Signatures of Recombination in Clonal Lineages of the Citrus Brown Spot Pathogen, *Alternaria alternata* sensu lato

Jane E. Stewart, Kalyn A. Thomas, Christopher B. Lawrence, Ha Dang, Barry M. Pryor, L. M. (Pete) Timmer, and Tobin L. Peever

First, second, and seventh authors: Department of Plant Pathology, Washington State University, Pullman; third and fourth authors: Virginia Bioinformatics Institute and Department of Biological Sciences, Blacksburg; fifth author; Division of Plant Pathology and Microbiology, School of Plant Sciences, University of Arizona, Tucson; and sixth author: Citrus Research and Education Center, University of Florida, Lake Alfred, FL.

Current address of J. E. Stewart: U.S. Department of Agriculture–Agricultural Research Service, Horticultural Crops Research Laboratory, Corvallis, OR.

Accepted for publication 19 February 2013.

ABSTRACT

Stewart, J. E., Thomas, K. A., Lawrence, C. B., Dang, H., Pryor, B. M., Timmer, L. M., and Peever, T. L. 2013. Signatures of recombination in clonal lineages of the citrus brown spot pathogen, *Alternaria alternata* sensu lato. Phytopathology 103:741-749.

Most *Alternaria* spp. are considered asexual but recent molecular evolution analyses of *Alternaria* mating-type genes show that the mating locus is under strong purifying selection, indicating a possible role in sexual reproduction. The objective of this study was to determine the mode of reproduction of an *Alternaria alternata* sensu lato population causing citrus brown spot in central Florida. Mating type of each isolate was determined, and isolates were sequenced at six putatively unlinked loci. Three genetically distinct subpopulations (SH1, SH4A, and SH4B) were identified using network and Bayesian population structure analyses. Results demonstrate that most subpopulations of *A. alternata* associated with citrus are clonal but some have the ability to extensively recombine through a cryptic sexual cycle or parasexual cycle. Although isolates were sampled in close physical proximity (≈2,500-m^2 area), we were able to reject a random mating model using multilocus gametic disequilibrium tests for two subpopulations, SH1 and SH4B, suggesting that these subpopulations were predominantly asexual. However, three recombination events were identified in SH1 and SH4B and localized to individuals of opposite mating type, possibly indicating meiotic recombination. In contrast, in the third subpopulation (SH4A), where only one mating type was present, extensive reticulation was evident in network analyses, and multilocus gametic disequilibrium tests were consistent with recombination. Recombination among isolates of the same mating type suggests that a nonmeiotic mechanism of recombination such as the parasexual cycle may be operating in this subpopulation. The level of gene flow detected among subpopulations does not appear to be sufficient to prevent differentiation, and perhaps future speciation, of these *A. alternata* subpopulations.

Additional keywords: diversity, index of association.

Many fungi have the capacity for both sexual and asexual development, and fungi with mixed reproductive modes are generally more genotypically diverse and may adapt more rapidly to novel environments compared with exclusively asexual organisms (44,79). Although teleomorphs have been described for many plant-pathogenic ascomycetes, it is not clearly known how often the teleomorph occurs and to what extent meiosis contributes to the natural biology of the pathogen. Many putatively asexual fungi have been shown to have recombined population structures or recombinant genomes, possibly indicative of an active sexual stage and meiotic recombination (17,41,44,51,75,79). This growing list of putatively asexual fungi bearing signatures of recombination highlights the need for a more thorough investigation of the mode of reproduction of putatively asexual fungi.

The majority of *Alternaria* spp. are considered exclusively asexual or clonal in their reproductive mode (26,73,74). Evidence for asexuality in *Alternaria* spp. includes a lack of teleomorph connections, lack of reports of the sexual stage from nature, and lack of success in inducing laboratory crosses (8,12). Despite the apparent lack of meiotic recombination, high levels of genotypic diversity have been found in some *Alternaria alternata* populations using random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism, and microsatellite markers sampled from various hosts, including tomato (47), pistachio (5), and *Pinus* sp. (23), and the closely related *A. tenuissima* on wheat (22) and *A. helianthi* on sunflower (65). Similarly, a recent population study of *A. brassicicola* found a large number of multilocus haplotypes using AFLP and microsatellite markers, possibly indicative of meiotic or mitotic recombination within this putatively asexual species (9,50). Both meiotic and mitotic recombination can lead to populations with high genotypic diversity and linkage equilibrium, whereas asexual populations are characterized by strong associations among loci and multiple occurrences of the same haplotype composed of unique combinations of alleles (35,44). The recent discovery of a teleomorphic stage for the putatively asexual fungus *Aspergillus fumigatus* (54) suggests that the high levels of genotypic diversity observed in populations of *Alternaria alternata* could be derived from cryptic sexual reproduction or through mitotic mechanisms of recombination such as the parasexual cycle (62).

Several studies have examined the role of mating genes in putatively asexual *Alternaria* spp. Arie et al. (6) demonstrated that mating genes are expressed in *A. alternata*, and Stewart et al. (76)

Corresponding author: J. E. Stewart;
E-mail address: stewajan@science.oregonstate.edu

*The *e*-Xtra logo stands for "electronic extra" and that the online version contains one supplemental table.

http://dx.doi.org/10.1094/PHYTO-08-12-0211-R

showed that over half the codon sites in both mating genes were under strong purifying selection. Together, these results suggest that these loci are not evolving neutrally, as might be expected in a species where sex does not occur and where mating genes might be predicted to be redundant and nonfunctional. These patterns of selection could be explained by the existence of a cryptic contemporary sexual cycle, a recent sexual past, or the involvement of the mating type loci in other critical cellular functions.

Previous population studies of the citrus brown spot pathogen, *A. alternata*, strongly supported an asexual or clonal reproductive model (57,60). Isolates sampled from a single grove of 'Minneola' tangelo in central Florida could be differentiated into two well-supported genetic groups and, subsequently, into distinct phylogenetic lineages named SH1 and SH4 based on sequence data from an endo-polygalacturonase (*endoPG*) gene (58,61). Currently, it is unclear whether these phylogenetic lineages should be considered population diversity or discrete species (3). Previous population structure studies of *Alternaria* spp. infecting citrus have suggested asexuality (57,60); however, the mode of reproduction of this fungus has not been critically tested. Within the SH1 and SH4 groups, both mating types were present but in highly skewed ratios (T. L. Peever, *unpublished data*), supporting a clonal reproductive model. A 1:1 ratio of mating types was rejected in population SH1 but could not be rejected in the SH4 population, likely due to small sample size and type II statistical error. Interestingly, *MAT1-1* isolates predominated in the SH1 population, whereas *MAT1-2* isolates predominated in the SH4 population. Mating types were perfectly correlated to multilocus haplotype (16 putative loci) in both populations, again providing strong support for a lack of contemporary recombination in this sample. Limitations of the Peever et al. (57) study included lack of information concerning linkage relationships among RAPD loci and between the RAPD loci and the mating type locus and small sample sizes. Furthermore, phylogenetic studies of small-spored *Alternaria* taxa, including isolates causing brown spot of citrus, have revealed putative intragenic recombination events within particular regions of the *A. alternata* genome (3) suggesting that recombination may be more common than previously thought.

Citrus are long-lived, evergreen species, and the longevity of the interaction between these hosts and *Alternaria* spp. makes it an ideal system for exploring the mating system and potential recombination in a single population from a single geographic location. The objective of this study was to ascertain the mode of reproduction and detect recombination in a large sample of *Alternaria* isolates infecting citrus. The development of several physically unlinked variable genomic regions allowed for the assessment of the mating system and potential recombination using multilocus sequence typing in a large, well-characterized population of *Alternaria* spp. from citrus.

MATERIALS AND METHODS

Sampling. In all, 105 isolates of *A. alternata* sensu lato were randomly sampled from a 2,500-m^2 area within a single citrus grove (Shinn Grove) in central Florida planted to Minneola tangelo (*Citrus reticulata* × *C. paradisi* (Macfad.)) in 1999. This same location was sampled previously, in 1996 (57). A single infected leaf was sampled from each tree and a single hyphal isolate of the pathogen was obtained from each leaf. A single-conidial isolate was derived from each mass-hyphal isolate and stored following the procedures of Peever et al. (57,61).

DNA extraction. Fungi were cultivated in potato dextrose broth (VWR International, Radnor, PA) for 5 to 7 days at room temperature on an orbital shaker at 150 rpm. Phenol and chloroform were used to extract genomic DNA from powdered, lyophilized mycelium following the methods of Peever et al. (57), quantified using a NanoDrop spectrophotometer ND-1000 (NanoDrop, Wilmington, DE), and used as template for polymerase chain reaction (PCR).

Selection of subsample for sequencing and analysis. Prior to sequencing and phylogenetic analyses, all 105 isolates were characterized for mating type (*MAT1-1* or *MAT1-2*) using mating type-specific PCR. Primers used were ALMAT-L/AA-MAT1-867 and AA-MAT2-1691/AsM1-8 (Table 1) designed to the *MAT1-1* (GenBank AB009451) and *MAT1-2* (GenBank AB009452) idiomorphs, respectively, of the Japanese pear pathotype of *A. alternata* (6). Sampled isolates were assigned to one of two previously defined phylogenetic lineages, SH1 and SH4 (59), using the sequence characterized amplified region (SCAR) marker OPB-4-1 (2) (Table 1). The OPB-4-1 primer set was designed to a cloned RAPD-PCR amplicon previously described by Andrew et al. (3) and resulted in amplification of a 648-bp PCR product from isolates of the SH1 lineage and no amplicon from isolates of the SH4 lineage. PCR reaction mixtures (20 µl) contained 20 ng of genomic DNA, 1× PCR buffer (New England Biolabs [NEB], Ipswich, MA], 4 nmol each dNTP (NEB), 50 pmol primer, and 1 U of Taq polymerase (NEB). Cycling conditions consisted of denaturation at 94°C for 4 min; 44 cycles of 94°C for 1 min, 58 or 55°C for 30 s, and 72°C for 2 min; and final extension at 72°C for 7 min, optimized slightly for each primer set. Amplification products were visualized in 2% ethidium bromide-stained agarose gels. Among 105 isolates originally sampled, a subsample of 50 representative isolates was selected for further characterization

TABLE 1. Primers used to amplify genomic regions for phylogenetic and population genetic analyses of lineages of the citrus brown spot pathogen, *Alternaria alternata*

Locus	Location	Primers	Primer sequence	Reference
MAT1-1-1	Mating type gene	ALMAT-L/AA-MAT1-867	GCAAGATTCTAGGCCCAACG/ TGCGGTGGGGAGTAGTGT	This study
MAT1-2-1	Mating type gene	AA-MAT2-1691/AsM1-8	CAGCACCCCGACTACAAGTAT/ GGTCGTGAGTCGTGATCG	This study
OPB-4-1	Non-protein coding region	OPB4-1F/OPB4-1R	GGACTGGAGTTGAAAAGTTCAGA/ GGACTGGAGTGGAGACTTCCT	2
Endo-polygalacturonase	Protein coding-region	PG3/PG2b	TACCATGGTTCTTTCCGA/ GAGAATTCRCARTCRTCYTGRTT	58
AA-Flank-F3	Microsatellite flanking region	Flank3F/Flank3R	AGCCAAAACACGTTGATACC/ ATCCGCAGCGAAAAGAACT	This study
AA-Flank-F13	Microsatellite flanking region	Flank13F/Flank13R	CGGGTTCGCTATGTAAAAAG/ ACTGGAATTGCGACCAAAAC	This study
OPA-5a	Non-protein coding region	SCAR5aF/SCAR5aR	GGTGACCGTATCGGTACTAGTGATT/ TGACCGTGACTCAGGTGAAC	2
OPA-9a	Non-protein coding region	SCAR9aF/SCAR9aR	CAAACGTCGGCAACTGTAGTG/ CAAACGTCGGTGTCATAAACA	2
OPA-11a	Non-protein coding region	SCAR11aF/SCAR11aR	CAAACGTCGGCTGGACAGT/ CAAACGTCGGGCACACAA	2

and will be referred to hereafter as the "population". Based on mating type and lineage characterization with the SCAR marker, these 50 isolates represented the entire spectrum of genetic diversity present in the larger original sample. Sequence data can be assessed in GenBank (accessions JN122026 to JN122275) and TreeBASE (accession number 11709).

Sequencing. Six fast-evolving loci, including a gene coding for an *endoPG*, three anonymous noncoding regions (OPA-5A, OPA-9A, and OPA-11A) (3), and two noncoding microsatellite flanking regions (Flank-F3 and Flank-F13), were selected for sequencing the 50 isolates. Microsatellite flanking regions were identified and primers designed based on GenBank accessions DQ272483 to DQ272487 containing microsatellite motifs (82). Primers were designed to amplify the microsatellite flanking regions using Primer 3 (67) and the primers used to amplify these genomic regions are listed in Table 2. For PCR, 20-µl PCR reaction mixtures contained 20 ng of genomic DNA, 1× PCR buffer (NEB), 4 nmol each dNTP (NEB), 50 pmol primer, and 1 U of Taq polymerase (NEB). Cycling conditions consisted of denaturation at 94°C for 4 min; 44 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 2 min; and final extension at 72°C for 7 min, depending on the optimal conditions for each primer set. PCR products were sequenced directly on both strands following treatment with EXOSAP-IT (USB, Cleveland) using the Big Dye terminator kit (Applied Biosystems, Foster City, CA). Sequence reads were performed on a PE Biosystems model 3700 automated DNA Sequencer by the Laboratory for Biotechnology and Bioinformatics at Washington State University, Pullman.

Physical linkage of sequenced loci. Because no genetic map is available for *A. alternata*, we attempted to maximize the probability that the sequenced loci represented a physically unlinked set of genetic loci by BLASTing each genomic region to contigs of the newly sequenced genomes of *A. tenuissima* and *A. alternata* (C. Lawrence, *unpublished data*). These contigs (<250,000 bp) were then BLASTed to the larger scaffolds of *A. brassicicola* genome (http://www.jgi.doe.gov/) to infer linkage relationships by synteny among the six genomic regions and to select a set of genetic loci with high probability of mapping to different chromosomes (Table 3).

Bayesian population structure inference and phylogenetic analyses. Sequence data were aligned, edited, and concatenated manually and using ClustalW (80) as implemented in BioEdit v7.0.53 (24). Population structure was inferred for the concatenated data set using BAPS v5.3 (78). Cluster analyses at the individual level were completed using the clustering with linked loci function, allowing for linked and unlinked loci within a population. Gene flow among genetically distinct subpopulations was also estimated using an admixture analysis as implemented in BAPS (15). Haplotype networks were estimated using concatenated data sets independently for each genetic subpopulation defined by BAPS using statistical parsimony analysis as implemented in TCS using a 92% parsimony criterion (14).

Associations of alleles among loci. Genetic diversity and associations of alleles among loci were estimated within the population and the three subpopulations as defined by BAPS above. DnaSP v.5 (66) was used to estimate the number of unique multilocus sequence types and also number of segregating sites (S) for each locus and the combined data set for each subpopulation (Table 2). Linkage disequilibrium was tested using the index of association (I_A). I_A is based on the observation that a sexually recombining population will have pairwise genetic distances between loci that are normally distributed but, in a clonal population, the variance of genetic distances is skewed (1,39, 40,79). I_A was estimated using concatenated data sets for the population and within subpopulations, including SH1, SH4, SH4A, and SH4B, using the standardized I_A (I^s_A and $\bar{r}_d$) (11,40) for allelic profiles as implemented in LIAN 3.0 (25) (http://pubmlst.org) and as single-nucleotide polymorphisms (SNPs) using Multilocus version 1.3b (1). The null hypothesis is that $V_{obs} = V_{exp} = 0$. I_A is dependent on number of loci analyzed, and $\bar{r}_d$ was introduced by Apagow and Burt (1) to remove this dependency. SNPs from each locus were treated as a single linkage group, and comparisons were made between linkage groups. Tests of significance were performed by comparing the original observed data set to 1,000 simulated randomly recombining data sets.

Tests for recombination. Putative intragenic and intergenic recombination events in the population were identified using the concatenated data set with the Recombination Detection Program (RDP) v3.44 (33,37) and Bayesian recombination analyses tracker (BRAT) (39). RDP implements several recombination detection methods and allows for comparable searches to be performed on sequence data. Recombination events were identified using five recombination algorithms: RDP (37), maximum χ^2 test (MaxChi) (38), maximum mismatch χ^2 (Chimaera) (64), gene conversion (GENECONV) (69), and Seq3 methods (10). The analysis options for RDP were adjusted to the following general settings: sequences were considered linear, the *P* value cutoff was set to 0.05, the Bonferroni correction was applied, consensus daughter sequences were found, breakpoints were polished, and only recombination events detected with two or more methods were listed.

To further validate our inferences of recombination, phylogenetic analyses were conducted to test for topological incongruence between the concatenated data set and one of the putative intergenic recombination segments. Maximum parsimony and Bayesian phylogenies were estimated with two different data sets.

TABLE 3. Location of sequenced regions in *Alternaria alternata, A. tenuissima*, and *A. brassicicola* genomes

Locus	Contig (size, bp)		
	A. alternata	*A. tenuissima*	*A. brassicicola*
endoPG[a]	00436 (774,102)	00686 (253,069)	3 (1,771,757)
AA-Flank-F3	00333 (128,142)	00079 (666,611)	6 (1,122,016)
AA-Flank-F13	00405 (395,009)	00059 (336,922)	2 (2,633,681)
AA-SCAR-5A	00052 (556,629)	00764 (557,574)	1 (3,441,849)
AA-SCAR-9A	00103 (203,451)	00647 (244,188)	2 (2,633,681)
AA-SCAR-11A	00148 (11,514)	00087 (205,636)	9 (591,262)

[a] Endo-polygalacturonase gene.

TABLE 2. Sequence variability of multilocus sequence type loci for the population of 50 isolates (P) and SH1 (S1) and SH4 (S4) subpopulations of *Alternaria alternata*

	Subpopulation[b]																				
	Combined			*EndoPG*			OPA-5A			OPA-9A			OPA-11A			Flank-F3			Flank-F13		
Statistic[a]	P	S1	S4	P	S1	S4	P	S1	S4	P	S1	S4	P	S1	S4	P	S1	S4	P	S1	S4
Sequence	2790			435			684			676			586			146			263		
Var. sites	135	55	83	11	1	10	50	29	27	39	12	29	21	9	12	5	3	2	4	1	3
Haplo	42	13	29	9	2	7	14	5	9	20	6	14	14	5	9	5	3	3	5	2	3

[a] Sequence = sequence length; Var. sites = number of variation nucleotide sites, with gaps and missing data removed; and Haplo = number of haplotypes.
[b] Sample sizes for each population or subpopulation are P (50 isolates), SH1 (14 isolates), and SH4 (36 isolates). *endoPG* = endo-polygalacturonase gene.

The first was of the concatenated data set without the putative intragentic recombination segment and the second data set consisted of only the recombination segment. Comparison of phylogenies estimated from these two data sets allowed an assessment of incongruence between the data sets and the effect of this recombination even on phylogenetic reconstruction. DT-ModSel (46) was used to estimate the best-fit nucleotide substitution models for each data set. Analyses were performed in PAUP*4b 10 (77) and MrBayes 3.0 (32). Maximum parsimony analyses were performed using the heuristic search option with 1,000 random addition sequences using the TBR branch swapping with the MULPARS option on, and all characters were weighted equally. Robustness and support for clades were assessed using 1,000 bootstraps with random addition (10 replicates) for each heuristic search. The Kishino-Hasegawa (KH) (36) and Shimodaira-Hasegawa (SH) (72) tests of topological congruence were conducted on the maximum likelihood phylogenies as implemented in PAUP with RELL 1000 resampling replicates. Bayesian analyses were performed with settings suggested by the selected models. For Bayesian analyses, the Markov chain Monte Carlo search was run with four chains for 3,000,000 generations, generating 30,001 trees, and the first 6,000 trees were discarded as "burnin" of the chains.

RESULTS

Sequencing population. Of 105 isolates originally sampled, 50 were selected for sequencing and detailed genetic analyses (Supplemental Table 1). Isolates that belonged to SH1 represented 13% (14 of 105) of isolates. Of the 92 remaining SH4 isolates, 15% (14 of 92) carried *MAT1-2*, whereas 85% (78 of 92) carried *MAT1-1*. Because of the highly skewed ratio of SH1 to SH4 isolates, all isolates from SH1 were included and all isolates from SH4 carrying *MAT1-1* were included. The remaining SH4 isolates (≈36) carrying *MAT1-2* were selected at random. Therefore, the studied population consisted of 50 isolates, including 14 isolates from SH1 lineage (13 *MAT1-1* and 1 *MAT1-2* isolates) and 36 from the SH4 lineage (4 *MAT1-1* and 32 *MAT1-2* isolates) (Table 4).

Nucleotide variation. In total, 2,790 bp was sequenced from six loci (*endoPG*, OPA-5A, OPA-9A, OPA-11A, Flank-F3, and Flank-F13) from 50 isolates. The concatenated data set exhibited 135 (4.8%) polymorphic nucleotide sites within the population. The three SCAR loci were the most variable, including OPA-5A, which had 50 of 684 (7.3%) polymorphic sites (Table 2); and OPA-9A and OPA-11Am which had 39 of 676 (5.7%) and 21 of 586 (3.6%) polymorphic sites, respectively. *endoPG* and the two microsatellite flanking regions (Flank-F3 and Flank-F13) were the least variable, with 11 of 435 (2.5%), 5 of 146 (3.4%), and 4 of 263 (1.5%) polymorphic sites, respectively (Table 2).

Marker linkage. The six loci were mapped to the *A. alternata*, *A. tenuissima*, and *A. brassicicola* genome assemblies. All loci were located to unique contigs of *A. alternata* and *A. tenuissima*. Four of six loci were located to unique scaffolds of *A. brassicicola* (Table 3). Flank-F13 and OPA-9A were both located to *A. brassicicola* scaffold 2 (2,633,681 bp), and further investigation revealed that these loci were separated by 51,108 bp when aligned to scaffold 2 of the *A. brassicicola* genome.

Population assignment, gene flow estimates, and sequence typing. BAPS and network analyses similarly identified three genetic subpopulations within the population with high posterior probabilities. These subpopulations will subsequently be referred to as SH1, SH4A, and SH4B (Fig. 1A and B). Subpopulation SH1 included 14 isolates and corresponded to phylogenetic lineage SH1 identified previously by Peever et al. (59). Subpopulations SH4A and SH4B were represented by 19 and 17 isolates, respectively and corresponded to the phylogenetic lineage SH4 identified previously by Peever et al. (59). As highlighted by the Bayesian population admixture analyses, low levels of gene flow were detected among subpopulations. Sequenced loci of isolate 52 were partitioned into all three subpopulations, with 90% of the total sequenced region partitioned to SH1, 9% to SH4A, and 1% to the SH4B subpopulations (Fig. 1A).

Network analysis. Subpopulations SH1 and SH4B comprised isolates of both mating types. The network revealed genetic differentiation between isolates of different mating type (Fig. 1B). For the SH1 subpopulation, the putative recombinant isolate 52 (*MAT1-2*) was separated from the other *MAT1-1* isolates by 20 mutational steps. Isolates 133, 161, 162, and 163 from the SH4B subpopulation with *MAT1-1* were separated by *MAT1-2* isolates by 13 steps.

All isolates in the SH4A subpopulation were *MAT1-2* and separated from SH4B subpopulation by 32 mutational steps. Five loops were observed in the SH4A network (Fig. 1C), and these reticulation patterns, suggestive of putative recombination, were localized to six nucleotide sites at three loci, including OPA-5A (one site), OPA-9A (two sites), and OPA-11A (two sites).

Multilocus gametic disequilibrium. The null hypothesis of random mating was rejected ($P < 0.001$) using the I_A tests I^s_A and $\bar{r}_d$ for the population and within the SH1 and SH4 subpopulations (Table 4). When the SH4 subpopulation was further subdivided into SH4A and SH4B, the null hypothesis of random mating was not rejected for the SH4A subpopulation but was rejected for SH4B.

Recombination analyses. Options for RDP were set so that only recombination events detected by two or more methods were considered significant. From these analyses, four putative recombination events were detected. The best-supported recombination event was an intragenic recombination identified within the OPA-5A locus in isolate 52 from the SH1 subpopulation. This event was detected with four statistical methods: RDP, MaxChi, Chimeara, and BRAT (Table 5). In addition, two well-supported intergenic recombination events were detected between the *endoPG* locus and the OPA-5A locus for four isolates from the SH4B subpopulation (Table 5). In all cases, only one putative parent could be identified, suggesting that the unknown parent was likely external to the sampled population.

To further explore the *endoPG*/OPA-5A recombination event, phylogenetic analyses were conducted on the concatenated data

TABLE 4. Multilocus tests of associations among loci in the population and subpopulations (SH1, SH4, SH4A, and SH4B) of *Alternaria alternata*[a]

Population	Isolates	Haplotypes	Number of sites[b]	MT ratio[c]	I^s_A (P)[d]	$\bar{r}_d$ (P)[e]
Population	50	42	135	1:1	16.58 (0.001)	0.121 (0.001) 0.128 (0.001)
SH1	14	13	55	13:1	10.53 (0.001)	0.139 (0.001) 0.154 (0.001)
SH4	36	29	83	4:32	8.56 (0.001)	0.098 (0.001) 0.093 (0.001)
SH4A	19	18	35	0:19	**0.134 (0.28)**	**0.004 (0.28) 0.008 (0.187)**
SH4B	17	12	12	4:17	8.306 (0.001)	0.144 (0.001) 0.172 (0.001)

[a] Bold text indicates failure to reject the null hypothesis of random mating.
[b] Number of segregating sites for each population.
[c] Mating type ratio for each population (*MAT1-1*: *MAT1-2*).
[d] The *P* value comparing the estimated index of association (I_A) value for each subpopulation against a null hypothesis of random mating. I^s_A = standardized I_A (1).
[e] Clone-corrected; $\bar{r}_d = I_A$, less dependent on the number of loci (1).

set and compared with the phylogeny produced using only a recombination segment of the partial *endoPG*/OPA-5A region that was detected in isolates 161, 162, 163, and 133 from the SH4B subpopulation. The best model of evolution for the concatenated data set with the recombination segment removed (base pairs 1 to 384 and 931 to 2,790) was TrNef+I and HKY+I for the data set containing only the putative recombination segment (base pairs 385 to 930). Both phylogenies showed evidence for two major clades, SH1 and SH4, identical to the subpopulations. However, several isolates exchanged positions, highlighting topology incongruence between the two phylogenies, as supported by the KH and SH topological congruence tests ($P > 0.001$). In the non-recombinant phylogeny (Fig. 2A), isolate 52 grouped in subpopulation SH1 and isolates 133, 161, 162, and 163 were in population SH4. However, in the recombinant phylogeny (base pairs 385 to 930) (Fig. 2B), isolate 52 clustered within subpopulation SH4 and isolates 133, 161, 162, and 163 grouped in subpopulation SH1.

DISCUSSION

A population of *A. alternata* sensu lato sampled from a small, defined area within a single citrus grove in central Florida could be subdivided into three genetically distinct subpopulations with little evidence of gene flow among them. The mode of reproduction in two of these subpopulations was predominantly asexual based on several lines of evidence. I_A tests identified significant associations between polymorphic sites using methods that accounted for both linkage of the polymorphic sites within a locus and small sample size and were supported by strong correlations between multilocus haplotype and mating type. Isolates of opposite mating type were genetically differentiated in two subpopulations (SH1 and SH4), with several fixed alleles at six additional loci indicating a lack of gene flow between mating types. Although asexuality appeared to be the primary reproductive mode in these subpopulations, signatures of genetic recombination were detected in both. In contrast, individuals in the third subpopulation, SH4A, appeared to be freely recombining, and we were unable to reject a random mating model despite the fact that all members of this subpopulation were of a single mating type. Recombination among isolates of the same mating type suggests that a nonmeiotic mechanism of recombination such as the parasexual cycle may be operating in this subpopulation.

Several studies have reported the signatures of recombination in populations of putatively asexual fungi (17,20,51,74,79), suggesting that (i) these fungi are not really asexual (i.e., have a "cryptic" sexual stage that remains to be discovered) or (ii) that nonmeiotic mechanisms facilitate recombination of genomes that really do not produce a sexual stage. Population studies of species within the genera of *Penicillium* and *Aspergillus* inferred recombination (19,27,28) even though these groups of fungi have been assumed asexual for >100 years. Just recently, the teleomorphic stages of *Aspergillus fumigatus* (54), *A. flavus* (29), *A. parasiticus* (31), *A. nomius* (30), and *Penicillium pinophiulum* (51) were induced in laboratory setting. These fungi have cryptic sexual cycles that are difficult to discern or induce in laboratory and field settings (19).

Similar to the recombination events observed within the *Alternaria alternata* subpopulations studied here, recombination has been inferred in other asexual phytopathogens, including *Fusarium oxysporum* (4,53), *Verticillium dahliae* (7), and *V. albo-altrum* (13). Mitotic recombination through a parasexual cycle may explain some or all of the recombination observed in these putatively asexual fungi. The parasexual cycle, first described by Pontecorvo et al. (63) in the laboratory using *Aspergillus nidulans*, allows fungi to recombine in the absence of a sexual cycle. This cycle offers an evolutionary alternative for the introgression of novel genetic material and complementation of dysfunctional gene copies in asexual species. After the formation of a heterokaryon from two genetically distinct haploid mycelia, recombination via crossovers and haploidization can generate genetically variable progeny (62). Even in fungi with a known teleomorph, the occurrence of the parasexual cycle has been documented extensively in the laboratory (49,70,82) and there is accumulating evidence that this cycle may occur in natural populations as well (42,45,83). Studies of the fungus *Cryphonectria parasitica* have detected recombination among individuals in different vegetative compatibility groups (42,43,45). McGuire et al. (42) inferred the presence of parasexual recombination in a predominantly clonal field population of *C. parasitica* from Wisconsin, and further evidence

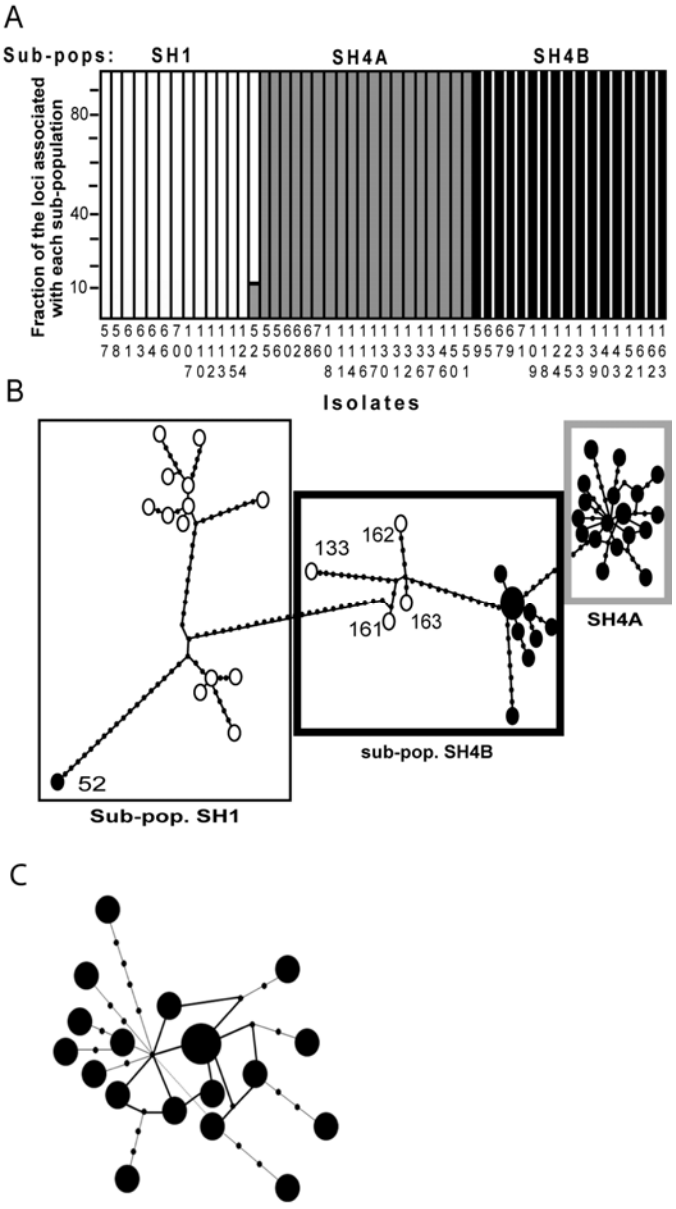

Fig. 1. A, Bayesian population clustering of 50 brown spot isolates, representing three distinct subpopulations: SH1 (white), SH4A (gray), and SH4B (black). Isolate 52 was assigned to all three clusters with 90, 9, and 1% assignment to the SH1, SH4B, and SH4A subpopulations, respectively. **B,** Parsimony network (92% criterion) among 50 isolates of *Alternaria alternata* sampled from Florida using the combined data set. Open circles represent isolates of *MAT1-1* mating type, whereas solid black circles represent *MAT1-2* isolates. Boxes circumscribe the SH1 (white), SH4A (gray), and SH4B (black) subpopulations inferred by Bayesian population clustering and phylogenetic analyses. **C,** Parsimony network (98% criterion) estimated within the SH4A subpopulation of *A. alternata* and showing reticulation. Small dots indicate missing haplotypes. Circle size is proportional to the number of sampled haplotypes. All isolates in the network are *MAT1-2* mating type.

suggested that stable heterokaryons formed between vegetatively incompatible individuals. Recently, Milgroom et al. (45) found additional evidence of parasexual recombination in two European populations of *C. parasitica*, dominated by one mating type. Two isolates, one from each population, had haplotypes that were a composite of the two clones in each population. Our results show similarities to the *C. parasitica* populations studied by Milgroom et al. (45) and McGuire et al. (42,43). The recombination observed in the SH4A subpopulation, where all individuals were of one mating type, highlights the potential significance of the parasexual cycle or some other nonmeiotic recombination process in *Alternaria* populations.

The intragenic and intergenic recombination events identified in this study would not have been identified had not population structure first been inferred within the larger sample. This suggests that careful delimitation of genetic populations with appropriate markers is a necessary prerequisite to identify and localize fine-scale evolutionary events. Peever et al. (57) genetically

TABLE 5. Identification of recombination events within subpopulations of *Alternaria alternata*[a]

Subpopulation	Isolates	Loci	RDP[b]	MaxChi[b]	Chimaera[b]	GENE[b]	Seq3[b]	BRAT[c]
SH1	52	OPA-5a[d]	2.2×10^{-03}	1.6×10^{-03}	1.4×10^{-02}	No	5.4×10^{-05}	99
SH4B	161, 162, 163	*endoPG*/OPA-5a[e]	No	1.4×10^{-02}	1.4×10^{-02}	No	1.4×10^{-02}	99
SH4B	133	*endoPG*/OPA-5a[e]	No	1.4×10^{-02}	1.4×10^{-02}	No	2.7×10^{-02}	99

[a] Methods used to test for recombination were the Recombination Detection Program (RDP) (37), maximum χ^2 method (MaxChi) (39,63), CHIMAERA (63), GENECONV (Padidam et al. 1999 [55]), Seq3 (10), and Bayesian recombination analysis tracker (BRAT) (Tang et al. 2007 [78]).
[b] *P* value listed as significant finding.
[c] Posterior probability.
[d] Intragenic recombination event.
[e] Intergenic recombination event; *endoPG* = endo-polygalacturonase gene.

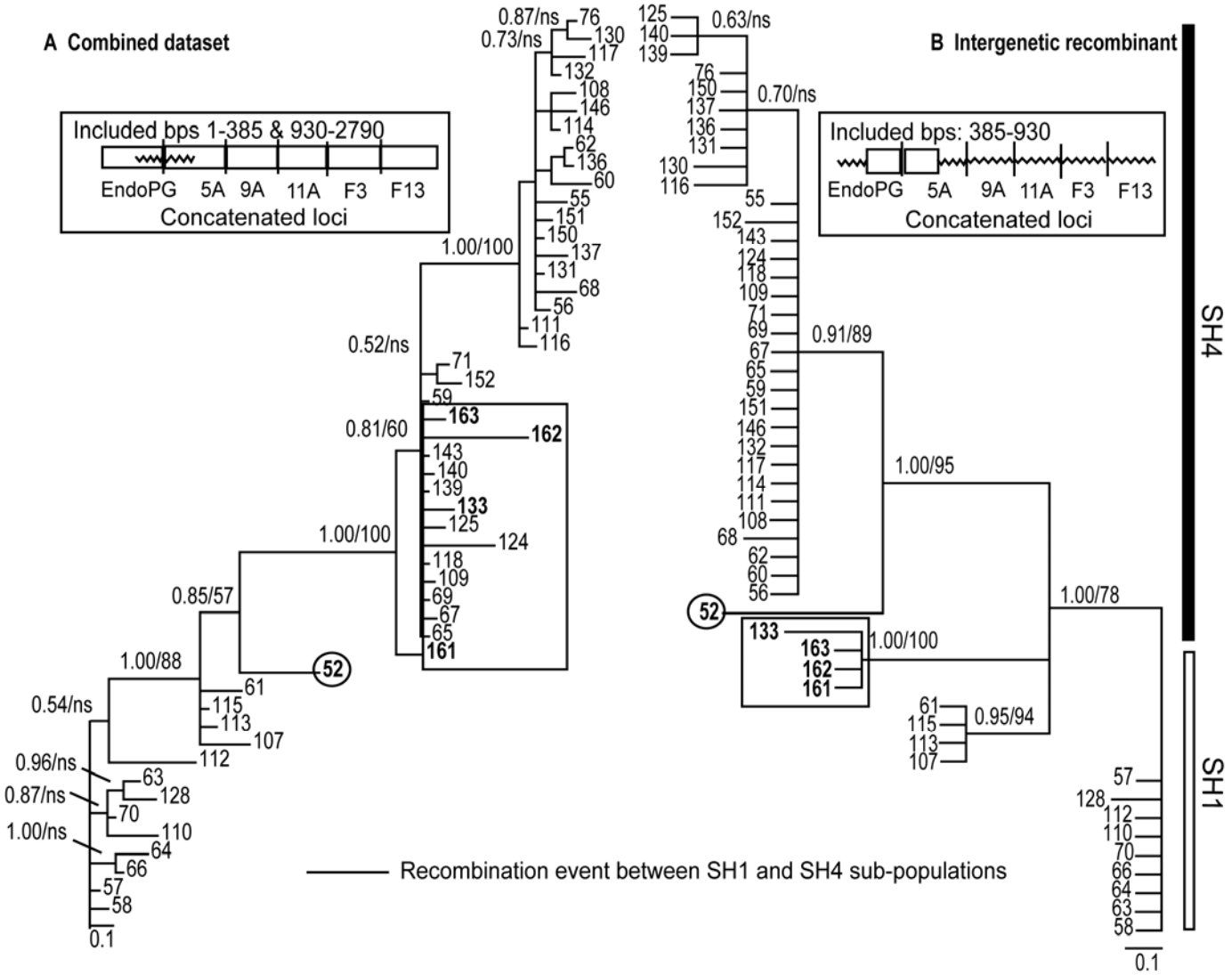

Fig. 2. Phylogenetic incongruence resulting from an intergenic recombination event (endo-polygalacturonase [*EndoPG*]/OPA-5a) detected in isolates 161, 162, and 163 of *Alternaria alternata* within the SH4B subpopulation. Node support was estimated using posterior probabilities and bootstrap values based on 1,000 bootstrapped replicates (pp/bs). Open boxes in the diagram above each phylogeny highlight the base pairs included in the data set. **A,** Phylogeny for the non-recombinant segment (base pairs 1 to 384 and 931 to 2,790). **B,** Phylogeny for the recombination segment (base pairs 385 to 930) only. Isolates in bold represent putative recombinants.

characterized a smaller sample of *Alternaria alternata* from this same Florida population using RAPD-PCR markers. Two genetic groups were identified that correspond to two morphologically and phylogenetically supported species, *A. citrimacularis* and *A. tangelonis* (57,74). With additional, sequence-based data and Bayesian inference of population structure, as implemented in BAPS, we inferred an additional subpopulation within SH4. Identification of this additional subpopulation likely resulted from the application of highly variable loci utilized in this study. Significant gametic disequilibrium was observed among loci for the overall population and for the SH1 and SH4 subpopulations. However, when the SH4 population was split into SH4A and SH4B, the random mating model for the SH4A subpopulation could not be rejected. Perhaps, when the two subpopulations were combined, there was population admixture with fixed alleles for members of each subpopulation. Similarly, Douhan et al. (18) detected recombination of *Cenococcum geophilum* at the subpopulation level but not at the population level, highlighting the importance of examining for recombination on multiple genetic levels within a population. Of the eight parsimoniously informative characters, reticulation was found among six phylogenetically incompatible sites in three different loci. Further analysis of these six characters highlighted possible intrapopulation recombination, visualized by loops in statistical parsimony network analyses. All isolates were *MAT1-2*, possibly suggestive of mitotic recombination events occurring through a parasexual cycle.

A putative reciprocal recombination event at the intragenetic region of the partial *endoPG* gene and locus SCAR-5A was found between subpopulations SH1 and SH4B and was supported by recombination, phylogenetic, and population genetic analyses. This event appears to have occurred through a meiotic recombination event between isolate 52 from subpopulation SH1 and several members of SH4B but we were unable to detect which sequences are recombinant and which are parental. Further, because asexual *Alternaria* spp. are thought to be derived from sexual lineages (8), it is difficult to discern whether these events occurred recently or are signatures from a sexual past.

To better characterize the mechanism, timing, and significance of recombination events in *A. alternata* populations, comparisons of entire genomes using hierarchical comparative genomics (71) could be useful. Differences between closely related taxa separated by a limited number of evolutionary events may allow resolution of various issues related to genomic evolution, such as determining the gain and loss of genes, detecting positive natural selection, and providing an evolutionary context for observed differences between taxa (71). Ruderfer et al. (68) used genomic analyses to infer the evolutionary history of the budding yeast, *Saccharomyces cerevisiae*. The availability of whole genome sequences of several isolates and one closely related outgroup, *S. paradoxus*, allowed Ruderfer et al. (68) to ascertain that outcrossing occurs infrequently but is sufficient to recombine the genome, such that ancestral segments remained linked at the level of only a few kilobases. When similar data become available for *A. alternata* and closely related species, these approaches could be utilized to distinguish between contemporary mitotic or meiotic events or signatures from a sexual past.

We observed high levels of genetic divergence between individuals from different subpopulations of *A. alternata* collected from a small area (2,500 m^2) within a single citrus grove. Evidence provided by the network analyses suggest that these subpopulations (SH1, SH4A, and SH4B) are differentiated by as many as 20 SNPs at six loci, indicating little gene flow and recombination among these subpopulations. In asexual taxa, it is hypothesized that loss of recombination associated with asexual reproduction dramatically reduces diversification (34), impeding speciation among asexual taxa, increasing the number of deleterious mutations, and, therefore, increasing extinction rates among asexual taxa (48,56). Conversely, we found evidence of significant genetic diversification among *A. alternata* subpopulations. Significant differentiation has also been observed among asexual lineages of the evening primrose, *Oenothera humifusa*, when compared with a sexual variety of this species (34). There was little evidence for a reduced rate of diversification and speciation in the asexual species and no clear differences in extinction rates were evident between the asexual and sexual *Oenothera* spp. (34). Perhaps the limited ability of *A. alternata* individuals to recombine within each subpopulation through nonmeiotic mechanisms, such as the parasexual cycle, as observed in the SH4A subpopulation, has allowed *A. alternata* to form new genetic populations and, eventually, species.

Speciation is a process driven by mechanisms that enhance genetic divergence and reduce gene flow between individuals within populations (16). Such mechanisms include behavioral, ecological, or habitat isolation, and ecological divergence was found to be the driving force of speciation in asexual lineages of bdelloid rotifers (21). It seems likely that similar mechanisms are operating in populations of asexual fungi, which may lead to speciation. Subpopulations SH1 and SH4 correspond to two morphological and phylogenetic species, *A. citrimacularis* and *A. tangelonis* (57,74), but these taxa cannot be separated by any obvious biological criteria such as host specificity. In this study, we observed further diversification within the SH4 lineage but have yet to associate this divergence with a specific phenotype. Three subpopulations co-exist within a small geographic area on the same host in the same environment, suggesting that these subpopulations may represent incipient species. However, the mechanisms promoting and maintaining divergence within this population remain elusive.

Gaining insight into the mode of recombination for a pathogen is important for understanding speciation, the processes of adaptation to new hosts or environments, the evolution of virulence, and fungicide resistance. Further studies will attempt to identify the mechanism, timing, and biological significance of recombination and divergence in *A. alternata* subpopulations.

ACKNOWLEDGMENTS

Funding was provided by National Science Foundation award DEB-0918668. PPNS Number 0568, Department of Plant Pathology, College of Agriculture, Human, and Natural Resource Sciences, Agricultural Research Center, Project Number WNP03061-0300, Washington State University, Pullman 99164-6430. We thank L. Carris, M.-S. Kim, and N. Klopfenstein for critically reading previous versions of this manuscript.

LITERATURE CITED

1. Agapow, P.-M., and Burt. A. 2001. Indices of multilocus linkage disequilibrium. Mol. Ecol. Notes 1:101-102.
2. Andrew, M. 2006. Molecular systematics of small-spored *Alternaria* species. MS thesis, Washington State University, Pullman.
3. Andrew, M., Peever, T. L., and Pryor, B. M. 2009. An expanded multilocus phylogeny does not resolve species within the small-spored *Alternaria* species complex. Mycologia 101:95-109.
4. Appel, D. J., and Gordon, T. R. 1994. Local and regional variation in population of *Fusarium oxysporum* from agricultural field soils. Phytopathology 84:786-791.
5. Aradhya, M. K., Chan, H. M., and Parfitt, D. E. 2001. Genetic variability in the pistachio late blight fungus, *Alternaria alternata*. Mycol. Res. 105:300-306.
6. Arie, T., Kaneko, I., Yoshida, T., Nouchi, M., Nomura, Y., and Yamaguchi, I. 2000 Mating-type genes from asexual phytopathogenic ascomycetes *Fusarium oxysporum* and *Alternaria alternata*. Mol. Plant-Microbe Interact. 13:1330-1339.
7. Atallah, Z. K, Maruthachalam, K., du Toit, L., Koike, S. T., Davis, R. M., Klosterman, S. J., Hayes, R. J., Krishna, V., and Subbarao, K. V. 2010. Population analyses of the vascular plant pathogen *Verticillium dahliae* detectrecombination and transcontinental gene flow. Fungal Genet. Biol. 47:416-422.
8. Berbee M. L., Payne B. P., Zhang G., Roberts R. G., and Turgeon B. G. 2003. Shared ITS DNA substitutions in isolates of opposite mating type

reveal a recombining history for three presumed asexual species in the filamentous ascomycete genus Alternaria. Mycol. Res. 107:169-182.
9. Bock, C. H., Thrall, P. H., and Burdon, J. J. 2005. Genetic structure of populations of *Alternaria brassicicola* suggests sexual recombination. Mycol. Res. 109:227-236.
10. Boni M. F., Posada, D., and Feldman, M. W. 2007. An exact non-parametric method for inferring mosaic structure in sequence triplets. Genetics 176:1035-1047.
11. Brown, A. H. D, Feldman, M. W., and Nevo, E. 1980. Multilocus structure of natural populations of *Hordeum spontaneum*. Genetics 96:523-536.
12. Chelkowski, J. A., and Visconti, A., eds. 1992. *Alternaria*: Biology, Plant Diseases and Metabolites. Elsevier, Amsterdam.
13. Clarkson, J. M., and Heale, J. B.1985. Pathogenicity and colonization studies on wild-type and auxotrophic isolates of *Verticillium albo-atrum* from hop. Plant Pathol. 34:119-128.
14. Clement, M., Posada, D., and Crandall, K. A. 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9:1657-1660.
15. Corander, J., and Marttinen, P. 2006. Bayesian identification of admixture events using multi-locus molecular markers. Mol. Ecol. 15:2833-2843.
16. Coyne, J. A., and Orr, H. A. 2004. Speciation. Sinauer Associates, Sunderland, MA.
17. Dodgson, A. R., Pujol, C., and Pfaller, M. A. 2005. Evidence for recombination in *Candida glabrata*. Fungal Genet. Biol. 42:233-243.
18. Douhan, G. W., Martin, D. P, and Rizzo, D. M. 2007. Using the putatively asexual fungus *Cenococcum geophilum* as a model to test how species concepts influence recombination analyses using sequence data from multiple loci. Curr. Genet. 52:191-201.
19. Dyer, P. S., and O'Gorman, C. M. 2011. Sexual development and cryptic sexuality in fungi: Insights from *Aspergillus* species. FEMS Microbiol. Rev. Online publication. doi:10.1111/j.1574-6976. 2011.00308
20. Ehrlich, K. C., Montalbano, B. G., and Cotty, P. J. 2007. Analysis of single nucleotide polymorphisms in three gene shows evidence for genetic isolation of certain *Aspergillus flavus* vegetative compatibility groups. FEMS Microbiol. Lett. 268:231-236.
21. Fontaneto, D., Herniou, E. A., Boschetti, C., Caprioli, M., Melone, G., Ricci, C., and Barraclough, T. G. 2007. Independently evolving species in asexual bdelloid rotifers. PLoS Biol. 5:e87. Online publication. doi:10.1371/journal.pbio.0050087
22. Gannibal, P. B., Klemsdal, S. S, and Levitin, M. M. 2007. AFLP analysis of Russian *Alternaria tenuissima* population from wheat kernels and other host. Eur. J. Plant Pathol. 119:175-182.
23. Guo, L.-D., Li, X., Zheng, W.-H., and Hyde, K. D. 2004. Genetic variation of *Alternaria alternata*, an endophytic fungus isolated from *Pinus tabulaeformis* as determined by random amplified microsatellites (RAMS). Fungal Divers. 16:53-65.
24. Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis. Nucleic Acids Symp. Ser. 41:95-98.
25. Haubold, B., and Hudson, R. R. 2000. LIAN 3.0: Detecting linkage disequilibrium in multilocus data. Bioinformatics 16:847-848.
26. Hawksworth, D., Kirk, P., Sutton, B., and Pegler, D. 1996. Ainsworth's and Bisby's D of the Fungi, 8th ed. CAB International, Wallingford, UK.
27. Henk, D. A., Eagle, C. E., Brown, K., van den Berg, M. A., Dyer, P. S., Petertson, S. W., and Fisher, M. C. 2011. Speciation despite globally overlapping distributions in *Penicillium chrysogenum*: The population genetics of Alexander Fleming's lucky fungus. Mol. Ecol. Online publication. doi:10.1111/j.1365-294X.2011.05244.x
28. Henk, D. A., and Fisher, M. C. 2011. Genetic diversity, recombination, and divergence in animal associated *Penicillium dipodomyis*. PLoS ONE 6:e22883. 27.
29. Horn, B. W., Moore, G. G., and Carbone, I. 2009. Sexual reproduction in *Aspergillus flavus*. Mycologia 101:423-429.
30. Horn, B. W., Moore, G. G., and Carbone, I. 2011. Sexual reproduction in aflatoxin-producing *Aspergillus nomius*. Mycologia 103:174-183.
31. Horn, B. W., Ramirez-Prado, J. H., and Carbone, I. 2009. The sexual state of *Aspergillus parasiticus*. Mycologia 101:275-280.
32. Huelsenbeck, J. P., and Ronquist, F. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17:754-755.
33. Huson, R. R., and Bryant, D. 2006. Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. 23:254-267.
34. Johnson, M. T. J., FitzJohn, R. G., Smith, S. D., Rausher, M. D., and Otto, S. P. 2011. Loss of sexual recombination and segregation is associated with increased diversification in evening primroses. Evolution 65:3230-3430.
35. Keller, S. M., Wolfe, M. S., McDermott, J. M., and McDonald, B. A. 1997. High genetic similarity among populations of *Phaesophaeria nodorum* across wheat cultivars and regions in Switzerland. Phytopathology 87:1134-1139.
36. Kishino, H., and Hasegawa, M. 1989. Evaluation of the maximum likelihood estimates of the evolutionary tree topologies from sequence data, and the branching order in Hominoidea. J. Mol. Evol. 29:170-179.
37. Martin, D., and Rybicki, E. 2000. RDP: detection of recombination amongst aligned sequences. Bioinformatics 16:562-563.
38. Marttinen, P., Baldwin, A. Hanage, W. P., Dowson, C., Mahenthiralingam, E., and Corander, J. 2008. Bayesian modeling of recombination events in bacterial populations. BMC Bioinf. 9:421.
39. Maynard Smith, J. 1992. Analyzing the mosaic structure of genes. Mol. Evol. 34:126-129.
40. Maynard Smith, J., Smith, N. K., O'Rourke, M., and Spratt, B. G. 1993. How clonal are bacteria? Proc. Natl. Acad. Sci. USA 90:4384-4388.
41. McDonald, B. A. 1997. The population genetics of fungi: Tools and techniques. Phytopathology 87:448-453.
42. McGuire, I. C., Double, J. E., MacDonald, W. L., Rauscher, J. T., McCawley, S., and Milgroom, M. G. 2005. Heterokaryon formation and parasexual recombination between vegetatively incompatible lineages in a population of the chestnut blight fungus, *Cryphonectria parasitica.* Mol. Ecol. 14:3657-3669.
43. McGuire, I. C., Marra, R. I., and Milgroom, M. G. 2004. Mating-type heterokaryosis and selfing in *Cryphonectria parasitica*. Fungal Genet. Biol. 41:521-533.
44. Milgroom, M. G. 1996. Recombination and the multilocus structure of fungal populations. Annu. Rev. Phytopathology 34:457-477.
45. Milgroom, M. G., Sotirovski, K., Risteski, M., and Brewer Talbot, M. 2009. Heterokaryon and parasexual recombinants of *Cryphonectria parasitica* in two clonal populations in southeastern Europe. Fungal Genet. Biol. 45:849-854.
46. Minin, V., Abdo, Z., Joyce, P., and Sullivan, J. 2003. Performance-based selection of likelihood models for phylogeny estimation. Syst. Biol. 52:674-683.
47. Morris, P. F., Connolly, M. S., and St Clair, D. A. 2000. Genetic diversity of *Alternaria alternata* isolated from tomato in California assessed using RADPs. Mycol. Res. 104:286-292.
48. Neiman, M, Hehman, G., Miller, J. T., Logsdon, J. M., Jr., and Taylor, D. R. 2010. Accelerated mutation accumulation in asexual lineages of a freshwater snail. Mol. Biol. Evol. 27:954-963.
49. Noguchi, M. T., Yasuda, N., and Fujita, Y. 2006. Genetic exchange by parasexual recombination and genetic pathogenicity and mating type of parasexual recombinants in the rice blast fungus, *Magnaporthe oryzae*. Phytopathology 96:746-750.
50. Linde, C. C., Liles, J. A., and Thrall, P. H. 2010. Expansion of genetic diversity of randomly mating founder population of *Alternaria brassicicola* infecting Cakile maritime in Australia. Appl. Environ. Microbiol. 76:1946-1954.
51. Litvintseva, A. P., Marra, R. E., Nielsen, K., Heitman, J., Vilgalys, R., and Mitchell, T. G. 2003. Evidence of sexual recombination among *Cryptococcus neoformans* serotype A isolates in sub-Saharan Africa. Eukaryot. Cell 2:1162-1168.
52. Lopez-Villavicencio, M, Aguileta, G, Giraud, T, de Vienne, D. M., Lacoste, S., Couloux, A., and Dupont, J. 2010. Sex in *Penicillium*: Combined phylogenetic and experimental approaches. Fungal Genet. Biol. 47:693-706.
53. O'Donnell, K., Cigelnik, E., and Nirenberg, H. I. 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90:465-493.
54. O'Gorman, C. M., Hubert, T. F., and Dyer, P. S. 2009. Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. Nature 457:471-474.
55. Padidam, M., Sawyer, S., and Fauquet, C. M. 1999. Possible emergence of new geminiviruses by frequent recombination. Virology 265:218-225.
56. Paland, S., and Lynch, M. 2006. Transitions to asexuality result in excess amino acid substitutions. Science 311:990-992.
57. Peever, T. L, Canihos, Y., Olsen, L., Ibanez, A., Liu, Y.-C., and Timmer, L. W. 1999. Population genetic structure and host specificity of *Alternaria* spp. causing brown spot on Minneola tangelo and rough lemon in Florida. Phytopathology 92:794-802.
58. Peever, T. L., Carpenter-Boggs, L., Carris, L. M., Timmer, L. W., and Bhatia, A. 2005. Citrus black rot is caused by phylogenetically distinct lineages of *Alternaria alternata*. Phytopathology 95:512-518.
59. Peever, T. L., Ibanez, A., Akimitsu, K., and Timmer, L. W. 2002. Worldwide phylogeography of the citrus brown spot pathogen, *Alternaria alternata*. Phytopathology 92:794-802.
60. Peever, T. L., Olsen, L., Ibanez, A., and Timmer, L. W. 2000. Genetic differentiation and host specificity among populations of *Alternaria* spp. causing brown spot of grapefruit and tangerine × grape fruit hybrids in Florida. Phytopathology 90:407-414.
61. Peever, T. L., Su, G., Carpenter-Boggs, L., and Timmer, L. W. 2004. Molecular systematics of citrus-associated *Alternaria* species. Mycologia 96:119-134.
62. Pontecorvo, G. J. 1956. Trends in Genetic Analysis. Columbia University Press, New York.

63. Pontecorvo, G. J., Roper, A., and Forbes, E. 1953. Genetic recombination without sexual reproduction in *Aspergillus niger.* J. Gen. Microbiol. 8:198-210.
64. Posada, D., and Crandall, K. A. 2001. Evaluation of methods for detecting recombination from DNA sequences: Computer simulations. Proc. Natl. Acad. Sci. USA 98:13757-13762.
65. Prasad, M. S. L., Sujatha, M., and Rao, S. C. 2009. Analysis of cultural and genetic diversity in *Alternaria helianthi* and determination of pathogenic variability using wild *Helianthus* species. J. Phytopathol. 157:609-617.
66. Rozas, J. J., Sanchez-DelBarrio, J. C., Messeguer, X., and Rozas, R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19:2496-2497.
67. Rozen, S., and Skaletsky, H. 2000. Pages 365-386 in: Primer3 on the WWW for General Users and for Biologist Programmers in Bioinformatics Methods and Protocols. S. Krawetz and S. Misener, eds. Humana Press, Totowa, NJ.
68. Ruderfer, D. M., Pratt, S. C., Seidel, H. S., and Kruglyak, L. 2006. Population genomic analysis of outcrossing and recombination in yeast. Nat. Genet. 38:1077-1081.
69. Sawyer, S. 1989. Statistical tests for detecting gene conversion. Mol. Biol. Evol. 6:526-538.
70. Schoustra, S. E., Debets, A. J. M., Slakhorst, M., and Hoekstra, R. F. 2007. Mitotic recombination accelerates adaptation in the fungus *Aspergillus nidulans*. PLoS Genet. 3:e68.
71. Sharpton, T. J., Stajich. J. E., Steven D. Rounsley, S. D., Gardner, M. J., Wortman, J. R., Jordar, V. S., , R., Kodira, C. D., Neafsey, D. E., Zeng, Q., Hung, C.-Y., McMahan, C., Muszewska, A., Grynberg, M., Mandel, M. A., Kellner, E. M., Barker, B. M., Galgiani, J. N., Marc, J. Orbach, M. J., Kirkland, T. N., Cole, G. T., Henn, M. R., Birren, B. W., and Taylor, J. W. 2009. Comparative genomic analyses of the human fungal pathogens *Coccidioides* and their relatives. Genome Res. 19:1722-1731.
72. Shimodaira, H., and Hasegawa, M. 1999. Multiple comparisons of log likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16:1114-1116.
73. Simmons, E. G. 1986. *Alternaria* themes and variations (22–26). Mycotaxon 25:287-308.
74. Simmons, E. G. 1999. *Alternaria* themes and variations (226–235): Classification of citrus pathogens. Mycotaxon 70:263-323.
75. Stergiopoulos, I., Groenewald, M., Staats, M., Lindhout, P., Crous, P. W., and DeWit, P. J. G. M. 2007. Mating-type genes and the genetic structure of a world-wide collection of the tomato pathogen *Cladosporium fulvum*. Fungal Genet. Biol. 44:415-429.
76. Stewart, J. E., Kawabe, M., Abdo, Z., Arie, T., and Peever, T. L. 2011. Contrasting codon usage patterns and purifying selection at the mating type loci in putatively asexual fungal *Alternaria* species. PLoS One 6:e20083.
77. Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4. Sinauer Associates, Sunderland, MA.
78. Tang, J., Hanage, W. P., Fraser, C., and Corander, J. 2009. Identifying currents in the gene pool for bacterial populations using an integrative approach. PLoS Comput. Biol. 5:e1000455.
79. Taylor, J. W., Jacobson, D. J., and Fisher, M. C. 1999. The evolution of asexual fungi: Reproduction, speciation, and classification. Annu. Rev. Phytopathol. 37:197-246.
80. Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acid Res. 22:4673-4680.
81. Tran-Dinh, N., and Hocking, A. 2006. Isolation and characterization of polymorphic microsatellite markers for *Alternaria alternata*. Mol. Ecol. Resour. 6:405-407.
82. Weinreich, D. M., Watson, R. A., and Chao, L. 2005. Sign epistasis and genetic constraint on evolutionary trajectories. Int. J. Org. Evol. 59:1165-1174.
83. Zeigler, R. S., Scott, R. P., Leung, H., Bordeos, A. A., Kumar, J., and Nelson, R. J. 1997. Evidence of parasexual exchange of DNA in the rice blast fungus challenges its exclusive clonality. Phytopathology 87:284-294.