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A comprehensive phylogeny of *Neurospora* reveals a link between reproductive mode and molecular evolution in fungi

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ABSTRACT

The filamentous ascomycete genus *Neurospora* encompasses taxa with a wide range of reproductive modes. Sexual reproduction in this genus can be divided into three major modes; heterothallism (self-incompatibility), homothallism (self-compatibility) and pseudohomothallism (partial self-compatibility). In addition to the sexual pathway, most of the heterothallic taxa propagate with morphologically distinct, vegetative dissemination propagules (macroconidia), while this feature is undetected in the majority of the homothallic taxa. In this study, we used sequence information of seven nuclear gene loci from 43 taxa (295 of the possible 301 locus-by-taxon combinations) to create a phylogeny of *Neurospora*. The results suggest that transitions in reproductive mode have occurred at multiple times within this group of fungi. Although a homothallic ancestor would imply fewer switches in reproductive mode, we argue that the ancestor of *Neurospora* was likely heterothallic and that homothallism has evolved independently at least six times in the evolutionary history of the genus. Furthermore, the two pseudohomothallic taxa of *Neurospora* (*N. tetrasperma* and *N. tetraspora*) represent two independent origins of pseudohomothallism. Likelihood ratio tests of substitution rates among branches in the phylogeny indicate that reproductive mode is an important factor driving genome evolution in *Neurospora*. First, an increased level of non-synonymous/synonymous substitutions in branches delineating homothallic taxa was found, suggesting a reduced efficiency of purifying selection in these taxa. Furthermore, elevated nucleotide substitution rates were found in heterothallic, conidia-producing, lineages as compared to the homothallic non-conidiating lineages. The latter finding is likely due to the presence of conidia, i.e., a higher rate of mitotic divisions inducing mutations, and/or that the homothallic taxa have evolved a lower mutation rate to avoid genomic degeneration.

1. Introduction

The estimated one and a half million fungal species of the world (Hawksworth, 2001) possess a great diversity of reproductive strategies. While sexual reproduction is the most common reproductive mode among plants and animals (Bell, 1982), fungi have highly complex life cycles and reproduce via various pathways; in addition to sexual reproduction with a variable rate of outcrossing, fungi often propagate asexually with both vegetative fragmentation and morphologically distinct dissemination propagules (Heitman et al., 2007). Furthermore, the different reproductive pathways are commonly decoupled in fungi, making them effective model systems to study the evolutionary consequences of these alternative modes of reproduction independently (Xu, 2004).

An organism's reproductive mode is likely to greatly influence the evolutionary trajectory of its genome. Sexual outcrossing is widely accepted to be advantageous, as it results in a higher efficiency of selection than a reproductive mode in which an individual inherits all genetic material from a single parent (Burt, 2000), i.e., asexuality or sexual selfing. This increased selective efficiency associated with outcrossing is expected because meiotic segregation and recombination allows natural selection to act independently on different genetic loci (Barton and Charlesworth, 1998; Kondrashov, 1988, 1993; Otto and Lenormand, 2002; Peck, 1994). Species with low effective recombination rates are predicted to suffer from a decreased rate of adaptive evolution (Goddard et al., 2005; Otto and Lenormand, 2002), and a higher rate of

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accumulation of mildly deleterious mutations (Keightley and Eyre-Walker 2000; Kondrashov 1988; Lynch et al., 1993) relative to their outcrossing counterparts.

In filamentous ascomycetes, sexual reproduction can be divided into three major modes: heterothallic, homothallic and pseudohomothallic. In heterothallic species, each haploid individual can be of either one of the two mating-types, and has to find a mate of the opposite mating-type in order to go through the sexual cycle (Coppin et al., 1997). Thus, a heterothallic individual is able to outcross and/or undergo mating with a meiotic segregant of the same parental cross (i.e., to inbreed). Homothallic species contain all necessary genetic information for sexual reproduction within the haploid genome, and can pass through the sexual cycle without finding a mate (Casselton, 2002; Coppin et al., 1997). It is noteworthy that such intra-haploid mating makes selfing under homothallism a reproductive strategy equivalent to asexuality (Nauta and Hoekstra, 1992). Pseudohomothallic species are heterokaryotic for mating-type, i.e., nuclei of both mating-types coexist in the tissue throughout the life cycle, and thus they may autonomously proceed through the sexual cycle. In contrast to the true homothallic species, the pseudohomothallic species occasionally produce individuals that are homokaryotic and self-sterile, i.e., only containing nuclei of a single mating-type (Raju, 1992; Raju and Perkins, 1994).

Here we present a study on reproductive mode shifts and their genomic consequences among taxa in the filamentous ascomycete model *Neurospora*, the genus of the orange bread mold. The taxa within *Neurospora* exhibit a diverse set of sexual reproductive modes, including heterothallism, homothallism and pseudohomothallism. In *Neurospora*, most heterothallic taxa readily produce both sexual ascospores, by mating among strains of opposite mating-types, and asexual dissemination propagules, the conidia. There are two types of conidia in *Neurospora*, macro- and microconidia, which are produced by separate processes and are assumed to have different ecological roles in the life cycle; while both types of conidia are believed to function in fertilization, macroconidia are assumed to also be important for dispersal and colonization (Springer, 1993). In contrast to the heterothallic taxa, multiple observations suggest that homothallic taxa of *Neurospora* reproduce in nature exclusively by sexual selfing. First, the homothallic *Neurospora* are degenerate, the taxa that have been studied extensively lack microconidia, macroconidia and trichogynes (the female receptive hyphae) (Howe and Page, 1963; Perkins, 1987). Only two reports on conidia-formation in homothallic *Neurospora* are known to us: *N. kobi* has been reported to produce mainly imperfectly developed conidia (Arx, 1982), and *N. pseudoreticulata* has been reported to produce arbuscular, immature conidia (Cailleux, 1971). Second, although mating-type (*mat*) genes (the master regulators of sexual outcrossing; Shiu and Glass, 2000) are present in the genome of homothallics, they evolve under very low selective constraints and most studied taxa have accumulated stop-codons or frame shift mutations that disrupt the open reading frames (Wik et al., 2008). These two observations suggest that, in general, homothallic taxa neither reproduce by asexual spores nor outcross in nature. Taken together, we assume that heterothallic taxa of *Neurospora* are able to reproduce by a combination of sexual outcrossing, inbreeding and asexuality, while homothallic taxa of *Neurospora* are limited to sexual selfing, i.e., to intra-haploid mating.

Although the relationship between the heterothallic and homothallic *Neurospora* has not yet been fully resolved, previous reports indicate that independent shifts in reproductive mode have occurred in the genus (Dettman et al., 2001; Cai et al., 2006). The aim of the present study was to collect and use sequence information for multiple nuclear gene loci to establish a robust phylogeny of *Neurospora*. Using this phylogeny, our goal was to infer ancestral reproductive modes throughout the genus, and to reveal how many times and in which lineages shifts in reproductive mode have occurred. Furthermore, we used this phylogeny to test hypotheses regarding genome evolution and reproductive mode in *Neurospora*.

2. Materials and methods

2.1. Fungal isolates and growth conditions

A total of 49 taxa (one strain/taxon) were used in this study (Table 1). Of these, 43 belong to the ingroup, and include the majority of described and available *Neurospora* taxa and one newly described species, *N. minuta*. The members of *Neurospora* and its close genus *Gelasinospora* (Dowding, 1933) are considered as synonyms by fungal taxonomists (Garcia et al., 2004) and thus have been merged into *Neurospora* in this report. The outgroup consists of five *Sordaria* taxa and *Pseudoneurospora amorphoporcata* (Table 1).

Cultures obtained from FGSC (the Fungal Genetics Stock Centre, University of Missouri, MO, USA) and from CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) were cultivated in test tubes with Vogel's minimal medium with 1.5% sucrose (Vogel, 1956) or on Petri dishes with oatmeal agar (Crawford et al., 1986). Techniques used for sampling and isolation of the strains from the other culture collections (Table 1) have been described previously (Stchigel et al., 1998; Garcia et al., 2002).

2.2. Morphological characterization of Neurospora minuta

The macro- and microscopic morphological characteristics of *N. minuta* were studied on potato-carrot-agar (PCA; potato 20 g, carrot 20 g, agar 20 g, and 1000 mL of distilled water) at 15, 25, 35, 40, and 45 °C. Color notations in parenthesis follow Kornerup and Wanscher (1984). The measurements of the fungal structures were taken in cold lactophenol.

2.3. Molecular work and primer design

DNA was extracted by either of two previously described methods (Johannesson and Stenlid, 1999; Cano et al., 2002).

Partial sequences of a total of seven nuclear gene loci are used in this study; β-tubulin (*Bml*), translational elongation factor $1-\alpha$ (*tef-1*), protein kinase C (*pkc*), 28S rDNA, mitogen-activated protein kinase-2 (*mak-2*), nonidentical kinase-1 (*nik-1*) and a hypothetical protein-coding gene (NCU02332) from the *Neurospora crassa* gene list (www.broadinstitute.org). For *Bml* and *nik-1*, two regions were amplified with separate primer pairs, while for each of the other gene loci one region was amplified. Primers used for four of the gene loci were developed in previous studies; *Bml* (Bt1a, Bt1b, Bt2a, and Bt2b: Glass and Donaldson, 1995), *tef-1* and *pkc* (Johannesson et al., 2000) and 28S rDNA (LROR: Bunyard et al., 1994 and LR5: Vilgalys and Hester, 1990). In order to find additional phylogenetically informative gene loci, we searched the Pezizomycotina-specific genes identified by Kasuga et al. (2009) for candidate genes. The three gene loci *mak-2*, *nik-1* and a hypothetical protein (NCU02332) were selected by downloading and blasting genes from the list of Pezizomycotina-specific genes to other sequences deposited to GenBank (www.ncbi.nlm.nih.gov) and ultimately chosen depending on the properties of the alignment created with hits from related taxa. The hypothetical protein (NCU02332) is confirmed as constitutively expressed in heterothallic *Neurospora* in experiment using the long oligomer microarray for *N. crassa* (data not shown).

Primers were developed in regions of the alignment showing a high level of conservation between distantly related ascomycete taxa, flanking variable regions. The new primer names and