Deciphering the Relationship between Mating System and the Molecular Evolution of the Pheromone and Receptor Genes in *Neurospora*

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Abstract

Here, we present a study of the molecular evolution of the pheromone receptor genes (*pre-1* and *pre-2*) in *Neurospora* taxa with different mating systems. We focus on comparisons between heterothallic and homothallic taxa, reproducing sexually by outcrossing and by intrahaploid selfing, respectively. Our general aim was to use a phylogenetic framework to investigate whether the evolutionary trajectory of the pheromone and receptor genes in *Neurospora* differs between heterothallic and homothallic taxa, and among the homothallic lineages/clades previously indicated to represent independent switches from heterothallism to homothallism in the evolutionary history of the genus. We complemented molecular evolution analyses with an expression study of the *pre* genes and their upstream regulators, the mating-type (*mat*) genes, in homothallic taxa. Our analyses suggest that the pheromone receptor gene *pre-1* is functionally conserved in both heterothallic and homothallic taxa. Moreover, we found evidence of positive selection for a small fraction of codons in the cytoplasmic signal-transducing C-terminal region of the protein PRE-1. Distribution of positively selected codons differs between heterothallic and homothallic groups, suggesting functional divergence associated with mating system. The gene *pre-2* was shown to evolve under high selective constraints, with no strong evidence for positive selection. Although our data suggest that both *pre-1* and *pre-2* are overall functional in homothallic taxa, individual taxa display frame-shift mutations causing premature stop codons, which might indicate loss of function. Transcriptional patterns of *pre* and *mat* genes in six homothallic taxa, selected to represent six different switches from heterothallism to homothallism, do not support a universal pattern of regulation of these genes during reproductive tissue development. Taken together, our analyses suggest that the pheromone receptor genes *pre-1* and *pre-2* are in general functional in homothallic *Neurospora* taxa, in contrast with the situation for the *mat* genes that are generally degenerate in these taxa.

Key words: *Neurospora*, pheromones, receptors, homothallism.

Introduction

Cell-to-cell communication is a ubiquitous and essential function in both microbial and multicellular species. In super kingdoms of both prokaryotes and eukaryotes, different pheromone response systems have evolved. Peptide pheromones and their cognate receptors have been found in bacteria, mollusks, fungi, arthropods, and various vertebrates, which reflect the evolutionary importance of this type of signaling system (Altstein 2004).

In fungi, research on pheromone response systems has a long tradition (e.g., Kim et al. 2002; Jones and Bennett 2011). There is a plethora of mechanisms by which pheromones and pheromone receptors control sexual development in fungi, which emphasizes the need to investigate pheromone/pheromone receptor on a case-by-case basis (Stanton et al. 2010). Here, we present a study of the molecular evolution of pheromone receptors in members of the filamentous ascomycete model genus *Neurospora* exhibiting different mating systems: heterothallism (self-incompatibility), homothallism (self-compatibility), and pseudohomothallism (partial self-incompatibility).

Heterothallic taxa of *Neurospora* have two distinct mating types, A and a, with alternative sequences (idiomorphs) at the mating-type (*mat*) locus (Glass et al. 1988; Metzenberg and Glass 1990). For sexual reproduction to occur, strains of the two opposite mating types must meet. In contrast, the homothallic and pseudohomothallic species can complete the sexual cycle in isolation, that is, they are self-compatible (Coppin et al. 1997). The true homothallic taxa have one or, more commonly, both mating-type components in the same haploid nucleus (Glass et al. 1990; Beatty et al. 1994), whereas pseudohomothallic taxa carry nuclei of both mating types in the vegetative and sexual tissue (Dodge 1927).

A recent study on the *mat* locus of *Neurospora* (Gioti et al. 2012) provided strong support for a heterothallic ancestor of
the genus: the locus is conserved among heterothallic taxa representing basal and terminal clades in the genus phylogeny (Nygren et al. 2011), whereas different gene arrangements characterize homothallic taxa. Models for mating-type transitions presented in this study indicate that several independent switches from heterothallism to homothallism have occurred throughout the evolutionary history of Neurospora. The availability of this phylogenetic framework renders Neurospora an ideal system to trace the evolution of traits over transitions in mating system.

In contrast to other genera of filamentous ascomycetes, the homothallic taxa of Neurospora appear to lack the reproductive structures important for outcrossing, that is, trichogynes (the female receptive hyphae) and conidia (the male fertilizing units) (Perkins 1987) and are therefore not believed to be capable of mating in the classical sense (Howe and Page 1964; Raju 1978; Perkins 1987; Nygren et al. 2011). Furthermore, the mat genes in homothallic Neurospora show signs of degeneration with disrupted open reading frames in several taxa and relaxed selective constraints on codons compared to the heterothallic clade (Wik et al. 2008).

The interaction between potential sexual partners of heterothallic Neurospora is chemotropic (Bistis 1983) and mediated by two diffusible pheromone peptides, cgg-4 and mfa-1 (Bobrowicz et al. 2002), and two distinct G-protein-coupled receptors, pre-1 and pre-2 (Pöggeler and Kück 2001; Kim and Borkovich 2004). The expression of the pheromone precursor and receptor genes is directly influenced by transcription factors encoded by genes at the mat locus, so that cgg-4 and pre-1 are predominantly expressed in mat A strains, whereas mfa-1 and pre-2 are expressed in mat A strains (Debuchy 1999; Pöggeler and Kück 2001; Bobrowicz et al. 2002; Kim et al. 2002; Kim and Borkovich 2004, 2006; Karlsson et al. 2008; Kim et al. 2012). The interactions between pheromones and their cognate receptors have been shown to be essential for both male and female fertility in a number of heterothallic ascomycetes (Kim et al. 2002; Turina et al. 2003; Coppin et al. 2005; Kim et al. 2012). For example, in N. crassa, deletion of pre-1 or pre-2 results in female sterility of the A and a mating type, respectively, because the trichogynes are incapable of directional growth and fusion with spermata (Kim and Borkovich 2004, Kim et al. 2012), and deletion of either cgg-4 or mfa-1 results in male infertility in the corresponding mating type, because spermata can no longer attract female trichogynes (Kim and Borkovich 2006). Moreover, a recent gene deletion study indicates that to go through meiosis and ascospore production, at the minimum one of the mating partners needs to have one functional pheromone receptor gene (Kim et al. 2012). A study of the evolution of the pheromone receptor genes in heterothallic and pseudohomothallic Neurospora indicated that purifying selection is the major force shaping the genes, although the intracellular C-terminal domains of both genes evolve rapidly, with both stochastic and directional processes driving this divergence (Karlsson et al. 2008). However, little is known about the role of the pheromone response system in homothallic taxa of the genus. Because mate recognition does not seem to be essential for sexual reproduction in fungi with this mating system, conservation of a pheromonal system in homothallic fungi would support additional roles besides attracting a mate for these proteins.

The aim of this study was to investigate whether the evolutionary trajectory of the pheromone receptor (pre) genes in Neurospora differs depending on the mating system, that is, if there is a general difference in evolutionary constraints of the genes in heterothallic and homothallic taxa. In addition, we aimed to investigate whether the evolutionary forces shaping these genes differ between the homothallic lineages/ clades indicated to represent independent switches from heterothallism to homothallism in the evolutionary history of Neurospora (Nygren et al. 2011; Gioti et al. 2012). Specifically, we were interested to know whether the pre genes show signs of genetic decay in accordance with the mat genes in homothallic Neurospora (Wik et al. 2008), or alternatively, if they are functionally conserved or under positive selection for a new function in homothallic taxa. In addition, we performed expression studies of both pre and mat genes from six isolates of homothallic Neurospora, to investigate whether these genes are expressed and regulated between developmental stages.

Materials and Methods

Fungal Strains

We used 30 strains belonging to 16 homothallic, 13 heterothallic, and 1 pseudohomothallic taxon of Neurospora (table 1). The strains were obtained from the Centraalbureau voor Schimmelcultures (CBS; Utrecht, The Netherlands), Facultat de Medicina Reus (Universitat Rovira i Virgili, Spain), or the Fungal Genetics Stock Center (FGSC; University of Missouri, Kansas City, USA). Without making any taxonomic judgment, the taxon of Neurospora (Dodge 1927) and Gelasinospora (Dowding 1933) were merged into Neurospora in this study, following García et al. (2004). The homothallic taxon Sordaria macrospora was included in the study as outgroup in the phylogenetic analysis. For expression analysis of pre and mat genes, six homothallic isolates of Neurospora (FGSC 1740: N. africana, FGSC 959: N. cerealis, FGSC 7221: N. pannonica, FGSC 5508: N. sublineolata, FGSC 1889: N. tericola, and CBS 112 768: N. uniporata) were used (table 1).

Culture Conditions

Isolates for polymerase chain reaction (PCR) and sequencing were cultivated in test tubes with Vogel’s minimal medium with 1.5% sucrose (Vogel 1956). For the expression study, three different developmental stages of the homothallic life cycle were studied: “vegetative,” “early reproductive,” and “late reproductive” (defined later). With the aim of analyzing expression patterns related to developmental stages rather than growth medium, tissue for all these stages was grown in synthetic crossing media (Westergaard and Mitchell 1947) with 2% sucrose. The samples analyzed as vegetative were grown submerged in E-flasks with 50 ml of liquid media. The flasks were incubated shaking at room temperature in darkness for 3–6 days before harvest. The samples analyzed as
early and late reproductive stages were grown on 9 cm Petri dishes with solid media covered with porous cellophane (Hoefer, San Francisco, CA), for 6–8 and 15–24 days, respectively. After inoculation, the dishes were sealed with parafilm (Menasha, WI) and incubated at 25°C in the dark. At harvest, we confirmed that the vegetative samples primarily consisted of mycelia, the early reproductive constituted of protoperithecia and mycelia, and the late reproductive stages contained mature perithecia with ejected ascospores. By this approach, we minimize the growth conditions differing between the developmental stages to include only liquid versus solid media.
Extraction of DNA, RNA, and cDNA Synthesis

DNA extractions were performed from fresh tissue using either of two previously described methods (Johannesson and Stenlid 1999; Cano et al. 2002). Total RNA was extracted from approximately 100 mg of frozen fungal material using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol for filamentous fungi. The Buffer RLC supplemented with 10 μl/ml β-mercaptoethanol was used as lysis buffer. Traces of DNA were removed from the RNA with a DNase I (Fermentas, Vilnius, Lithuania) treatment according to manufacturer’s description. Synthetic kanamycin mRNA (Promega, Madison, WI) was included in reverse transcription as internal control of cDNA synthesis efficiency. cDNA synthesis was performed on 500 ng total RNA per sample using the iScript cDNA synthesis kit (Bio-Rad) according to manufacturer’s instructions. The cDNA was diluted 10X before use in quantitative PCR.

PCR and Sequencing of pre-1 and pre-2 from Homothallic Neurospora

The sequences of pre-1 and pre-2 from the homothallic taxa of Neurospora (table 1) were gathered specifically for this study. Sequences from four homothallic taxa (N. africana, N. pannonica, N. sublineolata, and N. terricola) were obtained from de novo assemblies of Illumina sequencing data (Gioti A, Stajich EJ, Johannesson H, unpublished) as follows: published N. crassa gene sequences for pre-1 and pre-2 were retrieved from the N. crassa genome database at the Broad Institute (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html) and used as templates in local BlastN queries against databases of predicted genes for each of the four assembled and annotated genomes. BlastN (Altschul et al. 1990) was run with default parameters, and the best hits were retained. The pre-1 and pre-2 sequences from the remaining homothallic taxa were amplified using the primers given in Karlsson et al. (2008). Additional primers were designed based on publicly available pre-1 and pre-2 sequences using Primer3 version 0.4.0 (Rozen and Skaltsky 2000) and are listed in supplementary table S1, Supplementary Material online. PCRs were either performed with ThermoWhite Taq DNA polymerase (Saveen Werner, Limhamn, Sweden) or Expand High Fidelity PCR System (Roche Applied Science, Indianapolis, IN) following the manufacturer’s recommendations. PCR products were purified with ExoSAP-IT reagent (Amersham Biosciences, Uppsala, Sweden). The amplicons were sequenced in both forward and reverse directions either at Macrogen Inc. (Seoul, Korea) or at the Department of Evolutionary Biology, Uppsala University, Sweden. For the latter, the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was used. The products were first cleaned using BigDye XTerminator Purification Kit (Applied Biosystems) and then sequenced on an ABI3730XL (Applied Biosystems). The raw sequences were edited using the software package Sequencher 4.1.4 (Gene Codes Corporation, Ann Arbor, MI).

Sequence Alignment and Phylogenetic Analyses

DNA sequences of pre-1 and pre-2 from homothallic taxa of Neurospora were aligned to publically available pre-1 and pre-2 sequences from heterothallic and pseudohomothallic taxa of Neurospora (Karlsson et al. 2008), using the program MAFFT (Katoh et al. 2005). The alignments were adjusted manually using BioEdit version 7.0.0 (Hall 1999). Sequences from S. macrospora were used to root the trees but were not included in codon-based likelihood and reverse conservation analyses (RCA). Sequence analyses were performed separately on the pre-1 and pre-2 data sets.

Gene trees for pre-1 and pre-2 were obtained from phylogenetic analyses using both a maximum likelihood (ML) and a Bayesian approach. ML bootstrap analyses were run in RAxML v.7.2.2 (Stamatakis 2006) using the GTR+GAMMA model (for both data sets) and performing 1,000 bootstrap replicates. Bayesian inference was performed using MrBayes 3.1 (Huelsenbeck and Ronquist 2001). We used the Akaike information criterion in MrModeltest 2.3 (Nylander 2004) to assess the best model of sequence evolution; the best model was GTR+G for pre-1 and GTR+I+G for pre-2. Two separate runs, each initiated from a random tree, were carried out with four Markov chains. The Markov chains were run for 1 million generations. Default cold and heated chain parameters were used, and at the end of each run, the sampling of the posterior distribution was considered to be adequate if the standard deviation of split frequencies was < 0.01. Markov Chain Monte Carlo runs were summarized using the “sump” and “sumt” commands in MrBayes, and 2,500 trees (25%) from each run were discarded as “burn-in.”

Molecular Evolution Analyses: Alignments and Trees

We used the ML-based program codeml, included in the PAML package Version 4.3 (Yang 1997, 2007), to test different hypotheses of how the molecular evolution of the pheromone receptor genes pre-1 and pre-2 is related to mating system in Neurospora. Assuming that the evolutionary histories of the two pre genes in this study are the same as the history of the taxa, we chose to base our analysis on the species phylogeny constructed on seven molecular gene loci by Nygren et al. (2011). The topology was adapted with slight modifications: nodes that were not supported by both Bayesian Posterior probabilities (BPPs) ≥ 0.75 and ML bootstrap support ≥ 0.75 in Nygren et al. (2011) were collapsed unless they had full support value from one of the analyses (BPP = 1.0 or ML = 1.00) (fig. 1). When only subsets of the taxa were used in the analyses, the topology of the corresponding part of the species tree was used. We have, in a previous study, reported conflicts between species tree and reproductive gene trees in heterothallic Neurospora, which may not be caused by chance alone (Strandberg et al. 2010), and therefore, when the genealogy of the gene differed from the species phylogeny inferred by Nygren et al. (2011), we performed parallel analyses using the gene tree as input topology in the analyses. The pseudohomothallic taxon N. tetrasperma was inferred as heterothallic, because it has been reported to produce functionally heterothallic propagules and
occasionally outcross in nature (Raju 1992; Powell et al. 2001; Menkis et al. 2009). We assumed a heterothallic ancestor for the genus based on results from Gioti et al. (2012).

For the division of homothallic branches into different switches in mating system, we followed the phylogeny of Nygren et al. (2011), with the exception of N. pannonica, that was recently shown to have a different mat locus architecture/composition than N. sublineolata (Gioti et al. 2012) and, therefore, was treated as an independent switch in the analysis. Before the analysis, we excluded intronic sequences from the alignments, regions for which sequence data for less than 20 taxa were available, and regions that were ambiguously aligned. In case of frame-shift mutations, sequences were adjusted to fit the reading frame of the alignment by removing extra bases or replacing incomplete codons with gaps. Parallel runs excluding the sequences of the taxa with frame-shift mutations were made to verify that the sequences from the taxa containing frame-shift mutations did not markedly affect the results.

Two different types of codon substitution models were analyzed using codeml in PAML 4.3 (Yang 1997, 2007): branch models and site models (see later). The fit to the data of the nested models was tested using a likelihood ratio test (LRT), where twice the difference in log likelihood values (−2 ln Δ) was compared using a χ² distribution and with the degrees of freedom equal to the difference in parameters between the two models.

**Molecular Evolution Analyses: Branch Models**

Using branch models, we tested for general variance in dN/dS among the branches in the phylogenetic tree. For both pre-1 and pre-2, we used the global dN/dS (one ratio) model in codeml, assuming the same dN/dS for all branches in the phylogeny and the free ratios branch model, where each branch in the phylogeny is allowed independent dN/dS rates. In addition, we analyzed different models of local dN/dS to investigate mating system or switch-dependent evolution. In the first local model, branches were divided in two groups depending on mating system of terminal taxa, that is, heterothallic or homothallic (solid and dashed lines in fig. 1, respectively), thus allowing for two ratios of dN/dS for branches in the tree corresponding to either group. To test for differences between the phylogenetic lineages/clades of the homothallic taxa representing independent switches from heterothallism to homothallism, we used a version of the local model where each homothallic clade, or separate lineage, deviating from the main backbone of the phylogeny (branches with different dash length in fig. 1) was allowed separate dN/dS. In total, this model allowed eight different ratios of dN/dS, including the ratio for the background branches (i.e., all branches not belonging to a homothallic clade) (fig. 1).

**Molecular Evolution Analyses: Codon Site Models**

We used codon site models to test for variance in dN/dS among codons and to identify individual codons of pre-1 and pre-2 likely to be under positive selection between taxa. Four models were analyzed: M1a, M2a, M7, and M8. The models constitute two nested pairs (M1a+M2a and M7+M8) with one model of each pair only containing site classes allowing dN/dS to vary between 0 and 1 (neutral models; M1a and M7), whereas the second model of each pair (selection models; M2a and M8) contains an additional site class in which dN/dS ≥ 1, that is, allowing positive selection. Codons likely to evolve under positive selection were identified using the Bayes empirical Bayes (BEB) calculation of posterior probabilities for site classes (Yang et al. 2005).

To investigate potential differences in selective constraints affecting different regions or codons of the pheromone receptor genes in the homothallic and heterothallic taxa, respectively, the analyses were divided into subsets including sequences from all taxa, from heterothallic taxa only, and from homothallic taxa only.

**Reverse Conservation Analyses**

We used RCA to investigate potential differences in amino acid conservation between different regions or sites of the pheromone receptors in homothallic and heterothallic taxa, respectively. RCA was performed on the same data sets as the codon site analyses above. In addition, we performed analyses on subsets of homothallic taxa representing independent switches from heterothallism to homothallism (fig. 1). These homothallic subsets were selected as individual clades with multiple taxa in the phylogeny by Nygren et al. (2011) and are referred to as the N. africana, N. hapsidophora, and N. retispora clades. The same analyses were performed on each of the data sets.

The RCA analyses were performed as described by Lee (2008). In short, the program Rate4Site (Version 2.01) (Pupko et al. 2002; Mayrose et al. 2004) uses an empirical Bayesian method to calculate the degree of conservation (S score) of amino acids for each position in the alignment, here of proteins PRE-1 and PRE-2 obtained by sequence translation. A high S score corresponds to a low degree of conservation. A sliding-window average (n = 7) of normalized S scores (mean = 0, standard deviation = 1) was applied and plotted in Excel (Microsoft). According to Lee (2008), significant peaks of nonconservation are defined by intensity (I) values of 0.5 or higher.

**Real-Time Quantitative PCR**

Real-time quantitative PCR was used to reveal differences in expression of the pheromone receptor genes (pre-1 and pre-2), between the vegetative, early reproductive, and the late reproductive stages in six homothallic taxa of Neurospora (N. africana, N. cerealis, N. uniporata, N. sublineolata, N. pannonica, and N. terricola). In addition, the expression of the mat genes (mat A-1 of the a idiomorph and mat A-1, mat A-2, and mat A-3 of the A idiomorph) was measured. Actin (act) was used as an endogenous control to normalize the expression of target genes (Karlsson et al. 2008). Primer pairs for pre and mat genes were designed at conserved regions between the homothallic taxa using Primer3 (Rozen et al. 2000).
**FIG. 1.** Evolutionary relationship of *Neurospora* and estimations of \( dN/dS \) among branches for *pre-1* (A) and *pre-2* (B). Topology is adapted from a previously reported species tree based on seven nuclear gene loci (*Bml-1, tef-1, pck, 28S rDNA, mak-2, nik-1, and a hypothetical protein-coding gene NCU02332*) (Nygren et al. 2011). The solid lines represent branches of heterothallic taxa, and dashed lines represent branches of homothallic taxa, under the assumption of a heterothallic ancestor of *Neurospora* (Gioti et al. 2012). Homothallic lineages, resulting from independent switches from heterothallism to homothallism, are indicated by different dash lengths. The values presented at the branches are \( dN/dS \) ratios obtained from running the free-ratio model, and values given in boxes are \( dN/dS \) ratios obtained form the local model of a switch-dependent evolution in codeml. Different letters within parenthesis (a and b) indicate statistically significant differences between model fit, using the \( \chi^2 \) test.
and Skaletsky 2000) and are listed in supplementary table S1, Supplementary Material online.

The real-time PCR assays were carried out using an iQ5 iCycler thermocycler (Bio-Rad, Hercules, CA, USA) using 5 μl cDNA solution, forward and reverse primers (187 nm final concentration), and the Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Fermentas). Each reaction was performed in triplicates and had the following PCR parameters: an initial 5 min denaturation at 95°C, followed by 40 cycles of 10 s denaturing at 95°C, 30 s annealing at 60°C, and 30 s of elongation at 72°C. To analyze reaction specificity, a melt curve analysis was performed after the PCR reaction, by increasing the temperature from 60°C to 95°C in 0.5°C intervals.

The cycle threshold (Ct) values for the PCR product accumulation curve were determined for three biological replicates per developmental stage. Amplification efficiency values were determined from amplifications of serial dilutions of genomic DNA for each gene. Relative gene expression levels were calculated according to the method described by Pfaf (2001). Reverse transcriptase reaction efficiency was evaluated by amplification of a kanamycin internal control using the primers Km-1 and Km-2 (Baeriswyl 2002).

Statistical Analysis of Expression Data
Analysis of variance was performed on gene expression data using a General Linear Model approach implemented in Statistica version 10 (StatSoft, Tulsa, OK). Pairwise comparisons were made using the Tukey method at the 95% significance level. In addition, gene expression data were analyzed by Student’s t-test implemented in Statistica.

Results
Sequence Data from pre-1 and pre-2 in Neurospora
We generated 16 and 15 sequences of the pheromone receptor genes pre-1 and pre-2, respectively, from homothallic taxa. GenBank accession numbers of the sequences are given in table 1. The majority of the generated sequences contain the full protein-coding region of the genes although a few sequences are missing parts of the 5’- and/or 3’-ends of the coding sequence (CDS) (supplementary fig. S1, Supplementary Material online). Together with the publicly available sequences from heterothallic and pseudohomothallic taxa (table 1), these data allowed to build two separate alignments (pre-1 and pre-2), shown in supplementary fig. S1, Supplementary Material online. The pre-2 alignment displayed three ambiguously aligned regions, partly consisting of repetitive sequences where a single amino acid is repeated up to 12 times, 1 glycine repeat (3–12 aa), 1 threonine repeat (2–8 aa), and 1 aspartic acid repeat (2–10 aa). The pre-1 sequences from the homothallic N. kobi and N. uniporata, as well as the pre-2 sequences from the homothallic N. minuta and N. cerealis, were found to contain premature stop codons due to frame-shift mutations in the gene sequence. In pre-1 from N. kobi, two base pairs were missing in the third membrane-spanning region of the protein at codon position 118 in the pre-1 alignment causing a premature stop codon at position 137, whereas a single base pair is missing in the C-terminal cytosolic part at codon position 594 for N. uniporata pre-1 (supplementary fig. S1, Supplementary Material online). Regarding pre-2, N. cerealis and N. minuta show three and four independent frame-shift mutations, respectively (supplementary fig. S1, Supplementary Material online). In N. cerealis, the first of these mutations is located at codon position 363 in the alignment in the C-terminal cytosolic part of the protein. For N. minuta, the first missing base pair is at codon position 29 in the N-terminal extracellular part causing a premature stop codon at position 56. Additional PCR and sequencing were performed to validate the sequences with disrupted reading frames.

Genealogies of Pre-1 and Pre-2
Topologies of pre-1 and pre-2 genealogies are shown in supplementary fig. S2, Supplementary Material online. Both genealogies for the individual pre genes contained supported nodes (≥75% bootstrap support and ≥0.95 BPPs) that were in conflict with the consensus phylogeny for the genus presented by Nygren et al. (2011). In the pre-1 genealogy, three major discrepancies were found. First, N. terricola was deeply nested in the pre-1 gene tree, whereas it was part of the most basal branch in the species tree. Second, pre-1 sequences from N. uniporata and N. pannonica clustered together, which was not the case in the species tree. Third, the clade containing N. hapsidophora, N. minuta, and N. cerealis formed the most basal branch in the pre-1 gene tree, whereas this clade was deeply nested in the species tree (supplementary fig. S2, Supplementary Material online). In pre-2, the most striking incongruence between the gene genealogy and the species tree presented in Nygren et al. (2011) was the placement of the monophyletic group N. africana, N. galapagosenesis, and N. lineolata, which formed the most basal branch of the pre-2 phylogeny, whereas it formed the sister group to the heterothallic clade in the species tree. In general, the pre-2 gene tree shows low node support values (supplementary fig. S2, Supplementary Material online).

Molecular Evolution of pre-1
For pre-1, branch analyses did not support either a mating system or a switch-dependent evolution of this gene (fig. 1 and supplementary table S2, Supplementary Material online). Specifically, although the free ratios model provided a significantly better fit to the data than all the other tested models, none of the local models of mating system or switch-dependent evolution fitted the data better than the global model (fig. 1 and supplementary table S2, Supplementary Material online). Figure 1A shows the dN/ds ratios for branches under the free ratio model and under the local model of a switch-dependent evolution of pre-1. The results from the branch analyses were consistent in parallel runs, under any of two alternative assumptions: 1) using the topology from the pre-1 gene tree or 2) removing taxa with stop codons or frame-shift mutations (data not shown).
The overall dN/dS obtained from the codon site models were very similar for the heterothallic and the homothallic data set (table 2). Furthermore, models allowing positive selection (M2a and M8) provided a significantly better fit for the pre-1 data than their nested neutral models (M1 and M7), and this result was consistent for data sets containing both the heterothallic and the homothallic taxa (table 2). Thus, our data suggest that codons of pre-1 evolve under positive selection in Neurospora taxa of both mating systems. By plotting the BEB estimation of dN/dS for each codon (from model M8), we further verified that the overall evolutionary constraints of regions and domains of the pre-1 gene were similar between heterothallic and homothallic taxa (fig. 2A). In both data sets, the highest evolutionary rate is found in the C-terminal, intracellular domain of the protein. Furthermore, the sliding-window average of S scores from the RCA analysis plotted along the PRE-1 protein (fig. 2B) indicated that regions of low selective constraints as indicated by dN/dS correlated with regions of high amino acid variability in both the heterothallic and homothallic data sets. However, specific codons under positive selection in the two data sets differed between hetero- and homothallic taxa (fig. 2A). For example, in the homothallic taxa, one codon in the extracellular domain was identified by the BEB estimation to be evolving under positive selection, whereas codons found to be evolving under positive selection in heterothallic taxa were restricted to the intracellular C-terminal part of PRE-1 (fig. 2A). To investigate whether the differential distribution of positively selected codons could be inferred to be mating-system dependent or switch dependent, a detailed inspection of the amino acid composition of the BEB identified sites in the Neurospora taxa was performed. The analysis showed that the three codons identified as positively selected in heterothallic taxa indeed displayed high amino acid variation in the heterothallic taxa but were, with a few exceptions, conserved for a single amino acid in homothallic taxa (supplementary fig. S1, Supplementary Material online). Hence, these sites displayed a distinctive pattern of type 1 functional divergence (i.e., site conserved in one lineage but variable in the other lineage; Cole and Gaucher 2011). In contrast, the five codons identified as positively selected in homothallic taxa displayed a switch-dependent pattern of amino acid variation, rather than mating-system dependent. This pattern was most pronounced in codons 275E, 518Q, and 625S (fig. 2A), which were completely conserved for a single amino acid in heterothallic taxa but conserved for different amino acids in at least two different homothallic groups (supplementary fig. S1, Supplementary Material online). Hence, these sites displayed signs of type 2 functional divergence (i.e., site conserved in one lineage and conserved in the other lineage but for a different residue; Cole and Gaucher 2011).

**Molecular Evolution of pre-2**

For pre-2, branch analyses supported a mating-system-dependent evolution of the gene. The free-ratio model and the two local models assuming a mating-system-dependent evolution or a switch-dependent evolution, respectively, all fitted the data significantly better than the global model (fig. 1 and supplementary table S2, Supplementary Material online). However, the model of a switch-dependent evolution did not fit the data significantly better than the mating-system-dependent model, and the free-ratio model did not provide a better fit than any of the local models (fig. 1 and supplementary table S2, Supplementary Material online). Thus, the model of a mating-system-dependent evolution was the simplest model providing a significantly good fit for the data. When performing the parallel branch analyses excluding taxa with frame-shift mutations or using the topology from the gene tree under study, the results did not change, with one exception: the local model of a mating-system-dependent evolution did not provide a significantly better fit for the data than the global model when gene trees were used as input phylogeny in the analyses. Because of our uncertainty about the true evolutionary history of the pre-2 gene in heterothallic Neurospora (Strandberg et al. 2010), we should thereby interpret the result on mating-system-dependent evolution in this gene with precaution.

Table 2 shows the summary statistics for codon site analysis of pre-2 for the heterothallic and homothallic data sets and a combined data set including all taxa. Codons of pre-2 evolved under an overall higher selective constraint than codons of pre-1: the overall dN/dS was approximately 3-fold smaller in pre-2 than in pre-1 for all data sets (fig. 1 and supplementary table 2, Supplementary Material online) and a higher proportion of sites in pre-2 were found in classes indicating strong negative selection, that is, dN/dS < 1 (table 2). For the heterothallic data set, none of the models allowing codons under positive selection (M2a or M8) provided a significantly better fit to the data than its nested neutral models (M1 or M7) (table 2), and thus the null-hypothesis of neutral evolution could not be rejected. For the homothallic data set, we found weak support for positive selection. The model allowing positive selection (M8) provided a significantly better fit to the data than its nested neutral model (M7), although the site class allowing a dN/dS higher than one was 1.14 (table 2), which was not strongly suggestive of positive selection. Using the BEB calculation of posterior probabilities for site classes, we identified two sites potentially evolving under positive selection (probability of >95%) in pre-2 in the homothallic data set (fig. 3A).

Codon site analyses did not show a strikingly different pattern of dN/dS between the heterothallic and homothallic data sets across the pre-2 gene (fig. 3A), and this was also supported by the S scores plotted along the protein (fig. 3B). However, a comparison between three different subgroups of homothallic taxa indicated a difference in amino acid conservation pattern between the N. africana, N. hapsidophora, and N. retispora clades (fig. 3C). Although PRE-2 amino acid distribution in the N. africana and N. hapsidophora clades were similar to each other, they were different from the N. retispora clade, which showed the most resemblance to heterothallic taxa. The differences were found not only in both the...
Table 2. Summary Statistics and Parameter Estimates of Analyses of dN/dS among Sites of pre-1 and pre-2.

<table>
<thead>
<tr>
<th>Codon Substitution Models</th>
<th>In λ</th>
<th>p*</th>
<th>No.</th>
<th>Estimated Parameters</th>
<th>dN/dS (ω0)</th>
<th>dN/dS (ω1)</th>
<th>dN/dS (ω2)</th>
<th>Overall dN/dS</th>
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<td>pre-1</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>M1a -5,955.30</td>
<td>-5</td>
<td>&lt;0.001</td>
<td>21</td>
<td>ω0: 0.081, p0: 0.712</td>
<td>ω1: 1.00, p1: 0.288</td>
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<tr>
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<td>&lt;0.001</td>
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<td>ω1: 1.00, p1: 0.260</td>
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<td>ω1: 1.371, p1: 0.175</td>
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<tr>
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<td></td>
<td>21</td>
<td>p: 0.094</td>
<td>q: 0.169</td>
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<tr>
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<td>ω: 0.222, q: 0.462</td>
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<td>&lt;0.001</td>
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<td>ω: 1.164, q: 3.598</td>
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<td>M1a -10,567.27</td>
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<td>&lt;0.001</td>
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<td>ω1: 1.00, p1: 0.252</td>
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<td>&lt;0.001</td>
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<td>ω1: 1.00, p1: 0.224</td>
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<td>55</td>
<td>ω0: 0.060, p0: 0.514</td>
<td>ω1: 0.553, p1: 0.446</td>
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<td>q: 0.630</td>
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<td>ω: 0.524, q: 1.216</td>
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<tr>
<td>M1a -6,447.84</td>
<td>-6</td>
<td></td>
<td>19</td>
<td>ω0: 0.032, p0: 0.917</td>
<td>ω1: 1.00, p1: 0.083</td>
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<tr>
<td>M2a -6,447.84</td>
<td>-6</td>
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<td>21</td>
<td>ω0: 0.032, p0: 0.917</td>
<td>ω1: 1.00, p1: 0.000</td>
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<td>M3 -6,447.75</td>
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<td></td>
<td>22</td>
<td>ω0: 0.030, p0: 0.704</td>
<td>ω1: 0.030, p1: 0.838</td>
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<td>M7 -6,450.47</td>
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<td>19</td>
<td>p: 0.087</td>
<td>q: 0.707</td>
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<tr>
<td>M8 -6,447.85</td>
<td>-6</td>
<td>NS (0.073)</td>
<td>21</td>
<td>p0: 0.918</td>
<td>ω: 3.365, q: 9.99</td>
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<tr>
<td>M1a -6,754.10</td>
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<td></td>
<td>30</td>
<td>ω0: 0.040, p0: 0.938</td>
<td>ω1: 1.00, p1: 0.062</td>
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<td>NS (0.354)</td>
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<td>ω0: 0.041, p0: 0.938</td>
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<td>M3 -6,733.55</td>
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<td>33</td>
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<td>ω1: 0.194, p1: 0.197</td>
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<td>M7 -6,747.92</td>
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<td></td>
<td>30</td>
<td>p: 0.175</td>
<td>q: 1.678</td>
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<tr>
<td>M8 -6,733.69</td>
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<td>&lt;0.001</td>
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<td>p0: 0.966</td>
<td>ω: 0.324, q: 5.514</td>
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<tr>
<td>M1a -9,836.09</td>
<td>-9</td>
<td></td>
<td>48</td>
<td>ω0: 0.044, p0: 0.920</td>
<td>ω1: 1.00, p1: 0.080</td>
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<tr>
<td>M2a -9,836.09</td>
<td>-9</td>
<td></td>
<td>50</td>
<td>ω0: 0.044, p0: 0.920</td>
<td>ω1: 1.00, p1: 0.080</td>
<td>0.120</td>
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<td>M3 -9,775.52</td>
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<td></td>
<td>51</td>
<td>ω0: 0.022, p0: 0.817</td>
<td>ω1: 0.310, p1: 0.169</td>
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<td>48</td>
<td>p: 0.216</td>
<td>q: 1.984</td>
<td>0.094</td>
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<tr>
<td>M8 -9,777.09</td>
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<td>&lt;0.001</td>
<td>50</td>
<td>p0: 0.986</td>
<td>ω: 0.261, q: 3.040</td>
<td>0.325</td>
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</tbody>
</table>

*pResult from likelihood ratio test (LRT) between models M2a and M8 and their nested neutral models M1a or M7, respectively. NS, nonsignificant; NA, nonapplicable.

N-terminal extracellular part and the C-terminal cytosolic part but also in the transmembrane domains (fig. 3c).

Regulation of Pheromone Receptor Genes in Homothallic Neurospora

We investigated gene expression regulation of the pre and mat genes in three different conditions: vegetative growth, early reproductive, and late reproductive stages. Gene expression data for pre-1 and pre-2 is presented in figure 4. For five of the six studied species, at least one of the two pre genes was differentially expressed between the tested tissues. In N. cerealis, both pre-1 and pre-2 were significantly induced in reproductive tissues, when compared with vegetative tissue. In N. uniporata and N. sublineolata, only pre-1 was significantly induced in reproductive tissues. In N. pannonica, the opposite pattern was observed, as only pre-2 was induced in late reproductive tissue compared with both vegetative and early reproductive tissue. For N. terricola, both pre-1 and pre-2 displayed unique expression patterns with downregulation in the early reproductive tissue. In N. africana, no significant differences in expression levels of either pre-1 or pre-2 were detected between the developmental stages. For this taxon, the late reproductive measurement was excluded for all tested genes due to problems with culture homeostasis.
Regulation of Mating-Type Genes in Homothallic Neurospora

The *mat* genes encode transcription factors that regulate the gene expression of both *pre-1* and *pre-2* in heterothallic Neurospora (Kim et al. 2012). In the homothallic taxa of the genus, the *mat* genes are found to be under genetic decay (Wik et al. 2008). It is thus highly interesting to investigate the expression patterns of *mat* genes in the homothallic taxa, in particular in relation to the expression patterns of the *pre* genes. Gene expression data for the *mat* genes (*mat a-1*, *mat A-1*, *mat A-2*, and *mat A-3*) are shown in figure 5. In five of the six tested taxa, *a*, *A*, or both *A* and *a* genes showed differential regulation between vegetative and reproductive tissues. With the exception of *mat a-1* in *N. sublineolata*, we observed a general trend of induction in the expression of *mat* genes in sexual tissues compared with vegetative growth. In *N. africana*, both *mat A-2* and *mat A-3* were significantly induced in the early reproductive tissue compared with the vegetative. In *N. cerealis*, *mat A-1* was induced in the early reproductive tissue, whereas *mat a-1* was induced in the late reproductive tissue when compared with the vegetative tissue. For *N. uniorpora*, the only regulated gene is *mat a-1* that was induced in the early reproductive tissue compared with the vegetative. In *N. sublineolata*, we observed a unique expression pattern, where both *mat A-1* and *mat A-2* were significantly induced in both reproductive tissues compared with the vegetative, whereas in contrast, the *mat a-1* gene was repressed in both reproductive tissues compared with the vegetative tissue. In *N. terricola*, both *mat A-1* and *mat a-1* were induced in both reproductive tissues. In *N. pannonica*, none of the *mat* genes appear to be differentially expressed in the tested tissues.

Discussion

In this study, we use a phylogenetic framework to study the effect of mating system on the molecular evolution of pheromone receptor (*pre*) genes in the model system *Neurospora*. Both our analyses of dN/dS and amino acids (RCA) suggest a functional conservation of *pre-1* or *pre-2* following a change to a homothallic life style. This result is in sharp contrast to the low selective constraints of the *mat* genes of the homothallic *Neurospora* taxa that was inferred by Wik et al. (2008) from a high proportion of neutrally evolving codons. Because mate recognition and attraction is expected to be unnecessary for the homothallic species of *Neurospora* (Perkins 1987), our data suggest that both PRE-1 and PRE-2 are important for sexual development beyond mate search in these species; a suggestion in accordance with a general conclusion from functional studies from several species of filamentous ascomycetes. For example, the recent study by Kim et al. (2012) indicates that both receptors and pheromones function in postfertilization events in *N. crassa*. Similarly, in the homothallic *S. macrospora*, deletions of the pheromone receptor genes *pre-1* and *pre-2* abolish both fruiting body development and ascospore formation (Mayrhofer and Pöggeler 2005; Mayrhofer et al. 2006), and in the homothallic *Aspergillus nidulans*, the deletion of both pheromone receptor genes *preA* and *preB* abolishes fruiting body and ascospore formation.
**Fig. 3.** Molecular evolution of codons and domains of *pre-2.* (A) The Bayes Empirical Bayes (BEB) estimation of $dN/dS$ over codons. The red, blue, and dotted lines indicate the $dN/dS$ over codons in the three data sets, including the heterothallic, homothallic, and the all sequences, respectively. Asterisks indicate a probability of $>95\%$ that the codon belong to a class with $dN/dS > 1$ (indictive of positive selection), with site number as in the amino acid alignment. (B) A sliding-window average ($n = 7$) of normalized $S$ scores ($W$ means; mean = 0, standard deviation = 1) obtained from the reverse conservation analyses (RCA) of heterothallic and homothallic taxa. (C) A sliding-window average ($n = 7$) of normalized $S$ scores ($W$ means; mean = 0, standard deviation = 1) obtained from RCA of three homothallic clades; the *N. retispora*, *N. africana*, and *N. hapsidophora* clades. (D) Extracellular, transmembrane (TM) spanning, and intracellular domains of *pre-1* are indicated by white, dark gray, and light gray horizontal bars, respectively.
to have frame-shift mutations causing premature stop codons: N. kobi and N. uniporata have disrupted open reading frames in pre-1 and N. minuta and N. cerealis in pre-2. This may indicate a loss of function for pre genes in these particular taxa. In N. kobi, the frame-shift mutation in pre-1 severely shortens the predicted peptide, whereas for N. uniporata, the premature stop codon lies in the C-terminal cytosolic tail, leaving most of the pre-1 gene intact (supplementary fig. S1, Supplementary Material online). In heterothallic N. crassa, a shorter splice version of pre-1 is present (Pöggeler and Kück 2001; Kim and Borkovich 2004), and N. uniporata might maintain the function of this variant. In support of this hypothesis, our transcriptional analysis of pre-1 in N. uniporata shows that the gene is both expressed and regulated (fig. 4). It is also noteworthy that both branches delineating N. kobi and N. uniporata pre-1 did not show signs of relaxed selective constraints (dN/dS ratio closer to 1) when compared with the other branches (fig. 1a). In pre-2, the number and distribution of frame-shift mutations in N. minuta suggest that the gene is no longer functional (supplementary fig. S1, Supplementary Material online), but there is no expression data to support or refute this suggestion. For N. cerealis, only the C-terminal cytosolic part of PRE-2 is affected by the frame shift (supplementary fig. S1, Supplementary Material online), and for this species, we found pre-2 to be expressed and induced in reproductive tissue compared with vegetative, indicating that the gene could still be functional (fig. 4). Neurospora minuta and N. cerealis are sister taxa in the phylogeny (fig. 1), which may indicate a reduced need for a fully functional pre-2 already in their most recent common ancestor. However, after adjusting the sequences of both taxa to be in frame with the other taxa, we do not detect a higher dN/dS or new stop codons in these branches. We interpret this as an indication of a recent and sudden relaxation of selective constraints for the pre-2 gene in these two taxa, but this indication needs to be confirmed experimentally.

For PRE-1, both dN/dS and RCA analyses show that most variation is found in the C-terminal, cytosolic tail, which is also the region in which all but one of the positively selected codons is found (fig. 2). The intracellular parts of fungal pheromone receptors physically interact with a heterotrimeric G-protein complex that mediates regulatory signals to downstream targets in the expression cascade (Casselton 1997). Mutational studies in N. crassa show that different G-protein complexes are involved to transduce the pheromone signal and influence the mating process and perithecial development (Kays et al. 2000; Kays and Borkovich 2004; Kim and Borkovich 2004). In Saccharomyces cerevisiae, the third cytoplasmic loop of Ste3p (orthologous to PRE-1) is involved in G-protein complex activation, whereas the C-terminal region is a target for a kinase that regulates receptor signaling and function (Dohlman and Thurner 2001). Phosphorylation of the C-terminal region of S. cerevisiae Ste3p regulates receptor turnover, but the C-terminal region is also a target for several proteins that regulate receptor signaling and function (Feng and Davis 2000). The fact that no positively selected codons of the C-terminal, cytoplasmic part of PRE-1 are found in common between heterothallic and homothallic development, whereas single-gene deletions of either receptor result in smaller cleistothecia and lower numbers of ascospores (Seo et al. 2004). However, cases are found in which pheromone receptors seem to be dispensable for sexual development beyond fertilization, for example, in the homothallic Gibberella zeae (Kim et al. 2008).

Although molecular evolution analyses suggest that the pre genes are under high functional constraints in homothallic taxa of Neurospora, individual homothallic taxa are found
taxa (fig. 2), and the distribution of amino acid variation in those positions, may indicate functional divergence of this region between heterothallic and homothallic taxa of *Neurospora*. Additional clades of heterothallic taxa have to be included to reveal whether this divergence is due to changes related to mating system, but one may hypothesize that structural and functional modifications of the C-terminus of PRE-1 influence signal transduction during sexual development. Accordingly, deletion of this region in *S. cerevisiae* Ste3p pheromone receptor results in a 20-fold increase in pheromone sensitivity (Boone et al. 1993), and a single amino acid substitution in *Coprinellus disseminates* CDSTE3.1 is suggested to result in self-compatibility (James et al. 2006).

In addition, one of the positively selected sites in homothallic taxa is found in an extracellular loop region, which may influence pheromone-binding properties of PRE-1 in the homothallic taxa. Additional experiments with the homothallic taxa proteins would be needed to confirm the hypothesis that the observed differences in *pre-1*, compared with heterothallic taxa, would influence either their pheromone binding or their intracellular signaling properties.

Although positively selected sites were found only in the homothallic data set for *pre-2*, the variation in dN/dS or amino acid variation across PRE-2 does not provide support for a general functional divergence associated with mating.

**Fig. 5.** Relative expression levels of mating-type genes (*mat A-1*, *mat A-2*, *mat A-3*, and *mat a-1*) during different developmental stages of six homothallic isolates of *Neurospora*. Transcription levels are determined with quantitative PCR and normalized against *act* expression according to the method described by Pfaffl (2001). Expression level is shown relative to the level in vegetative tissue (=1). Abbreviations on the x-axis represent the different developmental stages (V, vegetative tissue; ER, early reproductive tissue; LR, Late reproductive tissue). Different letters (a and b) indicate statistically significant differences (*P* < 0.05) between treatments for a single gene using the Tukey test. Error bars represent the standard deviations of three independent biological replicates.

In the three taxa where only one pheromone receptor gene is expressed, regulatory regions, and ubiquitination signals (orthologous to PRE-2) are functionally important and contain phosphorylation sites, regulatory regions, and ubiquitination signals (Dohlman and Thorner 2001).

In the homothallic taxon S. macrospora, a gene deletion study suggested that pheromones can be bypassed for sexual development, in contrast to the pheromone receptor genes (Mayrhofer et al. 2006), and a recent survey on the evolution of pheromone genes in Ascomycetes has provided evidence for both positive diversifying selection and relaxed selective constraints for the pheromone genes (Martin et al. 2011). When aligning the two pheromone genes mfa-1 and ccg-4 obtained from draft genome assemblies of N. africana, N. sublineolata, N. pannonica, and N. terricola (Gioti A, Stajich Ej, Johannesson H, unpublished data) with the publicly available sequences of N. crassa and S. macrospora, we found that mfa-1 is completely conserved among all these taxa and that ccg-4 is highly conserved. For ccg-4, the pheromone peptides that are predicted to be produced after proteolytic cleavage of the precursor protein are conserved, but the number of repeats differs between taxa (supplementary fig. 3, Supplementary Material online). This result implies that the changes we see in the pre genes are not driven by coevolution with the predicted ligands.

Transcriptional patterns of pheromone receptor genes (pre-1 and pre-2) and mating type genes (mat a-1, mat A-1, mat A-2, and mat A-3) in six homothallic taxa do not support a universal pattern of regulation of these genes in reproductive tissue development of homothallic Neurospora taxa. In taxa where pre and mat genes are regulated, inductions are, with a few exceptions, found in reproductive tissue as compared with the vegetative tissue (figs. 4 and 5), similarly to what was recently shown in the homothallic Aspergillus nidulans (Czaja et al. 2011). However, the detected expression patterns of these genes appear to differ between taxa representing separate switches from heterothallism to homothallism. For example, in N. cerealis, both the expression of mat genes and pheromone receptors are regulated (figs. 4 and 5). In the three taxa where only one pheromone receptor gene is significantly regulated (N. pannonica, N. sublineolata, and N. unipora), we found no consistent correlation between the regulation of this pre gene and the expression of the mat gene expected to regulate this receptor. This is in contrast to heterothallic taxa where pre gene expression is predominantly mating-type specific (Kim and Borkovich 2004, 2006; Karlsson et al. 2008; Kim et al. 2012). Furthermore, in N. africana, pre-2 may have a form of regulation that is different from N. crassa where the expression of this gene is primarily regulated by the mat a locus (Kim et al. 2012), which is absent in N. africana (Glass et al. 1990; Gioti et al. 2012). In N. sublineolata, only the mat A mating type and pheromone receptor 1 pair seems to be responsible for sexual development; mat A-1, mat A-2, and pre-1 are induced in reproductive tissue, whereas mat a-1 is repressed. Compared with all other species of Neurospora where the mat organization in the genome have been investigated, N. sublineolata distinguishes itself by having the mat A locus relocalized on linkage group VI, whereas the mat a locus resides in the original mat locus on linkage group I (Gioti et al. 2012). Thus, our data indicate that the new mat A locus has taken over the control of the sexual reproduction in this species and that the original mat (a) locus is repressed. In conclusion, our expression study of the mat and pre genes in homothallic Neurospora taxa do not support a general mat gene dependent regulation of the pre genes, as has been found in the heterothallic N. crassa (Kim and Borkovich 2004, 2006; Karlsson et al. 2008; Kim et al. 2012).

Increasing lines of evidence suggest that mat A is important for homothallism in Neurospora. First, only mat A gene components reside in the homothallic N. africana, N. dodgei, N. galapagosensis, and N. lineolata genomes. Second, the only gene in the study by Wik et al. (2008) that does not have a disrupted reading frame is mat A-1 (Wik et al. 2008). And third, although the mat A-1 gene was not found consistently regulated in our expression studies, at least one of the three mat A components was; the only exception was N. pannonica, where none of the mat genes were found to be regulated.

In all herein studied homothallic taxa, at least one of the pre genes is functionally conserved. This finding is in line with previous results for the heterothallic N. crassa, where at least one functional pheromone–receptor pair seems necessary for postfertilization development (Kim et al. 2012). Future studies should aim at a deeper investigation of where and when these genes are expressed during sexual development in homothallic taxa, as well as how the nucleotide changes associated with homothallism affect the protein function to further understand the roles of the mating type genes and the pheromonal signal system in sexual development.

Supplementary Material
Supplementary figures S1–S3 and tables S1 and S2 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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