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Evolution

Evolution of the fungal self-fertile reproductive life style from self-sterile ancestors

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ABSTRACT **In most fungal ascomycetes, mating is controlled by a single locus (*MAT*). Fungi requiring a partner to mate are heterothallic (self-sterile); those not requiring a partner are homothallic (self-fertile). Structural analyses of *MAT* sequences from homothallic and heterothallic *Cochliobolus* species support the hypothesis that heterothallism is ancestral. Homothallic species carry both *MAT* genes in a single nucleus, usually closely linked or fused, in contrast to heterothallic species, which have alternate *MAT* genes in different nuclei. The structural organization of *MAT* from all heterothallic species examined is highly conserved; in contrast, the organization of *MAT* in each homothallic species is unique. The mechanism of conversion from heterothallism to homothallism is a recombination event between islands of identity in otherwise dissimilar *MAT* sequences. Expression of a fused *MAT* gene from a homothallic species confers self-fertility on a *MAT*-null strain of a heterothallic species, suggesting that *MAT* alone is sufficient to change reproductive life style.**

Which mode of fungal sexual reproduction, heterothallism or homothallism, is derived and what genetic mechanism(s) mediates the change from one to the other? Some authors (1–7) have hypothesized that homothallism arises from heterothallism and others (8–10) have suggested the reverse. To address this issue, we have compared *MAT* sequences from heterothallic and homothallic species within the ascomycete genus *Cochliobolus,* using a combination of molecular genetic and phylogenetic methods. Because *MAT* genes control the reproductive process (7), comparison of their sequences should reflect life history and may reveal mechanisms underlying changes in reproductive mode. Indeed, we have discovered fused *MAT* genes in homothallic species that provide a snapshot of the genetic link between heterothallism and homothallism.

Alternate sequences at *MAT* are not alleles in the classic sense because they lack significant sequence similarity and encode different transcriptional regulators (11–14). The term idiomorph is used to describe this unusual genetic organization (3), which is common among all *MAT* loci from heterothallic ascomycetes investigated to date (7). Because they are dissimilar sequences, idiomorphs do not normally recombine and are inherited uniparentally, as is the mammalian Y chromosome (15–17). In this report, we offer evidence that homothallism is derived from heterothallism and that the vehicle for this change is, in fact, a recombination event between short islands of identity within the idiomorphs.

MATERIALS AND METHODS

Strains, Media, Crosses, and Transformation. *Cochliobolus heterostrophus* heterothallic strains C4 (*MAT-2*), C5 (*MAT-1*), CB7 (*MAT-1*; *alb1*), and CB12 (*MAT-2*; *alb1*) have been described (18). Homothallic strains *Cochliobolus luttrellii* 14643–1 and *Cochliobolus cymbopogonis* 88109–1 were provided by J. Alcorn (Department of Primary Industries, Queensland, Australia), *Cochliobolus kusanoi* Ck2 by T. Tsuda (Kyoto University, Japan), and *Cochliobolus homomorphus* 13409 was obtained from the American Type Culture Collection. *C. heterostrophus MAT*-deletion strain ΔNcoMAT-2 (isolation C4–41.7; *MAT-0*;*hygB*^{R}; refs. 19 and 20) was the recipient for heterologous gene expression. Growth conditions, storage of fungal strains (21), and mating (18) and transformation (22) procedures have been described.

DNA Preparation and PCR Primers. Isolation of fungal DNA (19, 23) and PCR amplification conditions (24) were described previously. Primers used to isolate the homothallic *MAT* genes included TP2, TP3 (24), AD2 (25), and ho1–ho24 (Fig. 1). Primer sequences are available on request. Primers GPD1 and GPD2, designed by using *C. heterostrophus GPD1* (GenBank accession no. X63516) and *Cochliobolus lunatus GPD* (GenBank accession no. X52718) sequences, generated a fragment of ≈600 bp (440 bp of coding sequence plus two introns). Ribosomal internal transcribed spacer (ITS) regions were amplified with primers ITS4 and ITS5, by using conditions described (26).

Cloning *MAT* Loci from Homothallic Species. *MAT* gene sequences appear to evolve rapidly (27, 28), making them difficult to clone from new species by heterologous hybridization (e.g., only two of the four homothallic *Cochliobolus MAT* genes hybridized to *C. heterostrophus MAT* DNA). Thus, a PCR approach was adopted (Fig. 1) and, for each new gene, if necessary, primers were redesigned based on consensus of already acquired *MAT* sequences.

C. homomorphus. A portion of the *C. homomorphus MAT* gene, the High Mobility Group (HMG) box (14), cloned originally by using PCR amplification (24), was used to probe a *C. homomorphus* subgenomic library constructed with an ≈3.7-kb *Xba*I-digested fraction that hybridized to both *C. heterostrophus MAT* probes. Sequencing of a positive clone insert revealed an ORF with >70% nucleotide identity to the *C. heterostrophus MAT-1* and *MAT-2* ORFs.

C. luttrellii. Primers ho1 and ho2, corresponding to conserved *MAT-2* regions of heterothallic *C. heterostrophus* and homothallic *C. homomorphus,* were used with *C. luttrellii* DNA as template to amplify a 584-bp fragment with 92% nucleotide identity to *C. heterostrophus MAT-2*. Sequencing of a 1.1-kb *C. luttrellii* TAIL-PCR (25) product obtained with primer TP2, *MAT-2*-specific primer ho7, and nested *MAT-2* primer ho8 revealed the 3′ end of *MAT-2* and flanking region, which showed 84% identity with the *C. heterostrophus* 3′ flanks. To

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Abbreviations: ITS, internal transcribed spacers; TAIL, thermal asymmetric interlaced PCR.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF129740–AF129745).

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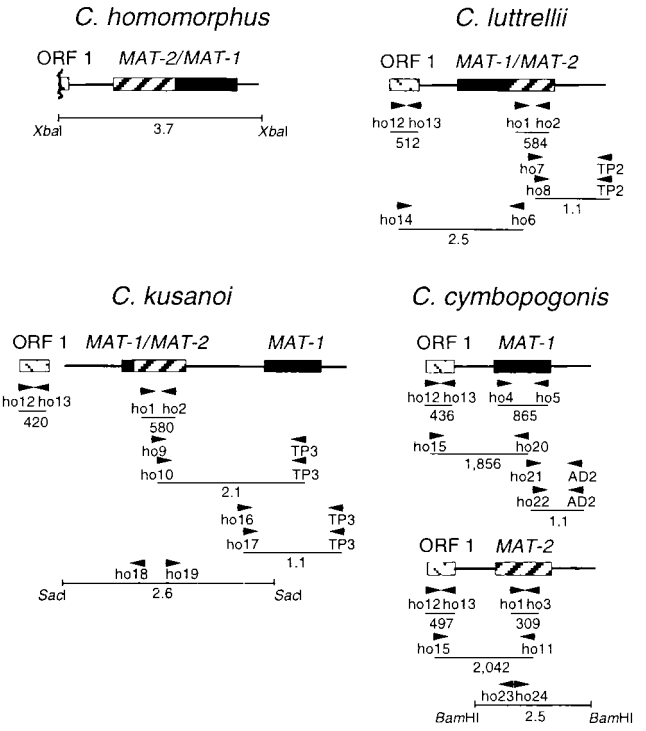

FIG. 1. Strategies to clone *MAT* genes from homothallic *Cochliobolus* species, as described in the text. Textures of boxes indicate *MAT-1* (black), *MAT-2* (hatches), *ORF1* (dotted diagonal lines); lines extending from boxes represent sequences flanking idiomorphs. Arrowheads identify locations and 5′ → 3′ direction of PCR primers. Numbers with decimals are in kb, those without are in bp.

clone the 5′ flank, degenerate primers ho12/ho13 corresponding to *ORF1* (a gene of unknown function found ≈1 kb 5′ of *MAT* in heterothallic *Cochliobolus* spp., homothallic *C. homomorphus*, and distantly related *Alternaria alternata* (Fig. 2) (20) were used with *C. luttrellii* DNA to amplify a 512-bp fragment, confirmed by sequencing to be the *C. luttrellii* homolog of *C. heterostrophus ORF1* (98.4% nucleotide identity). Primer ho14 specific to *C. luttrellii ORF1* was then used with *MAT-2*-specific primer ho6 to amplify a 2.5-kb fragment that, when sequenced, revealed part of *ORF1* and *MAT-1* fused to *MAT-2.*

C. kusanoi. Primers ho1/ho2 amplified a 580-bp fragment of *MAT-2* from *C. kusanoi.* TAIL-PCR primers ho9/TP3 followed by ho10/TP3 amplified a 2.1-kb region 3′ of the *MAT-2* fragment. Sequencing revealed that both *MAT* genes are arranged as shown in Fig. 2. Sequencing of a 1.1-kb TAIL-PCR product amplified with primers ho16/TP3 and ho17/TP3 revealed the 5′ end of *MAT-1* and 438 bp of 5′ flank. The 5′ region of the *MAT-2* PCR fragment, which could not be amplified by TAIL-PCR, was obtained by using inverse PCR (29, 30). Genomic DNA was digested with *Sac*I (an ≈2.6-kb *Sac*I fragment hybridizes to *MAT-2*), self-ligated, and used as template with primers ho18/ho19. Sequencing the product revealed the 5′ end of *MAT-2* and 2.0 kb of 5′ flank including part of *MAT-1* fused to *MAT-2. ORF1* primers ho12/ho13 amplified a 420-bp fragment.

C. cymbopogonis. *ORF1*-primers ho12/ho13 yielded a fragment; however, *MAT-2* primers ho1/ho2, which had been successful with *C. luttrellii* and *C. kusanoi*, did not work with *C. cymbopogonis.* Therefore a new *MAT-2* primer, ho3, corresponding to conserved *MAT-2* sequences of *C. heterostrophus, C. luttrellii*, *C. homomorphus*, and *C. kusanoi*, was used with ho1 to amplify a 309-bp fragment of *C. cymbopogonis MAT-2.* For *MAT-1,* primers ho4/ho5, based on *MAT-1* sequences of *C. heterostrophus, C. luttrellii*, *C. homomorphus*, and *C. kusanoi*, were successful (fragment = 865 bp). Combinations of *C. cymbopogonis ORF1*-specific (ho15) and *MAT-2* (ho11)- or *MAT-1* (ho20)-specific primers revealed two copies of *ORF1* (93% nucleotide identity), each linked to one *MAT* homolog. Sequencing of a 1.1-kb TAIL-PCR product amplified by using primer combinations ho21/AD2 and ho22/AD2 revealed the 3′ end of *MAT-1* and 0.8 kb of 3′ flank. Sequencing of a 2.5-kb PCR product amplified by inverse PCR on *Bam*HI-digested genomic DNA using primer pair ho23/ho24 revealed the 3′ end of *MAT-2* and 1.1 kb of 3′ flank.

Sequences of *C. heterostrophus MAT-1* (GenBank accession no. AF029913) and *MAT-2* (GenBank accession no. AF027687), *C. carbonum MAT-1* (GenBank accession no. AF032368), *C. victoriae MAT-2* (GenBank accession no. AF032369), and *C. ellisii MAT-1* (GenBank accession no. AF129746) and *MAT-2* (GenBank accession no. AF129747), as well as sequences of all homothallic species reported here [*C. luttrelli, C. homomorphus, C. kusanoi,* and *C. cymbopogonis* (GenBank accession nos. AF129740, AF129741, AF129742, and AF129744 and AF129745, respectively)], have been deposited. Sequencing details are available on request.

Expression of *C. luttrellii MAT* in *C. heterostrophus*. A 3.9-kb fragment carrying the entire *C. luttrellii MAT-1/2* ORF plus 1.6 kb of 5′ and 0.5 kb of 3′ flanking DNA was amplified from *C. luttrellii* genomic DNA by using primers LMAT/p1 (5′-CC*CTAGA*GGAACTTGGAATCGAACTCGCTTGTGTCTC-3′) and LMAT/p2 (5′-CC*TCTAGA*GGGACTACAACTGC-CAGGAGAAGCCAAGAA3-′), and *Pfu* DNA polymerase (Stratagene). Both primers included an *Xba*I site (italicized). The PCR product was purified (Qiagen, Chatsworth, CA), digested with *Xba*I, and ligated into the *Xba*I site of pBG, which carries the *bar* gene for resistance to bialaphos (31), creating pLMATB. *C. heterostrophus MAT* deletion strain ΔNcoMAT-2 was transformed with pLMATB. Transformants were selected on bialaphos, screened for resistance to hygromycin, and purified by single conidium isolation.

Phylogenetic Analyses. ITS and *GPD* sequences were spliced by using CAP2 (32), aligned first with CLUSTAL W (33) and then adjusted manually with SEQAPP (34). The alignment and list of all isolates analyzed are available on request (berbee@unixg.ubc.ca). Gaps were excluded, but all other positions were kept for the analysis. Each data set was initially analyzed by itself. We found 10,940 equally parsimonious trees for the 36 ITS sequences and 42 trees for the 36 *GPD* sequences in 50 replicated heuristic searches by using the TBR option and random addition of taxa with PAUP Version 4.0 d61a (35). The Kishino and Hasegawa test (36) indicated that the data sets were not substantially incongruent because the log likelihood of the fit of either the ITS or *GPD* data to the ITS + *GPD* trees was not significantly different. We found consensus trees for the ITS, the *GPD*, and the combined data sets by using 1,000 replicated parsimony searches without branch swapping. All branches receiving 55% or more bootstrap support in either data set were present in trees from both data sets, again indicating congruence between the ITS and *GPD* data sets. Also, 20 of the 22 nodes in the bootstrap trees received higher support from combined data than from either individual data set. Because the two data sets were substantially congruent, we combined them for further phylogenetic analysis. A maximum-likelihood tree was generated by using PAUP Version 4.0 d61a and default options. Using this tree, we estimated that 40% of the sites in the alignment were invariable and that, for the variable sites, substitution rates followed a γ-distribution with a shape parameter of 1. Using these estimates for substitution parameters, we found three equally likely trees, with log likelihoods of −8,235. The three trees differed only in the arrangement of near-zero-length branches. To test support for branches, PAUP was used to perform 500 bootstrap replicated parsimony searches with tree bisection and reconnection. All branches receiving 50% or more boot-

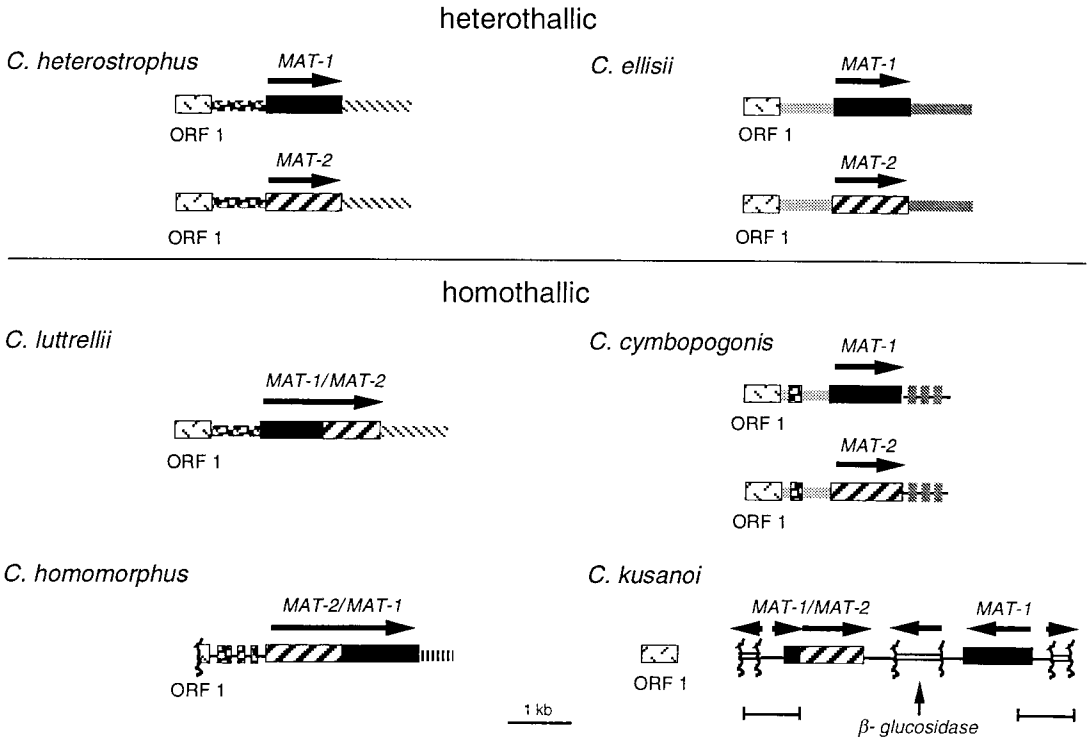

FIG. 2. Organization of *MAT* in heterothallic and homothallic species. The arrangement is identical in all heterothallic species examined to date, including *C. heterostrophus* and *C. ellisii* (shown here), *C. carbonum, C. victoriae, C. intermedius*, and asexual *B. sacchari* and *A. alternata* (not shown, see Fig. 5). Organization of each homothallic locus is unique, as described in the text. Textures of boxes are as in Fig. 1 for *MAT-1*, *MAT-2*, and *ORF1*; gene encoding β-glucosidase (open boxes); all other textures represent noncoding sequences 5′ or 3′ of *MAT* that are either unique to a particular species or common to more than one. Arrows indicate direction of transcription. Tightly linked to *MAT* in all species (except *C. kusanoi*) is a highly conserved ORF (*ORF1*) that shows similarity to a *Saccharomyces cerevisiae* ORF (GenBank accession no. U22383) of unknown function. Note that all genes are linked or fused, except that linkage has not yet been detected between the *C. cymbopogonis MAT* genes (which reside in the same nucleus unlike the heterothallic ones which reside in separate nuclei) or between *ORF1* and *C. kusanoi MAT*.

strap support were also present in the maximum likelihood tree.

RESULTS

Organization of Homothallic *MAT* Loci. The structural organization of the *MAT* loci of five heterothallic species (*C. heterostrophus*, *C. carbonum, C. victoriae, C. ellisii,* and *C. intermedius*) is highly conserved, with each strain carrying a single *MAT* gene; two examples are shown in Fig. 2. Furthermore, *MAT* loci from two asexual species, *Bipolaris sacchari* (*MAT-2* only, GenBank accession no. X95814), a close relative, and *A. alternata* (both *MAT* idiomorphs; ref. 37), a distant relative of *C. heterostrophus*, also have the same organization (data not shown). Together, these species represent most of the major branches of the phylogenetic tree (Fig. 5). In contrast, all homothallic species carry both *MAT* genes in one genome, but the structural organization of each locus is unique (Fig. 2). In two cases (*C. luttrellii* and *C. homomorphus*) the genes are fused into a single ORF; the gene order in *C. luttrellii* (5′ *MAT-1/MAT-2* 3′) is reversed in *C. homomorphus* (5′ *MAT-2/MAT-1* 3′). In the remaining two cases, the genes are not fused. In *C. kusanoi,* the organization is 5′ *MAT-2* 3′–3′ *MAT-1* 5′, and part of the sequence between the genes is similar to a portion of the β-glucosidase gene normally found 3′ of both *MAT* genes in heterothallic *C. heterostrophus* (GenBank accession nos. AF029913 and AF027687) (20). To the 5′ of *MAT-2* is a perfect inverted repeat of a 561-bp region containing 123 bp of the 5′ end of the *MAT-1* ORF fused to the 5′ end of *MAT-2* and 145 bp of a different fragment of the β-glucosidase gene, separated from each other by 293 bp. *C. cymbopogonis* carries both homologs of the heterothallic idiomorphs, but these are not closely linked; PCR reactions using various combinations of *MAT-1/MAT-2*-specific primers yielded no products, and gel blot analysis provided no evidence for linkage within 30 kb. Thus, the *MAT* genes have close physical association in three *Cochliobolus* homothallics but not in the fourth. *ORF1,* a gene with no apparent mating function (20) in heterothallic *C. heterostrophus,* is present in all homothallic species; in three of four cases it is ≈1 kb 5′ of the 5′ end of *MAT. C. cymbopogonis* has two copies of *ORF1*, each linked to a *MAT* gene. *ORF1* is not closely linked to *MAT* in *C. kusanoi*.

Recombination Converts a Heterothallic to a Homothallic Species. Insight into the genetic mechanism by which one reproductive life style evolves from the other was obtained by comparing *MAT* sequences from a closely related pair of species, heterothallic *C. heterostrophus* and homothallic *C. luttrellii* (Fig. 3). Inspection of the sequence at the *MAT* fusion junction in *C. luttrellii* revealed that 345 nt from the 3′ end of the *MAT-1* ORF and 147 nt from the 5′ end of the *MAT-2* ORF are missing, compared with the *C. heterostrophus* heterothallic homologs. The deletions are consistent with the hypothesis that a crossover event occurred within the dissimilar *C. heterostrophus* genes at positions corresponding to the fusion junction (Fig. 3). Inspection of the *C. heterostrophus* genes reveals 8 bp of sequence identity precisely at the proposed crossover site, which would explain this arrangement. A single crossover within this region would yield two chimeric products, one of which is identical to the fused *MAT* gene actually found in *C. luttrellii* (Fig. 3). A similar scenario can be proposed for *C. homomorphus*; in this case the fused gene is missing 27 nt from the 3′ end of *MAT-2* and 21 nt from the 5′ end of *MAT-1* compared with the *C. heterostrophus MAT* genes. Examination of the *C. heterostrophus MAT* sequences at positions corresponding to the *C. homomorphus* fusion junction reveals nine

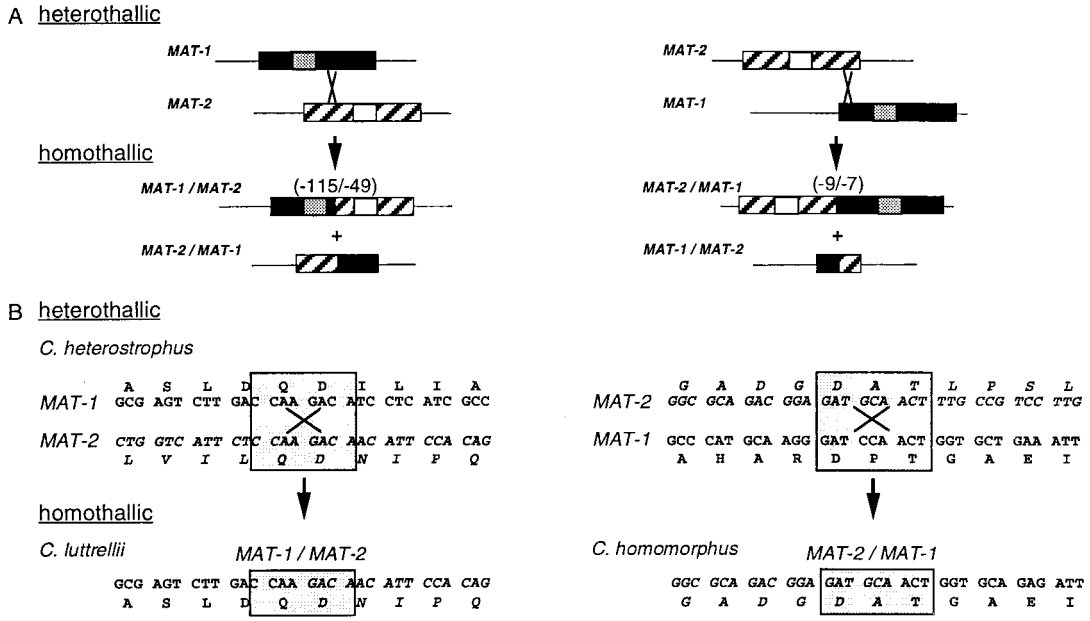

FIG. 3. Models for evolution, by recombination events, of fused homothallic *MAT* genes in *C. luttrellii* and *C. homomorphus* from opposite heterothallic *MAT* genes in *C. heterostrophus* (the heterothallic progenitor of *C. homomorphus* is unknown). (*A*) Misalignment of homologous flanking sequences could bring into register short islands of identity between the largely dissimilar *MAT* idiomorphs. A homologous recombination event at the point of identity would result in two fused *MAT* genes, both incomplete with respect to their heterothallic counterparts. If the crossover point were on either side of the DNA binding region (14), one fusion product would have both DNA-binding motifs and one would have neither. The number of amino acids eliminated (*C. luttrellii*, 115 from the 3′ end of *MAT-1,* 49 from the 5′ end of *MAT-2*; *C. homomorphus*, 9 from the 3′ end of *MAT-2,* 7 from the 5′ end of *MAT-1*) depends on the position of the crossover point (compare *A Left* with *A Right*). Textures are described in Fig. 1; small boxes within idiomorphs represent DNA-binding motifs; gray, α-box in *MAT-1*, white, HMG box in *MAT-2* (14). (*B*) Inspection of the actual nucleotide sequences of the *C. heterostrophus MAT-1* and *MAT-2* genes reveals an 8-bp region of complete identity (shaded box, *B Left*) and a 9-bp region, with one mismatch (shaded box, *B Right*), corresponding to the *C. luttrellii* and *C. homomorphus* fusion points, respectively. Recombination in the *Upper Left* box creates precisely the sequence found in the *C. luttrellii MAT-1*/*MAT-2* fused gene (*Lower Left* box); the left side of the fused sequence is similar to *C. heterostrophus MAT-1* and the right side to *MAT-2*. Recombination in the *Upper Right* box creates the *C. homomorphus MAT-2*/*MAT-1* fused gene (*Lower Right* box). Single letters above or below codons are standard amino acid abbreviations.

bp of identity (with one mismatch) and thus a putative recombination point (Fig. 3).

Conversion of a Heterothallic to a Homothallic Species. To determine whether *MAT* genes alone can control reproductive style, the sterile *C. heterostrophus MAT*-deletion strain ΔNcoMAT-2 was transformed with pLMATB carrying the fused *C. luttrellii MAT-1/2* gene. Transformants (*barR*;*hygR*) were purified, selfed, and crossed (18) to albino *C. heterostrophus MAT-1* and *MAT-2* tester strains.

Three transformants were analyzed in detail; all carried the transforming plasmid at ectopic sites. Abundant pseudothecia formed when the transformants were selfed or crossed (Fig. 4 *Top* and *Middle*), most of which showed some degree of fertility (1–10% of wild-type ascospore production). Pseudothecia and progeny from selfs were always pigmented, whereas approximately half the pseudothecia and half the progeny from crosses were albino and half of each were pigmented, indicating that heterothallic *C. heterostrophus* expressing a homothallic *MAT* gene can both self- and outcross (Fig. 4 *Bottom*). Thus, the *C. luttrellii MAT-1/2* gene alone conferred selfing ability to heterothallic *C. heterostrophus* without impairing its ability to cross.

Phylogenetic Analyses. To determine whether phylogenetic analyses support a convergent origin for homothallism, we used maximum likelihood and parsimony trees inferred from the ITS and *GPD* data set. All of the resulting trees (Fig. 5) show that homothallism is polyphyletic. None of the six homothallic species clustered together in any of the 15 most parsimonious trees or in maximum likelihood trees. How strong is the support for keeping the four homothallic species of *Cochliobolus* separated? To answer this question, we used the Kishino and Hasegawa test as implemented by PAUP Version 4.0 61a to compare the fit of the data to the maximum likelihood tree (Fig. 5) with the fit to the most likely of the parsimony trees constrained to show the homothallics as a monophyletic group. The log likelihood of the most likely parsimony tree constrained to show a monophyletic origin of homothallism was −8,530, compared with −8,235 for the most likely unconstrained tree. The constrained tree was more than 10 SD worse than the unconstrained tree, a difference significant at $P < 0.0001$. Thus, phylogenetic evidence clearly supports independent evolution of homothallism in the four homothallic *Cochliobolus* species.

DISCUSSION

In earlier reports, it was speculated that heterothallic fungal species are ancestral to homothallic species (1–7) and that homothallics may arise by unequal crossover events (2). Our analysis of extant *MAT* sequences provides direct evidence supporting these hypotheses. Comparison of *MAT* DNA from a closely related pair of species, one heterothallic, the other homothallic (Figs. 3 and 5), revealed a genetic mechanism that likely explains how a heterothallic species can become homothallic. Both *MAT-1* and *MAT-2* are truncated in the homothallic *C. luttrellii MAT* fusion, suggesting an unequal crossover event in the heterothallic *MAT* progenitor, resulting in the fusion; perusal of *MAT* ORFs revealed an 8-bp sequence that is identical between the *C. heterostrophus* genes, precisely where *MAT-1* becomes *MAT-2* in *C. luttrellii* (Fig. 3). Although the idiomorphs are largely dissimilar, as little as 8 bp may be enough to promote rare homologous recombination, because as little as 4 bp is sufficient for recombination in *Saccharomyces cerevisiae* (38). After recombination, one fusion product would

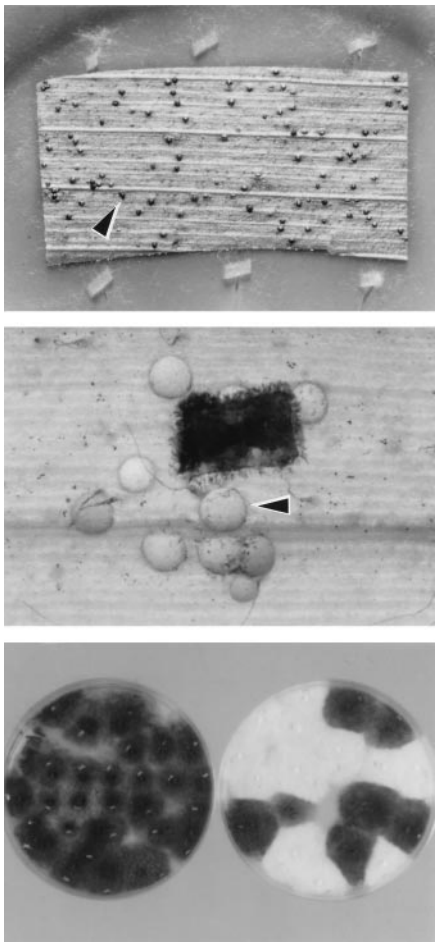

FIG. 4. The *C. luttrellii* homothallic *MAT* gene alone confers on heterothallic *C. heterostrophus* the ability to self and cross. (*Top*) Plate with a senescent corn leaf as substrate for mating (18) inoculated with *C. heterostrophus* carrying the fused *C. luttrellii MAT* gene. Black bodies (arrowhead) are pseudothecia, indicating selfing. (*Middle*) Part of a mating plate, inoculated first with an albino *C. heterostrophus MAT-1* tester strain, followed by inoculum (black rectangle) of a pigmented *C. heterostrophus MAT*-deletion strain carrying the fused *C. luttrellii MAT* gene. White pseudothecia (arrowhead) indicate crossing with the albino parent as female, because pseudothecial walls are of maternal origin. (*Bottom Left*) Progeny of a selfed transformant (*Top*), demonstrating that pseudothecia from selfed strains yield viable ascospores and that all progeny of a selfed pigmented strain are pigmented. (*Bottom Right*) Progeny of a cross (*Middle*), demonstrating that ascospores are viable and that alleles at the color marker *Alb1* segregate (1:1).

be the functional *C. luttrellii* gene (Fig. 2); the other is predicted to have no mating function because it would lack both DNA-binding domains (14). The recombination point alone suggests heterothallic-to-homothallic evolution. It is difficult to envision a mechanism for the reverse, e.g., that homothallic *C. luttrellii MAT,* lacking 164 aa, could acquire sequences necessary for two full-length *MAT* genes, distribute them in separate nuclei, and become heterothallic. Furthermore, all heterothallic *Cochliobolus* species have the same *MAT* organization, whereas each homothallic species is unique at *MAT.* It is unlikely that these different homothallic loci could give rise to the single arrangement found in the diverse collection of heterothallic species that we studied (Fig. 5).

A similar recombination mechanism can be proposed for evolution of homothallic *C. homomorphus,* despite the fact that a close heterothallic relative is not available (Fig. 5). There is a 9-bp island of identity in the *C. heterostrophus MAT* sequences, again precisely at the fusion junction (Fig. 3). A

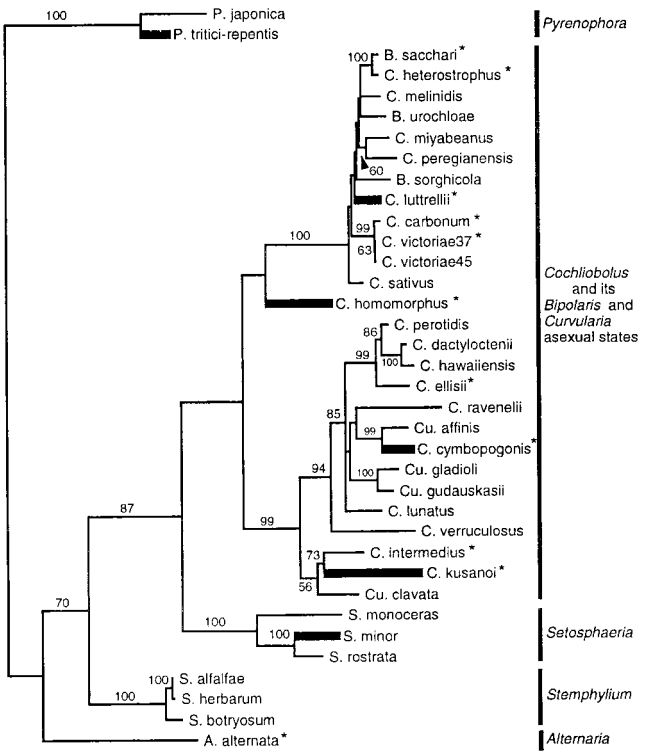

FIG. 5. Maximum likelihood tree generated from combined ITS and *GPD* sequence data by using PAUP Version 4.0 d61a (35). Homothallic species (thick lines) are scattered among heterothallic species, indicating their polyphyletic origin. Numbers are percentages (only those over 50 are shown) of times a group was found in 500 parsimony bootstrap replicates. * indicate species from which *MAT* loci were examined.

crossover at this point, in the hypothetical heterothallic progenitor, would eliminate 16 aa and create the *C. homomorphus MAT-2/MAT-1* chimera. Similarly, recombination between as-yet-unidentified regions of identity in the 5′ and 3′ flanks of heterothallic idiomorphs likely gave rise to the *MAT* gene arrangement in *C. kusanoi,* although there is no direct evidence for this in the available heterothallic sequences. The scrambled sequences in *C. kusanoi* suggest multiple recombination events. The mechanism by which *C. cymbopogonis* arose is difficult to assess until we determine whether the *MAT* genes are linked.

The hypothesis that homothallism derives from heterothallism is supported by data from heterologous expression studies. The ability of *C. heterostrophus* carrying *C. luttrellii MAT* to form fertile reproductive structures when selfed indicates that it has all of the genes needed to be homothallic except the "correct" configuration of the *MAT* gene itself. The same haploid *C. heterostrophus MAT*-deletion strain, expressing both *C. heterostrophus MAT* genes, can produce pseudothecia, but these are barren (20). The single variable in these experiments is *MAT,* indicating it alone can control differences in reproductive life style. We suggest that a recombination event is necessary to initiate the change from one reproductive mode to the other. Continued alterations in a new homothallic strain could optimize its homothallic fitness.

The organization and origin of homothallic *MAT* loci have been investigated in certain homothallic members of an unrelated family of fungi, the Sordariaceae. Most, like *Cochliobolus,* carry both *MAT* idiomorphs found in heterothallic species (2, 39, 40). An exception is the genus *Neurospora*, where certain species carry only one (2). All heterothallic and homothallic *Neurospora* spp. have a *MAT*-flanking region that

distinguishes heterothallic from homothallic species (39). We have not detected such a region flanking *Cochliobolus MAT* (Fig. 2). *Neurospora terricola,* one of two homothallic species that carries both *MAT* genes, has them on the same chromosome, although not closely linked (6). The *MAT* locus of homothallic *Sordaria macrospora* contains counterparts of all four *MAT* genes encoded by the two *MAT* idiomorphs of heterothallic *Neurospora crassa* (7); one gene is a *mat A/mat a* idiomorph fusion that is like *N. crassa mat A-3* at the 5′ end and like noncoding *mat a* idiomorph sequence at the 3′ end (40). Homothallic *MAT* genes from *Neurospora africana* (41) and *Sordaria macrospora* (40) act as mating activators in heterothallic genetic backgrounds, as does the *C. luttrellii* gene. Because the Sordariaceae genes were not expressed in *MAT*-deleted heterothallic strains, the ability to promote ascospore formation could not be evaluated, because of the ''interference'' phenomenon described earlier (7, 19).

The evolutionary origins of the dissimilar *MAT* idiomorphs of ascomycetes are unknown. It has been suggested (7) that the small pockets of identity in the otherwise unlike heterothallic *MAT* idiomorphs reflect common ancestry. Conceivably, these pockets are remnants of a series of mutagenic events in a single ancestral gene. The mutations, coupled with recombination suppression, might have led to the highly divergent extant *MAT* genes that now encode different products (14). A similar scenario has been proposed for evolution of the Y chromosome (15–17).

The importance of combining molecular genetic and phylogenetic approaches to understanding life style evolution cannot be overemphasized. Phylogenetic analysis allowed us, on the one hand, to choose diverse species for demonstrating that *MAT* is constant in heterothallics, and on the other hand, to pick a pair of closely related species for evaluating lifestyle evolution. The *C. luttrellii MAT* fusion led to detection of the 8-bp recombination point, the key to understanding events in the change from heterothallism to homothallism. Hypotheses regarding reproductive lifestyle evolution in any fungal group would be strengthened by examination of nucleotide sequences of genes controlling the sexual process. As *MAT* data are accumulated, we may find that some fungi evolve differently from *Cochliobolus*. Geiser *et al.* (10), for example, suggest (based on phylogenetic analyses using β-tubulin and hydrophobin sequences) that heterothallism is the derived state in *Aspergillus* species. A mechanism underlying evolution in this direction has not been established. Perhaps, in homothallic species that have two complete unfused *MAT* genes, either could be deleted independently, leaving a strain with a single *MAT* gene. If the opposite *MAT* gene were deleted from a different homothallic strain, the population would contain non-selfing pairs that might be capable of crossing with each other.

Although our molecular and phylogenetic data argue that, in nature, homothallism is derived from heterothallism, it is possible that this process can be reversed under laboratory conditions, thus providing a tool that would facilitate genetic analysis of homothallic species. For example, it may be possible to make a homothallic species heterothallic by replacing its *MAT* gene(s) with individual heterothallic *MAT* genes, each in a different strain, then crossing the strains. This may not work with the homothallic *Neurospora* species, which do not conidiate or form female receptive hyphae (41–43). All homothallic *Cochliobolus* species, however, are able to conidiate, although their ability to form trichogynes or outcross has not been evaluated for lack of genetically marked strains required as testers.

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