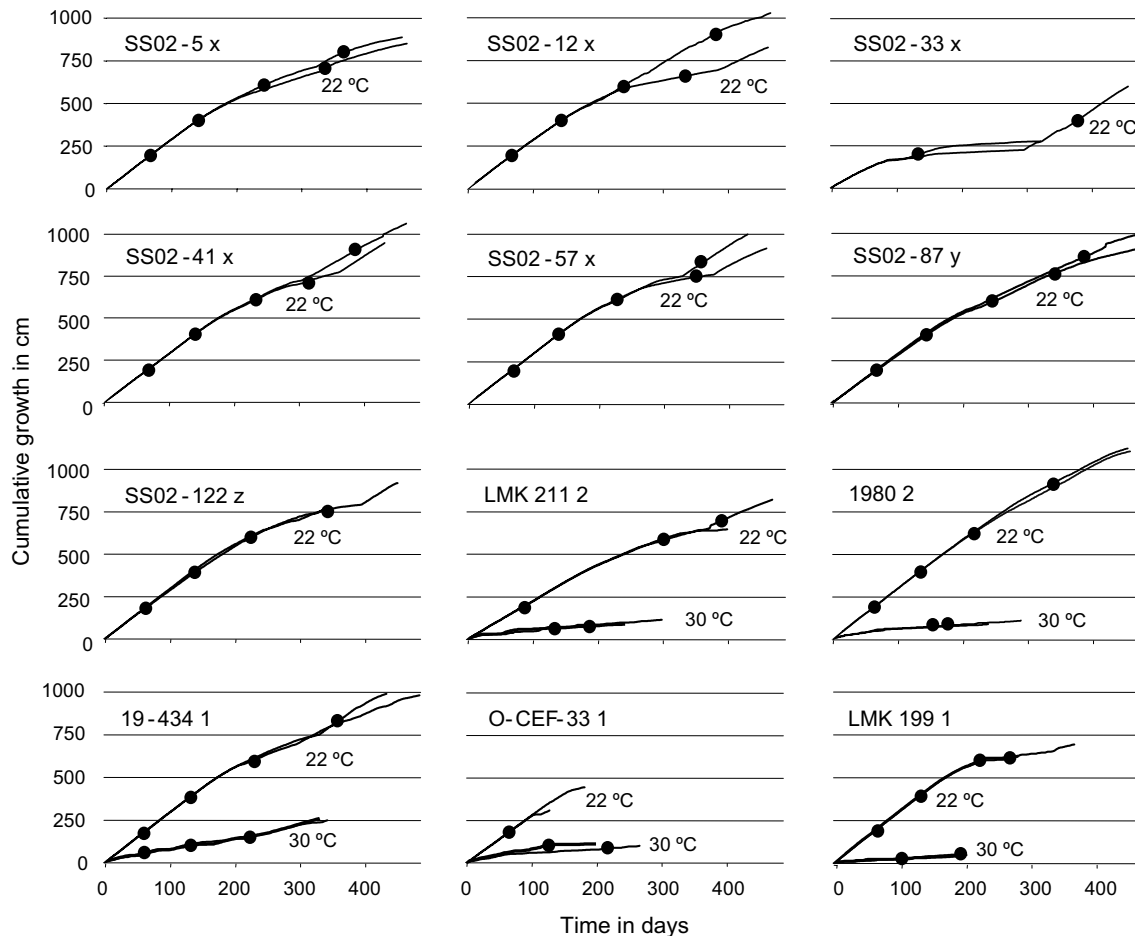


Fig. 1. Growth curves for 12 strains of *S. sclerotiorum* grown at 20 °C for approximately 400 days of serial transfer or at 30 °C for approximately 300 days or until death. SS-prefixed strains are from Washington with suffixes of x, y or z designating clonal lineages (Grünwald, Chen and Kohn, unpublished), 1980 is the genome sequence strain from Nebraska, and all remaining strains are from Ontario with suffixes 1 or 2 designating clonal lineages (Hambleton et al., 2002; Kohn et al., 1991). Strain 19-434 was recovered after 2 years of drought with high seasonal temperatures.

were run with 10 μ l loading buffer (9.8 μ l deionized formamide and 0.2 μ l GeneScan-500 size standard, Applied Biosystems) on a capillary sequencer (3100 Avant Genetic Analyzer, Applied Biosystems). Samples were run in a 36 cm μ m capillary with POP-4 polymer. Samples were injected for 12 s at 15 kV and run at 13 kV for 34 min at 60 °C. Electropherograms were analyzed using GeneMapper software (version 3.7; Applied Biosystems) to extract a matrix of presence and absence of alleles in a range of 100–500 bp after visual inspection for further downstream analysis. All AFLP analyses were replicated on independent DNA extractions and PCRs until all polymorphic alleles could be unambiguously called. AFLPs were also assayed at each point indicated in Fig. 1. AFLP analysis resulted in a total of 56 alleles with an average of 22 and a range of 19–25 bands per individual.

Seven microsatellite primer pairs (5–3, 7–3, 17–3, 55–4, 106–4, 110–4, 114–4) developed by Sirjusingh and Kohn (2001) were chosen based on markers with higher allelic diversity observed in *S. sclerotiorum* populations. Each strain had one characteristic fragment size for each microsatellite.

3. Results and discussion

No changes were observed for any of the markers systems at any point during the experiment. AFLP allele distributions, mycelial compatibility, and DNA fingerprints were completely associated, delimiting clonal lineages; microsatellite allele distributions did not resolve clonal lineages with all loci, consistent with previous observations (Sirjusingh and Kohn, 2001).

Incubation at 30 °C was stressful, as reflected in a reduced growth rate or death, but all markers remained stable through approximately 300 days of serial culture. Both replicates of the temperature tolerant strain, 19-434, when incubated at 30 °C, showed a sharply reduced growth rate at transfer 7, but recovered normal growth rate after approximately 4 weeks of refreshment at 22 °C. At 22 °C only one of the two replicates of 19-434 required refreshment and not until transfer 30. Compared with other strains, the relatively high growth rate at 30 °C as well as the high rate at 20 °C is striking. This strain was isolated from a drought-stressed soybean field with disease incidence below 1%, after two successive years with above

average degree days (≥ 30 °C). This strain appears to have an extended temperature tolerance range, likely through mutation, that is stably maintained, noting that the same temperature response is not evident in other strains from clonal lineage 1 included in this study and sampled before the period of drought. The frequency of high temperature tolerant isolates was not determined among the sample of five made from this field, nor among the total of 83 isolates made in 2002. Nevertheless, the existence of such a phenotype, with fitness within the normal temperature range and at usually limiting high temperatures, provides one model of adaptation to climate stochasticity, if not climate warming, for future studies in *S. sclerotiorum* and other plant pathogens.

Although the nuclear generation time of *S. sclerotiorum* is uncertain, our experiments represent a cumulative total of more than 30 years of continuous growth in culture (length in days from the start of the experiment to the final marker assay point for each replicate, two at 22 °C plus two at 30 °C for five strains, of each of the 16 strains in Fig. 1) with not a single genotypic change observed. The generation time for this fungus in nature is also unknown, but it can only be much longer than the generation time of a culture continuously growing in vitro. This study lends support to the assumption that the genotypic variation observed in field populations of *S. sclerotiorum* arises on a substantially different time scale in likely much larger populations. For example, in field populations these same markers have detected multiple DNA fingerprints or microsatellite alleles within one mycelial compatibility group, turn-over of DNA fingerprints and MCGs within a 2-year-period (Carbone and Kohn, 2001), as well as phenotypic diversity within clonal lineages (Hambleton et al., 2002; Kull et al., 2003; Malvárez et al., 2007). These field studies were not based on marked progenitor isolates, although the study of Carbone and Kohn (2001) was performed on marked quadrats resampled over two years. In other pathosystems, such evidence of mutation over time in field populations has also been observed. For example, recently Prospero et al. (2007) observed mutations in some microsatellite loci in a clonal population of *Phytophthora ramorum* in Oregon in natural infestations (2007). Out of 10 loci analyzed, only two (PrMS43a and PrMS43b) were very informative and differentiated most genotypes over the course of the study that ranged from 2001 to 2004.

Serial propagation of laboratory strains is quite different from propagation, dispersal, and interaction among genotypes in the wild. Nevertheless, the stability of strains in the laboratory is of substantial importance to workers conducting long-term research utilizing archived strains. We conclude that the probability of confounding effects due to genotypic changes during laboratory propagation of any strains is minimal in *S. sclerotiorum*. As a side note, without very large population sizes and high standing genetic variability in the progenitor (perhaps enhanced by mutagenesis), *S. sclerotiorum* is a poor candidate for experimental evolution.

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