The Evolution of the Pheromonal Signal System and Its Potential Role for Reproductive Isolation in Heterothallic Neurospora

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Comparative sequencing studies among a wide range of taxonomic groups, including fungi, provide the overall pattern that reproductive genes evolve more rapidly than other genes, and this divergence is believed to be important in the establishment of reproductive barriers between species. In this study, we investigated the molecular evolution of the pheromone receptor genes pre-1 and pre-2 of strains belonging to 12 and 13 heterothallic taxa, respectively, of the model genus Neurospora. Furthermore, we examined the regulatory pattern of both pheromone precursor and receptor genes during sexual crosses of Neurospora crassa and Neurospora intermedia, for which reinforcement of interspecific reproductive barriers in sympatry previously has been documented. We conclude that the part encoding the C-terminal intracellular domain of pre-1 and pre-2 genes evolves rapidly. Both stochastic and directional processes drive this divergence; both genes contain neutrally evolving codons, and in addition, pre-1 contains codons evolving under positive selection, whereas in pre-2 we found highly variable regions with numerous repeats encoding glycine, threonine, or aspartic acid. In addition, we found regulatory changes of the pheromone and receptor genes during crosses between N. crassa and N. intermedia with different reproductive success. Gene expression levels are higher in the interspecific sympatric crosses with low reproductive success than in their intraspecific and/or allopatric equivalents, both at the stage of initial communication and contact and later at postfertilization stages. Taken together, our data indicate that pheromones and receptors are important key players during reproductive isolation between Neurospora species, and this study provides a general framework for future studies on the role of reproductive proteins for reproductive isolation.

Introduction

Understanding the evolutionary dynamics of traits that contribute to reproductive success among species is vital to a deeper knowledge about how biological diversity is generated and has been a central focus of evolutionary biologists since Darwin (1859). Comparative sequencing studies among a wide range of taxonomic groups, including fungi, provide the overall pattern that reproductive genes evolve more rapidly than other genes (Brown and Casselton 2001; Pöggeler and Kuck 2001; Clark et al. 2006), and this divergence is believed to be important in the establishment of reproductive barriers between species. It is not clear what causes this rapid divergence, but several models have been proposed (e.g., Clark et al. 2006). One of the proposed driving forces for the positive selection often seen at gamete recognition loci is reinforcement of reproductive isolation between sympatric species, in response to the production of less fit hybrids (Dobzhansky 1937). Empirical data have provided numerous examples of the occurrence of reinforcement in natural populations (Capretti et al. 1990; Karlsson and Stenlid 1991; Noor 1995; Saetre et al. 1997; Rundle and Schluter 1998; Dettman, Jacobson, Turner, et al. 2003; Nosil et al. 2003). For example, the reproductive success between the 2 species of the filamentous ascomycete Neurospora, Neurospora crassa and Neurospora intermedia, is lower in sympatry than in allopatry (Dettman, Jacobson, Turner, et al. 2003).

Pheromones and their receptors are used in mate choice and discrimination in a wide range of organisms. With regard to reproduction, the specificity of pheromonal signals is of decisive importance and any change in the pheromonal signal can have dramatic consequences on species reproduction and formation; if the change in the signal is matched with the change in its reception, this variation could be the origin of a new species (e.g., Nosil et al. 2007). Molecular data demonstrate that the mating-type (mat) locus in heterothallic (outcrossing) Neurospora encodes transcription factors that initiate a regulatory cascade including the expression of mating-type–specific pheromones and receptors, which further mediates signal transduction to induce expression of mating genes. During the initial phase of mating in Neurospora, the trichogynes (female receptive hyphae) grow toward and fuse with male cells of the opposite mating type. Following fusion, a male nucleus migrates into a specialized cell in the protoperithecium (the female reproductive structure) in which nuclei of opposite mating types undergo several synchronous mitotic divisions and migrate in pairs to the developing asci. Here they undergo karyogamy and subsequent meiosis (Shiu and Glass 2000). True interspecific hybrids of Neurospora have not been found in nature, despite the fact that species have broadly overlapping ranges (Dettman, Jacobson, Turner, et al. 2003) and laboratory crossing experiments have revealed that reproductive isolation in this group of organism is prezygotic; crosses yielding barren perithecia due to genetic incompatibilities arrest between fertilization and just before, or at, karyogamy (Raju and Perkins 1978).

Two pheromone precursor genes, mfa-1 and cgg-4 (Bobrowicz et al. 2002), and 2 pheromone receptor genes, pre-1 and pre-2 (Pöggeler and Kuck 2001; Kim and Borkovich 2004), have been identified and characterized in N. crassa. All 4 genes are present in strains of both mating types, however, mat A strains express the cgg-4 pheromone and the pre-1 receptor, whereas mat a strains express the mfa-1 pheromone and the pre-2 receptor. MFA-1 and CCG-4 are the predicted ligands for PRE-1 and PRE-2, respectively. The pheromones have been shown to be essential for male fertility and sufficient to direct chemotropic, polarized growth of trichogynes during mating in N. crassa (Kim and Borkovich 2006). In addition,
accumulating evidence suggests that pheromone/pheromone receptor interactions in fungi function during later stages of the sexual cycle. These additional postfertilization events include induction of meiosis in Schizosaccharomyces pombe (Chikashige et al. 1997), nuclear migration and clamp cell fusion in basidiomycetes (Casselton and Olesnicky 1998), dikaryotic filamentous growth (Spellig et al. 1994), and internuclear recognition (Debuchy 1999), although postfertilization functions of pheromones and their receptors have not yet been thoroughly investigated in Neurospora.

Amino acid sequences of the pheromones MFA-1 and CCG-4 are completely conserved between N. crassa and the related Sordaria macrospora (Bobrowicz et al. 2002), indicating that differences in binding properties between pheromones and their receptors in early communication between individuals are not the manner by which hybridization is prevented between Neurospora species. However, comparatively low levels of between species nucleotide identity have been observed for the pheromone receptor genes in Neurospora and consequently they have been suggested to evolve at a faster rate than genes coding for metabolic enzymes (Pöggeler and Kuck 2001).

Here, we present a study of the molecular evolution of the pheromone receptor genes, pre-1 and pre-2, in heterothallic Neurospora. By using likelihood methods, we confirm that both genes evolve rapidly and that the region encoding the C-terminal intracellular domain of both PRE-1 and PRE-2 constitutes the rapidly evolving domain. Although the majority of the codons are conserved in both genes, pre-1 contains codons evolving under positive selection whereas around one-third of the codons evolve neutrally. In pre-2, we found 3 highly variable regions encoding numerous repeats of glycine, threonine, or aspartic acid, and a smaller proportion (7–8%) of neutrally evolving codons. In order to further investigate the role of pheromonal signals in reproductive success between Neurospora species, we studied the regulatory pattern of both pheromone precursor and receptor genes during sexual crosses of N. crassa and N. intermedia. Regulatory differences of the pheromone and receptor genes in connection to differences in reproductive success are detected; we found a higher expression of the genes in the nonsuccessful cross than in the corresponding successful cross where the male or female strain was kept constant.

Materials and Methods

Strains and Culture Conditions

Thirty-four strains, belonging to 13 taxa of heterothallic Neurospora, were used in this study, and their taxon affiliation and geographic origin are listed in table 1. These strains have previously been characterized for relatedness based on phylogenetic analyses of anonymous nuclear loci (Dettman, Jacobson, and Taylor 2003, 2006). All strains were obtained from the Fungal Genetics Stock Center (FGSC; Department of Microbiology, University of Kansas Medical Center, Kansas City, KS). Strains for polymerase chain reaction (PCR) and sequencing were cultivated in test tubes with Vogel’s minimal medium with 1.5% sucrose (Vogel 1956). To confirm intron splice patterns, N. crassa strain FGSC 8900 were grown for 16 days on solid synthetic crossing medium (SCM; Westergaard and Mitchell 1947) at 25 °C for subsequent sequencing of pre-1 cDNA. Strains used in expression studies were grown in 90-mm petri dishes on solid SCM with 2% sucrose.

Experimental Setup for Sexual Crosses

For expression analyses of crosses, we used the strain combinations of N. crassa and N. intermedia, all of which previously have been scored for reproductive success (Dettman, Jacobson, Turner, et al. 2003; table 2). For each cross, strains used as females were inoculated onto the Petri dishes and incubated at 25 °C for 10 days to induce production of protoperithecia. Subsequently, crosses were carried out as follows: 7 strokes, 6 cm long and 1 cm apart, were made in the mycelia in each dish using a sterile platinum thread, and a total of 30,000 conidia (30 μl of a 1 × 109 conidia/ml water solution) of the opposite mating type were added in each stroke. After incubating the cross in ambient light and temperature, fungal tissue within an area of a 5-mm distance from the strokes was harvested by scraping fungal tissue from the agar with a scalpel. Thus, the harvested material consisted of both male and female tissue. Harvesting was done at different time points during the mating process (0, 4, 10, 20, 30, and 45 h after application), and tissue was immediately frozen on dry ice. Five biological replicates were included for each cross. Controls were inoculated with water.

Nucleic Acid Manipulations

Frozen mycelia were ground, and nucleic acids were extracted with 3% hexadecyltrimethylammonium bromide and phenol/chloroform. Precipitation of nucleic acids was done with 3 M NaAc, pH 5.2, and 95% ethanol, whereas subsequent precipitation of RNA was done with 8 M LiCl. Nucleic acid concentrations were determined spectrophotometrically. DNase I treatment was performed on RNA according to the recommendations of the manufacturer (Fermentas, Burlington, Canada). Oligo(dT)-primed reverse transcription of 1,000 ng total RNA was performed in a total volume of 30 μl using moloney murine leukemia virus reverse transcriptase according to the manufacturer’s recommendations (Invitrogen, Carlsbad, CA). Kanamycin positive control synthetic mRNA (Promega, Madison, WI) was included in reverse transcription as internal control. The cDNA was diluted (30 → 200 μl) before use in quantitative PCR.

PCR and Sequencing of Genomic DNA

Published sequences for pre-1 and pre-2 of N. crassa and S. macrospora, as well as the N. crassa genome sequence (http://www.broad.mit.edu), were used as templates when designing primers for the amplification and sequencing of the genes. Primers were designed manually, and all primer sequences and GenBank accession numbers of template sequences are given in supplementary table 1 (Supplementary Material online). All PCRs were performed with
ThermoWhite Taq DNA polymerase following the manufacturer’s recommendations (Saveen Werner, Limhamn, Sweden), with a final concentration of 1 mM MgCl₂. All sequencing was performed by Macrogen Inc., Seoul, Korea (http://www.macrogen.com), using the same primers as used for amplification and additional internal primers. Sequence alignments were performed in BioEdit (Hall 1999) using nucleotide and amino acid sequences.

**Codon-Based Likelihood Analyses**

We excluded introns, and gaps present in 3 or more of the sequences, from further analyses, as well as 4 highly variable regions of pre-2 (see alignment in supplementary figure 1 [Supplementary Material online] for excluded codons). Three of these highly variable regions consist of single amino acid repeat where glycine, threonine, or aspartic acids are repeated up to 13 times.

**Table 1**

<table>
<thead>
<tr>
<th>Strain b</th>
<th>Geographic Origin</th>
<th>pre-1</th>
<th>pre-2</th>
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<tbody>
<tr>
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<td>AM749886</td>
<td>AM904734</td>
</tr>
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<td>AM749878</td>
<td>AM904735</td>
</tr>
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<td>AM749888</td>
<td>AM904736</td>
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<tr>
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<td>Louisiana</td>
<td>AM749891</td>
<td>AM904737</td>
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<td>AM749892</td>
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</tr>
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<td>AM904740</td>
</tr>
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<td>N. crassa Shear and Dodge (NcC) 8863</td>
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<td>AM749893</td>
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**Table 2**

<table>
<thead>
<tr>
<th>Strains and Crosses Used for Gene Expression Experiments of Both Pheromone Precursor and Receptor Genes</th>
<th>mat a Strain</th>
<th>mat A Strain</th>
<th>Cross</th>
</tr>
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<tr>
<td>Taxon b</td>
<td>FGSC Number</td>
<td>Taxon b</td>
<td>FGSC Number</td>
</tr>
<tr>
<td>N. crassa Shear and Dodge (NcA) 8848</td>
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<td>8903</td>
<td>Intraspecific/sympatric</td>
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<tr>
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<td>N. crassa A</td>
<td>8786</td>
<td>Interspecific/sympatric</td>
</tr>
<tr>
<td>N. crassa Shear and Dodge (NcB) 8830</td>
<td>N. crassa A</td>
<td>8786</td>
<td>Interspecific/allopatric</td>
</tr>
</tbody>
</table>

**Note.**—Reciprocal crosses were carried out, in which either the mat a or the mat A strain was used as the female parent.

* For reference, see Dettman, Jacobson, and Taylor (2003).

* Classification of interaction and mating success as in Dettman, Jacobson, Turner, et al. (2003). High-mating success means formation of perithecia and >15% of black ascospores; low-mating success means formation of no, or barren, perithecia.
To identify the evolutionary processes underlying the high divergence of the pheromone receptor genes, we used likelihood-based tests in the CODEML program of the PAML package, version 3.14 (Yang 1997; Nielsen and Yang 1998; Yang and Nielsen 1998; Yang et al. 2000). Models of variable ratios of nonsynonymous (d_\text{K}) to synonymous substitutions (d_\text{S}), \omega, among codons were used to test for the presence of codons under different evolutionary constraints and to identify them. We used 6 models outlined by Nielsen and Yang and implemented in the PAML package. The one-ratio model (Nssites 0) assumes one \omega for all codons, whereas the discrete model (Nssites 3) uses a general discrete distribution with 3 codon classes estimated from the data. The nearly neutral model (Nssites 1a) assumes 2 classes of codons in the protein, with \omega < 1 and \omega = 1. The positive-selection model (Nssites 2a) adds a third class of codons to the nearly neutral model, in which \omega > 1. The beta model (Nssites 7) uses a \beta distribution of \omega over codons: \beta(p,q), where, depending on the parameters p and q, can take various shapes in the interval (0,1). The beta&\omega model (Nssites 8) adds an extra class of codons, with \omega > 1, to the beta model. For each model, equilibrium codon frequencies were estimated from the average nucleotide frequencies at each codon position, amino acid distances were assumed to be equal, and the transition/transversion ratio (\kappa) was estimated from the data. For all other parameters, we used the default settings provided by Yang et al. (2000).

To verify which of the models best fits the data, likelihood ratio tests (LRTs) were performed by comparing twice the differences in log-likelihood values (−2 lnΔ) between 2 models using a \chi^2 distribution, with the number of degrees of freedom equal to the difference in the number of parameters between the models. First, the one-ratio model (Nssites 0) was compared with the discrete model (Nssites 3) to test for a variation of \omega among codons within each gene. To test whether the pheromone receptor genes evolve under positive selection, the 2 models including a class of codons with positively selected sites (i.e., \omega > 1; models Nssites 2a and Nssites 8) were compared with their corresponding neutral models (Nssites 1a and Nssites 7, respectively).

Finally, we identified particular codons in the genes that were likely to have evolved under positive selection by using the Bayes empirical Bayes (BEB) calculation of posterior probabilities for codon classes implemented in the positive-selection and beta&\omega models.

Real-Time Quantitative PCR

Primer pairs were used to be designed in real-time quantitative PCR were designed for mfa-1, ccg-4, pre-1, and pre-2 at regions conserved between N. crassa and N. intermedia, by using PrimerSelect (DNAStar, Madison, WI). Expression of actin (act) was used as an endogenous control to normalize the expression of the target genes (Lee and Ebbole 1998) and as an internal control of contaminating genomic DNA. Primer pairs for act were designed as above, and the amplicon was made spanning an intron, thus producing amplification products of different sizes from genomic DNA or cDNA, respectively. Primer sequences and GenBank accession numbers of template sequences are given in supplementary table 1 (Supplementary Material online).

The quantitative PCRs (25 μl) consisted of 5 μl diluted cDNA solution, gene-specific primers (80 nM final concentrations), fluorescent dye SYBR Green, and other components according to the SYBR Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA) protocol and performed in an Amp 7000 Sequence Detection System (Applied Biosystems). The thermal profile was: 1 cycle 50 °C, 2 min, 1 cycle 95 °C, 10 min, 40 cycles; 95 °C, 15 s and 60 °C, 1 min. Melting curve analysis was performed after the PCR to confirm that the signal was the result of a single product amplification and not due to primer dimers or arbitrary amplification. Cycle threshold (Ct) values for the PCR product accumulation curve were determined for a minimum of 3 biological replicates based on 2 technical replicates. Amplification of a kanamycin internal control was achieved with primers Km-1 and Km-2 (Baeriswyl 2002) and was used as a control of the efficiencies of the reverse transcriptase reactions. Ct values and amplification efficiency values, determined from amplifications of serial dilutions of genomic DNA or cDNA, were used to calculate relative expression levels according to the method described by Pfaffl (2001).

Statistical Analysis of Expression Data

Gene expression data from growth on solid SCM media were analyzed by Student’s t-test. Temporal expression data were analyzed using a General Linear Model (Minitab 14.1) to test for effects of time, strain combination in crosses, and the combination of time and strains on gene expression. Pairwise comparisons were made between time and strain combinations using the Tukey–Kramer method at the 95% significance level.

Results

Splicing Pattern of pre-1

Amplification of pre-1 cDNA from N. crassa strain 8900 grown for 16 days on SCM medium revealed that 2 introns had been spliced, in contrast to only 1 intron previously reported for pre-1 in N. crassa (Pöggeler and Kuck 2001; Kim and Borkovich 2004). The second intron reported here is identical to an intron previously predicted by automated annotation in N. crassa (Kim and Borkovich 2004) and homologous to an intron found in S. macrospora (Mayrhofer et al. 2006). Splicing of the second intron results in an open reading frame (ORF) with an alternative stop codon and consequently in a protein 323 amino acids longer than the originally described pre-1 ORF in N. crassa. We excluded the possibility of analyzing more than one splice variant of pre-1 in the sexual crosses performed in this study, by verifying that the expression levels found when using an oligonucleotide pair specifically amplifying mRNA where both introns had been spliced (Pre1Exon3ftr) did not differ from expression levels found when using an oligonucleotide pair that would amplify both these possible splice variants (Pre1Ffr) (data not shown).
Two of the 3 sequenced strains of PS2 had an alignment in supplementary fig. 1, Supplementary Material online). We found a rather substantial sequence variation within species (table 3). In the taxa where at least 3 strains were sequenced, we found an average of 3.9 nt substitutions per 1 kb within taxa in *pre-1* and of these 2.5 were nonsynonymous. After excluding the ambiguously aligned region in *pre-2*, we found an average of 31.8 nt substitutions per 1 kb where 8.3 were nonsynonymous.

Sequence Variability of *pre-1* and *pre-2*

The entire coding regions of the pheromone receptor genes, *pre-1* and *pre-2*, were sequenced from 13 and 12 *Neurospora* taxa, respectively (table 1). Alignments of DNA and translated amino acid sequences of both genes are available in supplementary figure 1 (Supplementary Material online). We found a rather substantial sequence variation within species (table 3). In the taxa where at least 3 strains were sequenced, we found an average of 3.9 nt substitutions per 1 kb within taxa in *pre-1* and of these 2.5 were nonsynonymous. After excluding the ambiguously aligned region in *pre-2*, we found an average of 31.8 nt substitutions per 1 kb where 8.3 were nonsynonymous (table 3).

For both genes, most of the variation, both within and between taxa, was found in the region encoding the intracellular C-terminus, whereas the regions encoding membrane-spanning domains showed less variation (see alignment in supplementary fig. 1, Supplementary Material online). Two of the 3 sequenced strains of PS2 had a mutation in the 738th codon, changing the arginine codon into a stop codon, which resulted in a protein 6 amino acids shorter than in all other strains. In *pre-2*, we found 3 highly variable parts each containing a repeat region where a single amino acid is repeated up to 13 times; 1 glycine repeat (8–13 aa), 1 threonine repeat (2–8 aa), and 1 aspartic acid repeat (3–7 aa). The number of repeats was not always fixed within taxa.

Molecular Evolution of *pre-1* and *pre-2*

Log-likelihood values and parameter estimates of both *pre-1* and *pre-2* under models of variable *ω* among codons are listed in table 4. Using the one-ratio model (NSsites 0), the averaged value of nonsynonymous (*dS*) to synonymous substitutions (*dS*), *ω*, for *pre-1* was 0.36, whereas the corresponding value for the nonrepetitive part of *pre-2* was 0.09. Among heterothallic *Neurospora*, *ω* for the housekeeping genes act, *tef-1* (encoding translation elongation factor-1), and *ccg-7* (encoding clock-controlled gene-7) were 0.35 and 0.39, respectively.

### Table 3

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Number of Strains</th>
<th><em>S</em></th>
<th><em>N</em></th>
<th>Number of Strains</th>
<th><em>S</em></th>
<th><em>N</em></th>
</tr>
</thead>
<tbody>
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<td><em>Neurospora crassa</em> (NcA)</td>
<td>3</td>
<td>1.8</td>
<td>2.3</td>
<td>3</td>
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**Note:**—n/a, not applicable.

### Table 4

<table>
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<tr>
<th>Gene</th>
<th>Model&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ln&lt;sup&gt;d&lt;/sup&gt;</th>
<th>2Δln&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Parameter Estimates&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>71.37**</td>
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<td><em>ω</em>: 1.08, <em>p</em>: 0.24</td>
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<td>−4825.81</td>
<td></td>
<td><em>ω</em>: 0.04, <em>p</em>: 0.92</td>
<td><em>ω</em>: 1.00, <em>p</em>: 0.08</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>−4828.15</td>
<td></td>
<td><em>p</em>: 0.14</td>
<td><em>q</em>: 1.13</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>−4825.90</td>
<td>4.50</td>
<td><em>p</em>: 0.93</td>
<td><em>p</em>: 4.76, <em>q</em>: 99.00</td>
</tr>
</tbody>
</table>

<sup>a</sup> 0: one-ratio model; 3: discrete model; 1a: nearly neutral model; 2a: positive-selection model; 7: beta model; 8: beta& model. See the text for a description of the models.

<sup>b</sup> Significance level indicated when LRTs revealed that the model fits the data significantly better than the corresponding model, that is, 0 versus 3, 1a versus 2a, 7 versus 8 (*P < 0.05, **P < 0.01, ***P < 0.001).

<sup>c</sup> *p*: is the proportion of the sites found in a class with *ω*.

<sup>d</sup> Average ratio of nonsynonymous to synonymous substitutions in the gene under the given model.
are considerably lower than for pre-1; 0.00, 0.02, and 0.10, respectively (see parameter estimates for these 3 housekeeping genes in supplementary table 2 [Supplementary Material online]), and none of these contain any highly variable repetitive domains as are found for pre-2. For both pre-1 and pre-2, the discrete model (Nsites 3) fitted the data sets significantly better than the one-ratio model (Nsites 0) (P < 0.001), thus there is a variation of ω among codons in both pheromone receptor genes. The values of parameters under the discrete model for the 2 genes indicate that the majority of the codons in pre-1 (75%) and pre-2 (92%) are under purifying selection (i.e., found in a class with ω = 0), whereas a smaller proportion, 24% in pre-1 and 9% in pre-2, evolves under relaxed selective constraints (i.e., ω > 1). One percent of the codons of pre-1 was found in a class with ω of 5.56, indicative of positive selection, and both positive-selection models (Nsites 2a and 8) provided a better fit to the data than their corresponding neutral models (P < 0.01, table 4). For pre-2, no site class with ω > 1 was found, indicating that the part of pre-2 included in the analysis does not contain codons under positive selection. When analyzing the parameter estimates for the 3 housekeeping genes (supplementary table 2, Supplementary Material online), none of the codons were found in a class with ω > 1 (indicating relaxed selective constraints). All codons of act and tef-1 were found to be conserved. In only cgg-7, we found a variation among sites; in addition to the conserved sites, 4.4% of the codons were found in a class with ω of 1.61 in this gene.

The BEB posterior probabilities that codons of pre-1 and pre-2 belong to one of the estimated classes of ω obtained from the positive-selection model (Nsites 2a) are shown in figure 1. Two codons in pre-1 with a probability higher than 0.95 to be found in the class with ω > 1 in the betaω model (Nsites 8) were identified (codon no. 391, P = 0.968, and no. 401, P = 0.988), whereas, under model Nsites 2a, only 1 of these 2 codons was found to be significantly under positive selection (codon no. 401, P = 0.959). Nineteen of the codons were found with a posterior probability greater than 0.5 to belong to a class with ω > 1 (5.21 or 4.34 for model 2a or 8, respectively). No difference was seen in selective pressure between the regions coding for extracellular, membrane-spanning, and intracellular domains of the proteins. However, the region encoding the C-terminal intracellular domain of both PRE-1 and PRE-2 constitutes the rapidly evolving domain; it contains the majority of the codons evolving under relaxed selective constraints of both genes, as well as 17 of the 19 presumably positively selected sites of pre-1 and the highly variable repetitive parts of pre-2 (fig. 1).

Mating Type and Strain-Specific Expression of Pheromone Precursor and Receptor Genes

Expression of the internal normalizer gene act, shown over time and by crosses in supplementary figure 2 (Supplementary Material online), was stable during premating conditions and during the first 20 h of sexual crosses, as well as between crosses. After 20 h, we found a small increase in the expression of act. However, as the expression difference that we use for our conclusions are most pronounced between 4 and 20 h of interaction, it is in our opinion that act expression is sufficient for internal normalization in this study.

When measuring the premating expression of mfa-1, cgg-4, pre-1, and pre-2 in mycelia of the 4 strains used in the expression analysis, we found mating-type and strain-specific expression differences (table 5). After growth of the mycelia for 10 days on SCM media, all 4 genes were regulated differently between strains with different mating type (P ≤ 0.03), pheromone precursor genes more pronounced than pheromone receptor genes. As expected, mat a strains produced higher levels of mfa-1 and pre-2 as compared with mat A strains, whereas the latter produced higher levels of cgg-4 and pre-1. Furthermore, the expression levels of mfa-1, cgg-4, and pre-1 were all higher in the mat A strain of N. crassa A than of N. intermedia A, with the difference for mfa-1 and pre-1 being statistically significant (P ≤ 0.004). In mat a strains, the expression of cgg-4 and pre-2 of N. crassa C was higher than in N. crassa A (P < 0.001).

Transcription Profiles of Pheromone Precursor and Receptor Genes during Sexual Crosses

Transcription profiles of mfa-1, cgg-4, pre-1, and pre-2 during sexual crosses involving N. crassa and N. intermedia strains are shown in figure 2. As outlined above, all these represent successful crosses except the interspecific sympatric cross that is of low reproductive success.

Measurements of gene expression represented the combined expression from both interacting partners of the cross. Under the assumption of mating-type–specific expression of these genes, the general results were as expected. For the pheromone precursor genes, there was a large increase of cgg-4 and mfa-1 expression when mat A and mat a conidia, respectively, were added to female tissues (fig. 2A and F). The difference between 0 and 4 h in these cases should be interpreted as with or without the presence of pheromone-producing conidia. The expression of the opposite pheromone during the same conditions (mfa-1 in mat a, female × mat A, male and cgg-4 in mat A, female × mat a, male) was not changed dramatically during sexual development but was stable at a high expression level that originates from the female tissue (fig. 2E and B).

The pheromone receptor genes, pre-1 and pre-2, showed small regulatory changes during sexual reproductive development. The regulatory response of the receptive pheromone receptor in females was noteworthy as pre-1 was downregulated in mat A females after the initial stage of trichogyne chemotaxis, whereas pre-2 in mat a females was upregulated during the same conditions (fig. 2D and G).

On a more detailed level, there was a trend of higher expression levels in interspecific (N. crassa × N. intermedia) or sympatric crosses as compared with intraspecific (N. crassa × N. crassa), or allopatric, equivalents. Isolate 8786 (N. intermedia, mat A) displayed higher levels of mfa-1 when crossed with the sympatric N. crassa isolate 8848 than with the allopatric N. crassa isolate 8863 (P = 0.001, fig. 2F). For cgg-4, there were higher expression levels in the interspecific, nonsuccessful cross.
FIG. 1.—Probabilities that codons of pre-1 and pre-2 belong to site classes with different selective pressures. Vertical bar height represents the BEB probabilities for each site to belong to 1 of the classes obtained by running model 2a in PAML3.15d. For pre-1, 3 classes were found (o of 5.21 [black], 1.00 [gray], and 0.10 [white bars]) and for pre-2, 2 classes (o of 1.00 [gray] and 0.04 [white bars]) (see table 4). For pre-1, asterisks indicate a probability of >95% that the site belongs to a class with \( \beta \) in the positive-selection and/or \( \beta \) models implemented in codeml package, with site number as in amino acid alignment in supplementary figure 1 (Supplementary Material online). Extracellular, membrane-spanning, and intracellular domains are indicated by white, black, and gray horizontal bars, respectively. The positions and sequence of the 3 repetitive, highly variable parts of pre-2 in the included taxa are indicated below the panel. The relationship of the taxa, as proposed by Dettman, Jacobson, and Taylor (2003, 2006), is indicated in the phylogenetic tree.
Table 5
Premating Expression* of Pheromone Precursor and Receptor Genes

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Isolate</th>
<th>Mating Type</th>
<th>mfa-1</th>
<th>pre-2</th>
<th>ccg-4</th>
<th>pre-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurospora crassa A</td>
<td>8848</td>
<td>a</td>
<td>958 ± 146</td>
<td>1.1 ± 0.30</td>
<td>1.1 ± 0.19</td>
<td>1.1 ± 0.20</td>
</tr>
<tr>
<td>N. crassa C</td>
<td>8863</td>
<td>a</td>
<td>1132 ± 293</td>
<td>5.9 ± 0.38</td>
<td>8.2 ± 0.29</td>
<td>1.2 ± 0.21</td>
</tr>
<tr>
<td>Neurospora intermedia A</td>
<td>8786</td>
<td>A</td>
<td>1.0 ± 0.18</td>
<td>Not detectable</td>
<td>2079 ± 786</td>
<td>14.5 ± 2.26</td>
</tr>
<tr>
<td>N. crassa A</td>
<td>8903</td>
<td>A</td>
<td>3.4 ± 0.33</td>
<td>Not detectable</td>
<td>3972 ± 483</td>
<td>35.7 ± 4.82</td>
</tr>
</tbody>
</table>

* Expression levels were normalized by act expression according to Pfaffl (2001), and each gene presented in relation to the isolate with lowest detectable expression (=1×), 8786 for mfa-1, 8848 for pre-2, ccg-4, and pre-1. The standard errors from 4 independent measurements are shown.

Discussion

The selective forces acting on reproductive proteins, and the functional consequences of reproductive protein evolution, are just beginning to be understood. Here we demonstrate that the pheromone receptor genes pre-1 and pre-2 evolve rapidly among heterothallic Neurospora, a result in agreement with Pöggeler and Kuck (2001) who reported low levels of between species nucleotide identity for the 2 pheromone receptors. The extracellular domains of PRE-1 and PRE-2 are relatively conserved, and the part encoding the C-terminal intracellular domain constitutes the rapidly evolving domain. In this region, both genes contain neutrally evolving codons, and in addition, pre-1 contains codons evolving under positive selection whereas in pre-2, we found highly variable regions with numerous repeats encoding glycine, threonine, or aspartic acid. These intracellular C-terminal parts of PRE-1 and PRE-2 are coupled to a heterotrimERIC G-protein complex consisting of 3 subunits, α (GNA1, GNA2, or GNA3), β (GNB1), and γ (GNG1) that transmit the signal to activate a mitogen-activated protein kinase signal transduction pathway. The 3 different α subunits are all involved in the mating process, but noticeably, in different ways (Kays et al. 2000; Kays and Borkovich 2004; Kim and Borkovich 2004). We hypothesize that minor changes in the cytoplasmic part of pheromone receptors in heterothallic Neurospora in this study have effect on gene function, as have been found for other fungal systems; for example, the substitution of glutamine for leucine in this part of the Saccharomyces cerevisiae receptor STE3 leads to a partially constitutive and hypersensitive receptor (Boone et al. 1993) and, in Coprinus disseminatus, a single amino acid substitution in the cytoplasmic part of the pheromone receptor CDSTE3.1 is suggested to render self-compatibility (James et al. 2006).

In addition to early communication between fungal strains, the PRE-1 receptor is suggested to be involved in regulation of pheromone gene expression and postfertilization events through a pheromone-independent signaling activity as the loss of pre-1 greatly reduces the expression levels of mfa-1 and ccg-4 (Kim and Borkovich 2004). If the evolutionary forces acting on the cytoplasmic part of PRE-1 have led to an adaptation of the regulatory patterns (spatial, temporal, or in absolute amounts) of the pheromone precursor genes in heterothallic Neurospora species, then this can potentially be involved in preventing hybridization between species.

Interestingly, we found regulatory changes of the pheromone and receptor genes during crosses between Neurospora crassa and Neurospora intermedia with different reproductive success. Gene expression levels are higher in the interspecific, successful cross with Neurospora intermedia (8786) than in the intraspecific, successful cross with Neurospora crassa (8903) (P = 0.001, fig. 2A).

The same pattern was evident for the pheromone receptor genes. Higher expression levels for both pre-1 and pre-2 were observed in interspecific, unsuccessful crosses where isolate 8848 (Neurospora crassa, mat a) was crossed with Neurospora intermedia (8786) than in the intraspecific, successful cross with Neurospora crassa (8903) (P ≤ 0.003, fig. 2C and G). In addition, isolate 8863 (Neurospora crassa, mat a) displayed higher levels of pre-1 when crossed with Neurospora intermedia (8786) than with Neurospora crassa (8903) (P = 0.002, fig. 2C).

Acknowledgements

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FIG. 2.—Temporal expression levels of pheromone precursor and receptor genes during reciprocal sexual crosses in *Neurospora*. Transcript levels are determined with quantitative PCR and normalized against *act* expression according to the method by Pfaffl (2001). Expression levels for each gene are displayed in relation to the expression on SCM agar (0 h = 1) for strain FGSC 8848 (*Neurospora crassa* A), and the other samples are expressed as the fold change over that sample. The boxed strain combination represents the only cross with low reproductive success; the interspecific cross between FGSC 8848 (*N. crassa* A) and FGSC 8786 (*Neurospora intermedia* A) of sympatric origin. Units on the x axis represent time of interaction in hours. Error bars represent the upper part of standard error of 5 independent samples. Note that *pre-2* transcripts are not detected in crosses where *mat a* acts as male in the current setup.
preferred subcellular sites or mRNA degradation. Similar, long 3′-UTRs are found in other fungal pheromone genes including MF1-1 in Magnaporthe grisea (Shen et al. 1999) and mf2-1 in C. parasitica (Zhang et al. 1998), again highlighting the importance of precise pheromone regulation and localization.

The exact role of the pheromone/pheromone receptor pairs during later stages in sexual development in Neurospora is not known, but we can speculate about a role as “conglutinin” that adheres fungal hyphae together during the formation of the perithecium (Kim et al. 2002). The distortion of pheromone/pheromone receptor gene expression in interspecific sympatric crosses that we observe may in the conglutinin case cause abnormal adhesion of hyphae that ultimately may result in an inability to complete the physical and morphological development of mature perithecia. Intracellular recognition is hypothesized to be a key to development of the ascogenous hyphae from the ascogonium and to depend upon pheromone/pheromone receptor interaction inside the ascogonium (Debuchy 1999). In light of this theory, our data can be interpreted as premature recognition between the N. crassa and N. intermedia nuclei in the ascogonium due to larger pheromone gradients as indicated by the expression data. Recognition between distantly located nuclei can potentially induce formation of ascogenous hyphae and result in a distorted ratio between parental nuclei in the ascogonium and eventually lead to an aberrant development.

In conclusion, we established that the cytoplasmic C-terminal parts of PRE-1 and PRE-2 in heterothallic Neurospora evolve rapidly and revealed that both stochastic and directional processes drive this divergence. Both genes evolve under low selective constraints, and in addition, pre-1 contains sites under positive selection. By transcriptional analyses of pheromone precursor and pheromone receptor genes during sexual crosses of N. crassa and N. intermedia, we found a higher expression in the investigated interspecific nonsuccessful sympatric crosses as compared with its successful intraspecific and/or allopatric equivalents. Taken together, our data suggest that pheromone and pheromone receptors may constitute key players during reproductive isolation between Neurospora species, and this study provides a general framework for future studies on the role of reproductive proteins for reproductive isolation.

Supplementary Material

Supplementary tables 1 and 2 and figures 1 and 2 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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Literature Cited


Boone C, Davis NG, Sprague GF. 1993. Mutations that alter the 3Rd cytoplasmic loop of the a-factor receptor lead to a constitutive and hypersensitive phenotype. Proc Natl Acad Sci USA. 90:9921–9925.


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