EVOLUTION OF THE CIRCADIAN CLOCK IN THE GENUS OF *NEUROSPORA*: CONSERVATION AND DIVERGENCE IN THE *NEUROSPORA DISCRETA* SPECIES

COMPLEX

by

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ABSTRACT OF THE THESIS

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Circadian rhythms are daily oscillations in biological activities that have a periodicity of approximately 24 hours. Underlying these rhythms are multiple molecular oscillators that interact to form the circadian clock of an organism. The filamentous fungi and eukaryotic model organism, *Neurospora crassa*, possesses a core circadian oscillator known as the FREQUENCY (FRQ)/WHITE-COLLAR (WC) oscillator (FWO). Recent studies have shown that homologous *Neurospora* clock genes are not universally conserved across the fungal kingdom. Here, we aimed to identify similarities and differences at the sequence, molecular, and macroscopic level of the circadian clock in divergently related species to *N. crassa*. The *Neurospora discreta* species complex contains numerous reproductive species with isolates that have been collected from all around the world, and extend the latitudinal boundaries of *Neurospora* by inhabiting regions as far North as Alaska, thus this clade provides an unique opportunity to study the evolution of the fungal clock to diverse local environments. Sequence comparison of the *Neurospora discreta* PS4b (8579) core clock homologs revealed a high degree of overall

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conservation with notable exceptions in the presence of additional WC complex consensus binding sites in *frequency*, suggesting possible differences in gene expression. Rhythms in asexual development in *Neurospora discreta sensu stricto* are overtly circadian regulated and underlying this were oscillations in FRQ abundance and phosphorylation were robust. Rhythmic conidiation in N. discreta PS4b 8579 was observed in cycling and free-running environments, however this expression was reliant upon several environmental conditions in order to be visualized. Molecular analysis of FRQ appears to reveal rhythms in abundance with decreased amplitude. An Alaskan strain, N. discreta PS4b 9981, demonstrated blue light mediated photo-responses, but was arrhythmic under free-running conditions and temperature cycling conditions. Phenotypes in asexual development of N. discreta species range from robustly clock controlled to arrhythmic. Western analysis of FRQ in *N. discreta sensu stricto* and *N.* discreta PS4b 8579, suggests that these phenotypes might reflect differences in the FWO. Based on data from the current study, we propose that these changes in clock regulation might have played a role for the *N*. *discreta* species to adapt to their local environments.

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INTRODUCTION

Circadian rhythms and clocks

Circadian rhythms are daily oscillations that occur in a diverse set of biological activities that are generated and controlled by an endogenous cellular timing mechanism known as the circadian clock. Circadian clocks serve to orient an organism with respect to time and are composed of one or more molecular oscillators (Dunlap et al., 2004). Biological clocks are considered to be an adaptive trait because it allows an organism to anticipate and coordinate the appropriate activity to prepare for the oncoming of events that occur daily at set times, such as dawn and dusk.

A principal characteristic of a circadian rhythm is that it persists in the absence of all temporal cues with a periodicity that is approximately 24 hours in length (Bell-Pedersen et al., 2005; Dunlap et al., 1999; Young and Kay, 2001). Held under these artificial conditions, in which all environmental conditions are held constant, an organism reveals its genetically determined periodicity of its circadian clock, termed the freerunning period (FRP). Importantly, the FRP is temperature compensated such that it remains relatively unchanged over a wide range of physiological relevant temperatures (Virshup and Forger, 2009). Additionally, a circadian rhythm must be able to synchronize itself relative to the cyclical external environment. In order to accomplish this, a circadian clock perceives signals that relate time information known as zeitgeibers (time givers), such as light and temperature. This process of synchronization by zeitgeibers, whereby the endogenous rhythm of organism is aligned with the external rhythm of the cyclical environment, is known as entrainment. Entrainment is the process

that aligns the phase a circadian clock with the 24-hour cycling environment. Phase is a expression that describes the instantaneous state of an oscillation, such as the peak or trough. Relative phase is a measurement of a time interval that occurs between a define portion of a cycle, such as a light to dark transition, and a particular point of an oscillation, such as the peak. A circadian rhythm is entrained if it establishes a specific and stable phase relationship with the cyclical environment, in other words the curve of the oscillation oscillates (e.g., the peak) occurs exactly the same time during the cycle on successive days. Typically, the peak of the rhythm serves as a reference point to define entrained. In a cycling environment, a rhythm with an entrained phase exhibits a period that matches the length of time of the cycle, and more importantly it will establish a specific and stable phase relationship to a cycling environment. This means that certain portions of the oscillation occur only during certain defined points of the cycle. In other words, each point in the continuum of the oscillation, such as the maximum or peak, will occur at the same time during the cycle each and every day. Light and temperature are the primary entraining agents and convey time information via input pathways to properly synchronize the phase with the external environment (Dunlap et al., 2004).

Circadian clocks of eukaryotic organisms have been shown to consist of two fundamentally distinct types of molecular oscillators, termed the genetic and a metabolic oscillator (Bass and Takahashi, 2011). The genetic oscillator consists of a complex network of interlocked transcription and translation based negative and positive feedback loops (Bass and Takahashi, 2011; Bell-Pedersen et al., 2005; Brunner and Kaldi, 2008; Lee et al., 2000). Metabolic oscillators are able to function independently of both transcription and translation, and therefore rely upon post-translational processes to track time (Bass and Takahashi, 2011; O'Neill and Reddy, 2011; O'Neill et al., 2011). Currently, the molecular components that constitute the metabolic oscillator remain unknown, however the two oscillators have been shown to communicate with one another (O'Neill and Reddy, 2011; O'Neill et al., 2011).

Neurospora crassa: A circadian model organism

Neurospora crassa has served as a model organism for research in circadian rhythms for nearly four decades (Dunlap et al., 2004). Asexual development (conidiation) in *N. crassa* is under circadian regulation and this phenotype is typically visualized in the racetube assay (Heintzen and Liu, 2007). A racetube is a long narrow tube, which allows for the fungus to be inoculated at one end and growth proceeds linearly to the other. As filamentous fungi *Neurospora* grows as an interconnected mass of hyphae or filaments and this tissue can differentiate a give rise to other structures, such as asexual spores, which are known as conidia.

In *N. crassa*, conidiation is under control of a circadian clock and this produces a rhythmic expression of conidial abundance that is an overt circadian phenotype commonly referred to as conidial banding or simply banding. Almost all circadian studies of *N. crassa* use a mutation known as *band* (*bd*), which dramatically enhances the banding phenotype. The closed system of the racetube (airflow is restricted) allows the accumulation of carbon dioxide, which is believed to be the reason that wild type strains cannot express robust rhythmic banding (Dunlap et al., 2004; Heintzen and Liu, 2007; Sohyun Park, 2004). For this reason, the utility of the *bd* allele cannot be understated in the development of *N. crassa* into a circadian model organism and interestingly this allele

was recently cloned and showed to be a dominant point mutation in the highly conserved *ras-1* gene (Belden et al., 2007)

The circadian clock of N. crassa, which consists of multiple molecular oscillators, controls rhythmic banding. The core circadian oscillator of *N. crassa*, is a genetic oscillator that contains the five core clock proteins: FREQUENCY (FRQ), FRQ-interacting RNA Helicase (FRH), WHITE-COLLAR-1 (WC-1), WHITE-COLLAR-2 (WC-2), and VIVID (VVD) (Heintzen and Liu, 2007). This genetic oscillator, known as the FRQ/WC dependent oscillator (FWO), operates through a network of interconnected positive and negative feedback loops that contribute to the robustness of circadian timing (Akman et al., 2010; Dunlap and Loros, 2004; Lee et al., 2000). Central to this circuit is an autoregulatory negative feedback loop, in which FRQ is the central negative feedback loops, which are interconnected with one another (Brunner and Kaldi, 2008; Heintzen and Liu, 2007; Lee et al., 2000).

The positive elements, WC-1 and WC-2, are GATA-type transcription factors and form the heteromeric WHITE COLLAR complex (WCC) via the PAS protein interaction domains that they contain. The WCC has an activation role and is the central player in the positive limb of the circadian oscillator (Ballario et al., 1996; Smith et al., 2010; Talora et al., 1999). In addition to being a component of the circadian clock, the WCC directly receives temporal input in the form of blue light (Cheng et al., 2003). Light information is perceived by WC-1, which is the primary blue-light photoreceptor, through a specialized PAS domain belonging to family of light-oxygen and voltage (LOV) sensing domains (He et al., 2002). Blue light is physically perceived via a FAD chromophore and

is bound to a LOV domain of WC-1 and light stimulates conformational changes in the WCC. Two forms of the WCC exist, a dark form (D-WCC) and a light form (L-WCC), and L-WCC has greater transcription factor activity. The WCC binds transiently to the promoters of hundreds of light inducible genes, a consensus-binding site (GATCGA) is present in these light regulatory elements (LREs). Amongst the first described LREs are those that exist in clock genes, such as *frequency* and *vivid* (Chen et al., 2010a; Chen et al., 2010b; Froehlich et al., 2003). LREs are vital to the role of WCC and via these elements this clock component oversees a transcriptional network that regulates ~20% of all genes (Smith et al., 2010).

FREQUENCY was identified as a central component when seven different alleles were mapped to this locus that impacted the circadian FRP, by either shorten or lengthen it (16h to 36h), causing an arrhythmic phenotype, and/or effecting temperature compensation (Baker et al., 2009). These alleles and their mutant phenotypes were shown to be the result of either single amino acid substitutions or frameshifts that resulted in a truncated protein. FRQ is a phosphoprotein that is progressively phosphorylated throughout the circadian cycle, and once hyperphosphorylated it is target for degradation. Rhythms in the abundance of FRQ and the phosphorylation profile exist and seventy-five serine and threonine residues on the FRQ protein have been unambiguously identified as phosphorylation targets (Schneider et al., 2009). Transcriptional activation of the negative elements of the clock by WCC leads to the inhibition of the WCC activity. As mentioned, activation of the WCC promotes the expression of FRQ and VVD and these negative elements feedback to the WCC, to suppress WCC activity via physical interactions, thereby these components actually inhibit their own synthesis (Hunt et al., 2010). VVD is also a blue light sensing protein and it serves to gate light induced responses by physically interacting with the WCC.

The WCC promotes the transcription of *frequency* (*frq*) via three LREs, two of these elements reside in the promoter region and one is downstream of the open-reading frame (ORF) that promotes the transcription of the antisense transcript (Smith et al., 2010). The first LRE present in the promoter region of *frq* is known as the distal LRE (dLRE) and the second LRE is referred to as the proximal LRE (pLRE). The dLRE is also known as the clock-box (C-Box) because it is rhythmically bound by the WCC in constant conditions in the dark, and without the dLRE *frq* is not rhythmically transcribed. The pLRE is bound primarily by L-WCC upon light induction (Heintzen and Liu, 2007). Importantly, all three of these LREs have the WCC consensus-binding site (GATCGA).

Transcription of frq (frq) is under complex regulation and multiple alternative transcription sites exist. In *N. crassa*, 13 different transcripts arise from the frq locus, some of which are translated into either a short or long isoform of FRQ protein (sFRQ and LFRQ, respectively) (Aronson et al., 1994; Liu et al., 2000). These different isoforms, which differ in length by 99 amino, are produced at different ratios at different temperatures and have been shown to be involved in the temperature compensation of the clock (Colot et al., 2005). Alternative splicing of the frq transcript determines the ratio of each present and there are two different translation start sites (AUGs and AUGL).

Fungal circadian evolution

Circadian rhythms are a nearly ubiquitous phenomenon amongst life on Earth, and in accordance to this statement, circadian regulated phenotypes have been documented in almost every fungal phylum, which include the phyla's of the Ascomycetes, Basidiomycetes, and Zygomycetes (Salichos and Rokas, 2010). Currently, *N. crassa* represents the only individual from this entire kingdom for which the molecular basis of the circadian clock is known (Salichos and Rokas, 2010).

Therefore, our only insights into the molecular basis of other fungal circadian clocks is restricted the current knowledge of the *N. crassa* circadian clock. On this premise, a recent study identified the distribution and origin of the five core *Neurospora* circadian clock homologues amongst 64 diverse fungal proteomes (Salichos and Rokas, 2010). By utilizing the known phylogenetic relationships amongst the surveyed fungi, Salichos et. al was not only able to identify homologs but also to deduce the evolutionary history of these proteins (Fig. 1, adapted from Salichos and Rokas, 2010).

This study revealed that the presence of all five clock proteins was restricted to three taxonomic classes within the Ascomycetes: the *Sordariomycetes*, *Leotiomycetes*, and *Dothideomycetes* (Salichos and Rokas, 2010). Therefore, other fungi must have evolved numerous mechanisms for biological timekeeping. Interestingly, not all genera within these three classes possessed single copies of all five clock homologs, which suggests that closely related species have distinct mechanisms in their circadian clockwork (Salichos and Rokas, 2010).

Neurospora: A genus as an evolutionary model circadian system

Within the heterothallic *Neurospora* species (outbreeding species) there are two major clades, which are reproductively isolated from one another (For a phylogenetic tree see Figure 3 from Dettman et al., 2006). The first major clade contains both the heterothallic species: *N. crassa, N. intermedia, N. sitophila, N. perkinsii* (PS1), *N. Hispaniola* (PS2), and *N. metzerbergii* (PS3), as well as nine pseudohomothallic

(inbreeding species capable of outbreeding) species that belong to the *N. tetrasperma* species complex (Menkis et al., 2009).

The *N. discreta* species complex comprises the entire second clade that contains eight species in total, this includes the type species *N. discreta sensu stricto* in addition to *N. discreta* phylogenetic species (PS) 4-10 (Villalta et al., 2009). Phylogenetic species are identified by genealogical concordance of multiple independent (non-linked) nuclear loci and have not yet been given formal taxonomic names (Dettman et al., 2006).

Thus far, circadian rhythms have been extensively studied in N. crassa, however few studies have included other species from this genus. A primary reason that circadian studies of Neurospora have been focused almost exclusively on N. crassa is because other species do not possess the *band* mutation, which allows the expression of rhythmic banding in racetubes. One study, which was investigating the effects of medium composition and carbon dioxide on rhythmic spore production, decided to include other species in their experiments (Dettman et al., 2003a; Dettman et al., 2003b). Sargent and Katlenborn reported the banding phenotype of *Neurospora* species that included individuals from N. crassa. N. intermedia, N. sitophila, and N. tetrasperma (Sargent and Kaltenborn, 1972). They demonstrated that a constant flow of fresh air was able to induce the banding phenotype in these wildtype strains, which was suppressed under the typical confined experimental conditions of a racetube (Sargent and Kaltenborn, 1972)). Interestingly, they report that the free-running period for one of the species, N. sitophila, was 13.5 hours, which is an example of an extreme lower limit of a circadian rhythm. This extreme FRP implies a dramatic mechanistic difference between N. sitophila and N. crassa, which has a FRP of \sim 22 hours with mutants known to shorten this to \sim 16 hours

Circadian research regarding other species of *Neurospora* is limited; therefore I focused my research on investigating the molecular and phenotypic evolution of circadian clocks amongst different *Neurospora* species. The research in this thesis was designed to compare the circadian clocks and circadian phenotypes of distantly related *Neurospora* species. The previously established phylogenetic relationship amongst *Neurospora* species was used as a natural variation guide to select divergent species for analysis (refer toSohyun Park, 2004). This previously established phylogenetic framework, in combination with the well-characterized molecular understanding of the *N. crassa* circadian clockwork, makes this genus an ideal platform to address evolutionary questions regarding circadian evolution.

Here I report my analyses of the macroscopic and molecular phenotypes of the type species, *N. discreta sensu stricto* (FGSC 8777), along with two strains within the *N. discreta Phylogenetic Species 4 subgroup B* (PS4b). The first strain from PS4b, *N. discreta* 8579, is currently being genomically sequenced, with the first draft currently available. The second individual from PS4b, *N. discreta* 9981, was isolated from Tok, Alaska that was chosen because of the extreme latitude and habitat that it was collected from(Dettman et al., 2003a, 2006; Dettman et al., 2003b).

MATERIALS & METHODS

Strains, culture conditions, and racetube experiments

Neurospora strains used in this study were: N. crassa ras-1^{band} (328-4), N. crassa frequency^{KO} (FGSC 11554) (Dettman et al., 2006), N. discreta sensu stricto (FGSC 8777), N. discreta phylogenetic species 4 subgroup B (PS4b) (FGSC 8579) and N. *discreta* PS4b (FGSC 9981) a strain collected from Tok, AK (Colot et al., 2006). Racetube experiments were performed with low-glucose racetube media (LGRT, 1X Vogel's salt, glucose, L-arginine, 1.5% agar, supplemented with 50 ng/ul of biotin, pH 5.8) in inverted race tubes for all phenotype experiments, unless otherwise noted (Dettman et al., 2006). Munich minimal medium is LGRT without glucose added and it was utilized in one set of experiments. Racetubes were inoculated with tissue grown in petri dishes with high glucose liquid medium (HGLM, 1X Vogel's salt, 2% Glucose, 0.17% L-Arginine, supplemented with 50 ng/ml biotin, pH 5.8) in the dark at 30°C overnight. A #5 cork bore was used to punch out individual inoculums from each mycelial pad to use as inoculum. The growth fronts for racetubes were marked daily (unless otherwise noted) and data were analyzed with ChronOSX 2.1 to determine period and phase. Q_{10} values Q_{10} values were calculated by the following equation: Q_{10} =(Length of time of the free-running period at 30°C in hours)/ (Length of time of the free-running period at 20°C in hours).

Bioinformatics

Sequences from *N. crassa* OR74A (NC10) FGSC 2489 were retrieved from the Broad Institute (www.broadinstitute.org). Sequences for *N. discreta* (Draft V. 1.0, FGSC 8579) and *N. tetrasperma* (Draft V. 2.0, FGSC 2508) were obtained from the Joint Genome Institute (www.jgi.org). Gene construction kit (GCK V 3.0, Textco Biosoftware Inc.) and was used to compare the genetic elements present in the *frequency* locus. Phylogenetic trees were constructed with PhylFr with the configurations present in the single click method (www.phylogeny.fr). Sequence alignments were generated with Jalview 2.6.1 and sequence statistics were generated with NCBI Blast (www.ncbi.nlm.nih.gov/BLAST/).

Western analysis

Time course samples were cultured in 125mL flasks while shaking at ~100 RPM at 25°C in 50mL of HGLM that were inoculated by the same method as racetubes. Samples were synchronized in constant light (LL, 100 mmol) for 12h prior to commencement of the time course. Cultures were transferred from LL to constant darkness (DD) every 4 hours for 48 hours. Tissue samples were frozen in liquid nitrogen at the conclusion time course. Protein extraction, western analysis, quantification were performed as described in (Schneider et al., 2009) with the following exceptions: a polyclonal FRQ antibody was used at a 1:100 dilution, the 2nd antibody was used at a 1:3000 dilution and it was an antichicken IgG-horseradish peroxidase conjugate (Sigma-Aldrich), the detection substrate was an Amersham ECL Plus Detection Reagent (GE Healthcare), and a Storm Phoshporimager (GE Healthcare) was used to detect the chemifluorescenct signal for immunodetection. Coomassie stained membranes are provided for one of the experimental replicates and in certain instances a non-specific band was used as a loading control because the coomassie stained membrane was not obtainable. For densitometric analysis of the time course in the dark, the samples were normalized only to the highest signal intensity on each immunoblot, which was set at 100%. For all immunoblots, the

control sample that was cultured in constant light (LL) produced the highest levels of FRQ.

RESULTS

Sequence comparison and phylogenetic analysis

Sequence comparisons of the core clock protein sequences (FRQ, FRH, WC-1 and -2) between *N. crassa* and *N. discreta* reveals a very high level of overall conservation (Table I). In terms of absolute conservation, blast analysis with the peptide sequences of these components revealed that the percent of identities between these four homologous clock proteins ranges from 91-96% (Table I). When accounting for positive amino acid substitutions or analogous amino acids, the range of percentage of similarity increased and narrowed to 94-96% (Table I). Phylogenetic trees constructed with the genetic sequences of the *frq* and *wc-1* open reading frames (ORFs) from *N. crassa*, *N. tetrasperma*, and *N. discreta* indicates that *N. crassa* and *N. tetrasperma* have less genetic distance between them (Figure 2B and Figure 4B).

A FRQ amino acid sequence alignment between *N. crassa* and *N. discreta* 8579 reveals conservation of crucial functional sites. Of the 75 known phosphorylation sites in *N. crassa*, two of these are not conserved in *N. discreta* (Fig. 3A). The amino acid substitutions are a threonine to alanine at position 471 (T471A) and a serine to asparagine at position 573 (S573N). Other than these two substitutions, all other crucial amino acids are conserved between *N. crassa* and *N. discreta* 8579 (Fig. 3A).

Comparison of the *frq* loci of *N*. *crassa* and *N*. *discreta* 8579 shows that both of the alternative translation sites, AUGL and AUGS, and all of the 5' and 3' alternative splicing sites (ss) are conserved between both species of *Neurospora* (Fig. 3A & B). Similarly, the *N*. *discreta* 8579 *frq* gene possesses two LREs in the promoter region (dLRE and pLRE) and one downstream of the open reading frame (aLRE). All three of

these LREs are present in the *N*. *discreta frq* loci and located in very similar positions (Figure 3A & B). Interestingly, in addition to the LREs the *N*. *discreta frq* possesses numerous GATCGA sequences that *N*. *crassa frq* does not (Fig. 3A & B).

Analysis of the WC-1 protein alignment also has a high degree of conservation too, however there is considerable sequence divergence contained in two domains the aminoterminal polyglutamine (NpolyQ) and the carboxyl-terminal polyglutamine/histidine (CpolyQH)(Fig. 4A). The PAS-C domain contains a single amino acid substitution (Met716->Leu), while the PAS-A (LOV), PAS-B, the GATA Zn-Finger, and the nuclear localization signal (NLS) domains harbor no amino acid changes (Fig. 4A). Circadian phenotypes of *N. discreta sensu stricto*

Under constant conditions, *N. discreta sensu stricto* reveals a free-running rhythm in conidiation with a periodicity of ~21h (Fig. 5). Importantly, this rhythm was robust and persisted for greater than 5 days (data not shown). Furthermore, this free-running rhythm was temperature compensated with a Q_{10} value of 0.972, which indicates the change in the rate of a reaction with a 10°C increase in temperature (Figure 6A & B).

The *N. discreta sensu stricto* rhythm was entrainable to white and blue light photocycles (Fig. 8) and also to temperature cycles (Fig. 9). The entrained rhythm in both cyclical environments was identical to *N. crassa*. Light experiments revealed that constant illumination with either blue or white light yielded an arrhythmic phenotype (Fig. 10A & B). Constant red light did not suppress rhythmic banding nor did cycling red light entrain the rhythmic banding in this species (Fig. 11A & B).

Circadian phenotypes of N. discreta PS4b strains

The more distant relative of N. crassa, N. discreta PS4b (8579), also exhibits a

circadian banding phenotype however it less robust (Fig. 6). In addition, this strain from this specie requires a prior entrainment regimen prior to release into free-running conditions for the banding phenotype to be visualized. When entrained to a light-dark (LD) photocycle of 12 h light and 12h dark (LD 12:12) prior to release into constant conditions, this strain exhibited a free-running rhythm in spore production with a period of ~21h (Fig 6, lower *N. discreta* 8579 racetube). Experiments that did not include this entrainment regimen consistently showed an absence of conidial bands (Fig. 6, *N. discreta* 8579* (without prior entrainment)).

Photocylces with white light are capable of entraining the rhythm of *N. discreta* 8579 (Fig. 8C). Constant illumination with white, blue, or red light resulted in an arrhythmic phenotype (Fig. 10 & 11). This response to red light is not observed in *N. crassa* or *N. discreta sensu stricto* and therefore is unique to this strain. Despite the ability of red light to mask rhythmic conidiation it was not able to entrain the rhythm. In a LD 12:12 photocyle with red light, the period of the rhythm was determined to be ~22 hours, indicating that it was not entrained. In contrast, LD 12:12 photocycles with white light entrained this phenotype, resulting in period of ~24 hours (Fig. 8C). Additionally, the rhythm showed a stable phase relationship within the LD cycle, in which the timing of the peak conidiation occurred just prior to lights on each day. Together, both the period and phase measurements indicate entrainment by white light. Additionally, temperature cycles of 12 hours low (23°C) and 12 hours high (23°C) produced stable entrainment in this strain, however robust conidial banding was not observed until the 2nd day (Fig. 9A & B).

The Alaskan strain included from the PS4 subgroup, N. discreta 9981, did not

produce the conidial banding under any condition tested. This strain was arrhythmic in constant conditions (Fig 6.) and in the temperature cycling experiment (Fig. 9). However, conidiation was in response to illumination by white and blue light as compared to racetubes cultured in constant darkness. (Compare Fig. 5 with Fig. 10). In contrast to *N*. *discreta* 8579, a specie from the same phylogenetic species and subgroup (PS4b), conidiation was not stimulated by constant red light in *N. discreta* 9981 (Fig. 11)

Western analysis of FREQUENCY

The molecular phenotypes of *N. discreta sensu stricto* FRQ protein expression in constant light and the overtime in constant darkness are indistinguishable to that of *N. crassa* (Figure 12A & B). In constant darkness, *N. discreta* FRQ displays robust oscillations in its phosphorylation profile and in total abundance with respect to time.

Western analysis of FRQ protein from *N. discreta* 8579 appears to exhibit differences in expression compared to *N. crassa*. In terms of abundance, *N. discreta* FRQ protein is light inducible (Fig. 13A, compare LL to DD samples) and oscillates in constant darkness but with a reduction in the amplitude of the oscillation (Fig. 13B). Loading controls for these immunoblots indicate that even loading of protein samples was not achieved. In constant illumination (LL), there are two bands present in *N. discreta* 8579 that represent the short and long isoforms of FRQ, corroborating the finding that the alternative translation sites are conserved (Fig. 2B). Protein levels of *N. discreta* 8579 FRQ and *N. crassa* differ significantly in constant light (Fig. 13A, compare the two LL samples, note that approximately equal levels of protein were loaded in these samples). The first time sample in constant darkness (Fig 13A, DD4) reveals a sharp band that is present at the upper limit of the mass range for the FRQ protein. Typically,

this would be the region where highly phosphorylated FRQ proteins would reside, however FRQ protein does not appear to be phosphorylated thoroughly phosphorylated in this immunoblot. (Compare Fig. 13A & C to the immunoblots in Fig. 12A & B).

DISCUSSION

Sequence comparisons of *N. crassa* and *N. discreta* 8579 demonstrate a very high level of conservation of the core clock components. All functional domains were present between these homologs and most domains were 100% conserved. Terminal domains within the WC-1 protein contained the two major exceptions to this statement, the NpolyQ and the CpolyQH regions. These domains contain simple sequence repeats (SSRs) and were expected to exhibit polymorphisms as these sites have been shown to be a substrate for phenotypic variation via evolutionary selection (Michael et al., 2007).

Mutations in crucial amino acids in FRQ can greatly alter the FRP of *N. crassa* and amino acids responsible for these circadian defects were conserved in *N. discreta* 8579. Only 2 out of 75 phosphorylation sites in the *N. discreta* 8579 FRQ amino acid substitutions, a threonine to alanine at position 471 (T471A) and a serine to asparagine at position 573 (S573N), neither have any known effects on circadian phenotypes (Baker et al., 2009). Other fungi that possess FRQ have shown that these amino acids are conserved. *Sordaria fimicola*, a distantly related fungus within the same class of *Neurospora*, possesses a FRQ homolog with an overall protein sequence identity of 85%. The open reading frame of *S. fimicola* FRQ is capable of rescuing the arrhythmic phenotype of a *N. crassa frq* null mutant (Merrow and Dunlap, 1994). This implies that FRQ homologs maintain their roles as the central negative component in fungal clocks that include this homolog.

Often, the regulation of a gene is the influenced by selection forces, therefore I investigated the regulatory elements of *frequency*. Alternative splicing sites present in the *frq* transcript gives rise to a large number of different transcripts. The ambient

temperature influences the choice of the splicing sites, however certain splice sites yield the majority of the transcripts whereas certain sites contribute only a small portion to the frq mRNA pool (Aronson et al., 1994; Colot et al., 2005). Prior studies have shown that this complex regulation of frq is conserved between *N. crassa* and *S. fimicoloa*, with the sites that give rise to major transcripts being conserved(Colot et al., 2005). This observation holds true for *N. discreta* and even more so because this species possesses the all the splicing sites and in the same the order as *N. crassa*.

A key difference found in the comparison of the frq loci between these two species was the number of WCC consensus binding sites. If the WCC was to bind to these sites, some of which reside within the promoter region of *N. discreta* 8579 frq, theoretically it could inhibit the transcription machinery from transcribing frq. This supposition could account for the reduced amplitude of FRQ protein oscillations in both the dark and light. If this were to be true, then one would expect that these additional WCC binding sequences would not be present in the *N. discreta sensu stricto frq* promoter, since the dynamics of its FRQ protein mirror that of *N. crassa*.

Data from the molecular and macroscopic phenotypes of *Neurospora discreta sensu stricto*, along with sequence data from *N. discreta* 8759, indicate that this species possesses a FREQUENCY/WHITE-COLLAR based circadian oscillator (FWO). This species possesses an overt circadian rhythm in conidiation, which has a free-running period that is temperature compensated, and is entrainable to both temperature and light. Western analysis of the *N. discreta sensu stricto* FRQ homolog dynamics revealed that the free-running rhythm in abundance and phosphorylation were identical to that of *N. crassa*. One significant difference was found between these two species, which was not directly relevant to the circadian clock but rather to the physiology of the organism.

Munich medium (MM) is identical to the typical racetube medium (low-glucose race tube medium, LGRT) with the one exception being that it does not have glucose. On MM, *N. crassa* presented a robust conidial banding albeit dampened in comparison to the strains grown on LGRT (Fig 12). The free-running rhythm of *N. crassa* is not only temperature compensated but is also nutritionally compensated (Heintzen and Liu, 2007; Sargent and Kaltenborn, 1972). The reason that *N. discreta sensu stricto* did not express a rhythm on MM is most likely due to the lack of overall spore production, which prevented the visualization of the rhythm.

Results from experiments with *Neurospora discreta* PS4b (8579) reveal that this organism has both similarities and differences with respect to *N. crassa* and *N. discreta sensu stricto*. Sequence comparisons of the clock genes with *N. crassa* demonstrated a high level of conservation. Based upon these results it was expected that molecular and macroscopic phenotypes would be very similar to *N. crassa*. However, results from phenotype experiments showed numerous differences in the expression of rhythmic conidiation. In contrast to *N. crassa* and *N. discreta sensu stricto*, the expression of conidial banding in this strain in free-running conditions is reliant upon several prerequisites. First, robust banding was only observed when an entrainment regimen was applied prior to release into constant conditions. Second, the expression of the rhythm was dependent on the ambient temperature with temperature changes of $\pm 5^{\circ}$ C masking the phenotype. Importantly, the lack of an identifiable rhythm in these experiments is not contributable to absence of spore production; in fact there is increase in abundance at 30°C. Another distinction that this strain demonstrated was its response to illumination

by red light. *N. crassa* possesses three red light photoreceptors PHYTOCHROME-1 (PHY-1), PHY-2, and VELVET-1 (VE-1) and molecular and macroscopic phenotypes have been reported in mutants deficient of these genes (Sargent and Kaltenborn, 1972). The observation that red light photoresponses differs implies that differences in the action of photoreceptors exist between *N. crassa* and *N. discreta* 8579. Results from the temperature entrainment experiment support the first statement that *N. discreta* 8579 requires an entrainment regimen to reveal its free-running period. In this experiment this strain displayed a delayed banding phenotype, whereby prominent conidial bands were not produced until the second day (Fig. 9B).

These phenotype results by themselves suggest that the circadian clock of N. discreta PS4b 8579 is a low amplitude free-running oscillator, meaning that the oscillator is weak. The FRQ western analysis of this species is difficult to interpret due to technical difficulties, however the available data does not contradict this statement. In addition, a key difference found in the comparison of the frq loci between these two species was the number of WCC consensus binding sites. If the WCC was to bind to these sites, some of which reside within the promoter region of N. discreta 8579 frq, the WCC could theoretically block the transcription machinery from transcribing frq. Further experimentation is required to test these hypotheses.

Temperature compensation of the *N. discreta* 8579 free-running rhythm is the only requirement of a circadian rhythm that was not demonstrated in this individual. Despite this lacking piece of evidence, it seems certain that the free-running rhythm observed in this species is the control of a circadian clock and this conclusion is based upon the following observations: (i) when conditions permit rhythmic spore production

under free-running conditions the periodicity is ~ 22 hours, which is the same as *N*. *crassa*; (ii) under cycling conditions the rhythm is entrainable to both light and temperature.

The other N. discreta PS4b species included in this study, N. discreta 9981, did not display any conidial banding under any experimental condition. This strain was isolated from Alaska, which has parts of the year that have daylight or darkness that extends to entire day for weeks. It has been shown that reindeer from high latitudes do not display rhythmicity in core clock genes of mammals (Olmedo et al., 2010). This strain was responsive to blue and white light in constant illumination experiments as indicated by increase in overall conidiation relative to constant dark and constant red light. Due to the lack of a circadian phenotype this strain was not included in certain experiments, however in retrospect this was an error in judgment. Intriguingly, temperature cycles did not influence the abundance of conidiation in comparison to the response to light, in fact the racetubes from this experiment resemble those cultured in constant darkness. Considering that abundant conidia was produced in the constant light experiments it seems that the media composition was not the causative agent behind the lack of the banding phenotype, as was the case for Munich minimal medium and N. *discreta sensu stricto*. It was shown that reindeer are arrhythmic during parts of the year where days consist entirely of constant illumination or darkness. However, during the summer and the winter equinoxes, when day and night length are equal, the reindeer exhibit rhythmicity which is driven by cyclical environment (Lu et al., 2010). Further experimentation is required to investigate the possibility that N. discreta 9981 possesses similar mechanics.

CONCLUDING STATEMENTS

In conclusion, experimental data demonstrates that *N. discreta sensu stricto* possesses a FWO of *N. crassa* that governs asexual development. This specie was indistinguishable from *N. crassa* at the molecular and macroscopic level, this means that the circadian clock and its properties were likely present in the ancestor that of gave rise to the two reproductively isolated clades that *N. crassa* and *N. discreta* belong to. Strains from the *N. discreta* PS4b group are globally distributed and represent the most genetically distant heterothallic species from *N. crassa* amongst the heterothallic *Neurospora* species (Lu et al., 2010). In addition, these PS4b strains chosen in this study have adapted to a specialized niche in their habitat (Dettman et al., 2006). In contrast to other *Neurospora* species, PS4b individuals from North America grow under the bark of burnt trees, whereas other *Neurospora* grow on the surface of burnt substrates (Jacobson et al., 2004)



Adapted from (Salichos and Rokas, 2010)

Figure 1. Evolutionary distribution of the core circadian clock proteins of *Neurospora crassa* in the fungal kingdom. This phylogram depicts the evolutionary history of the clock proteins FRQ, WC-1, WC-2, FWD-1, and FRH in the context of fungal evolution. FWD-1 and FRH are highly conserved proteins and were present in the fungal ancestor. WC-1 and -2 proteins arose after the divergence of early branching fungi with the rest fungal kingdom (Basidiomycetes and Ascomycetes). FRQ protein homologues are isolated to the classes highlighted in red triangles. These classes represent the only fungi that could possess all five clock proteins, however within these individuals species among them exist that either have single (majority), multiple (2 individuals), or no copies (2 individuals) of FRQ(Salichos and Rokas, 2010). The class that *Neurospora* belongs to is shaded in yellow.



- 0.02
- Figure 2. Sequence comparison and phylogenetic analysis of *Neurospora* FRQ. (A) FRQ protein alignment of *N. crassa* (upper) and *N. discreta* PS4b 8579 (lower). Conserved amino acids (aa) are shaded in blue, non-conserved aa are shaded in white, aa associated with circadian rhythm defects (*frq* alleles 1-7) are highlighted in red, conserved phosphorylation sites are colored in black, nonconserved sites are shaded in gold. Highlighted in light blue is the nuclear localization signal. (B) Phylogenetic tree based the genomic sequence of *frq* shows that *N. crassa* and *N. tetrasperma* have less distance between then *N. discreta*.



Figure 3. Comparison of the genetic elements present in the *frq* locus. (A) *N. crassa* and (B) *N. discreta* 8579. Sequence annotations: The open reading frames are shaded in red and indicated by the arrow, while sequences 7 kb upstream and 2kb downstream are shaded gray. Alternative splicing sites (ss) are colored and numbered (In *N. crassa* there are five 5' ss labeled red and 1-5 and three 3' ss labeled blue and 1-3. Alternative translation initiation sites are indicated by AUGs and AUGL. WCC consensus binding sites, GATCGA, are indicated in black and the reverse sites or antisense sequences are colored in green and denoted by REV for reverse. Empirically confirmed WCC light regulatory elements (LREs) in *N. crassa* and there corresponding sites in *N. discreta* 8579 are indicated in pink: the two LREs in the promoter pLRE and the C-Box (dLRE) and one binding site which promotes the expression of antisense *frq*, aLRE.





A.



0.6

Figure 4. Sequence comparison and phylogenetic analysis of *Neurospora* WHITE-COLLAR-1. (A) Protein sequence alignment between *N. crassa* (Top) and *N. discreta* 8579. Functional domains are highlighted in cyan and are appear in the following order: the NpolyQ, PAS-A (LOV), PAS-B, PAS-C, nuclear localization signal (NLS), GATA-Zn Finger, and CpolyQHt domains. Sequence divergence is present in both terminal domains and only a single amino acid substitution (Met716Leu), in the PAS-C domain, is present. (B) Phylogenetic tree constructed with the open reading frame of *white-collar-1*.



Figure 5. Phenotypic analysis of *N. discreta species* under free-running conditions. *N. discreta sensu stricto* (*ss*) exhibits a free-running rhythm in constant dark at 25°C that is less pronounced than the *N. crassa bd* strain, however the rhythm maintains robust periodicity for greater than >5 days (5 days shown). Two racetubes represent the phenotype of the sequenced strain of *N. discreta* 8579. The first racetube does not have an overt rhythm, however with a prior 48h entrainment regimen of consisting of a photocycle of 12h light and 12h dark (racetube denoted with an *). The PS4b strain from Tok, Ak (9981) did not exhibit a free-running rhythm under either condition.



В.

Temperature Compensation of the FRP





A.



Figure 7. Expression of the *N. discreta* 8579 banding rhythm is dependent on ambient temperature. Racetubes were held under constant conditions in constant darkness (DD) at three different temperatures 20°C, 25°C, and 30°C (A) Rhythmic conidiation is only permitted at 25°C in this strain. is there a rhythm present. (B) Densitometry tracings of conidial density plotted in respect with time. Each trace is an average of 5 or more replicates.







Figure 9. Temperature entrainment of the *N. discreta* circadian clock. Strains were entrained to temperature cycles of 12 hours 23°C (blue shading) and twelve hours 26°C (red shading). (A) Representative racetubes are shown and the calculated period and phases are listed. With the exception of *N. discreta* 9981, which displayed no rhythmic banding, all strains exhibited entrained periods (~24h) and phase. (B) Densitometry tracings were averaged for all strains, representing conidial density, plotted against time. Low and high temperatures are designated as mentioned above. *N. discreta sensu stricto* displays a phase delay relative to *N. crassa bd. N. discreta* 8579 did not exhibit robust bands until the 2nd day of the experiment. *N. discreta* 9981 displayed an arrhythmic phenotype with peaks occurring within both the low and high temperatures.

N. crassa (bd) N. discreta sensu stricto N. discreta (8579) N. discreta (9981)



В.

A.

N. crassa (bd) N. discreta sensu stricto N. discreta (**8579**) N. discreta (9981)



Figure 10. The effects of constant illumination of white and blue light on *N. discreta*. Light responses of *N. discreta* are similar to that of *N. crassa*. Constant illumination with either white or blue light suppresses rhythmic conidiation. Since red light is not detectable to *Neurospora* this allows the expression of the FRP.



Figure 11. Effects of constant and cycling red light on circadian phenotypes of *N. discreta*. (A) Constant illumination of red light results in a free-running rhythm in *N. crassa* and *N. discreta sensu stricto*. Constant red light causes an arrhythmic phenotype for *N. discreta* 8579. (B) Red light-dark photocycles (LD 12:12) do not entrain the rhythm in *N. crassa* or *N. discreta sensu stricto*, however the periods of dark allow the *N. discreta* 8579 to produce conidial bands. (C) Table summarizing period determinations with standard deviations in strains that were rhythmic in (A) and (B). Standard deviation could not be calculated for *N. discreta* 8579 due to technical reasons. Two different media types were used, see materials and methods.







Figure 12. Western analysis of FRQ in *N. discreta sensu stricto*. FRQ protein dynamics in (A) *N. crassa bd* and (B) *N. discreta sensu stricto*. Tissue samples were cultured under constant illumination (LL) and transferred at 4h intervals to constant darkness (DD). Replicate α -FRQ immunoblots are shown and coomassie stained membranes are shown as loading controls for one replicate. Averaged densitometric tracings representing the relative expression of FRQ in (C) *N. crassa bd* and (D) *N. discreta sensu stricto* during the time course in DD (Bold line; 3 replicates DD4-40, 2 replicates DD48). Light lines indicate the s.e.m.



Figure 13. Western analysis of FRQ in *N. discreta* 8579. (A) Immunoblots showing the expression of *N. discreta* 8579 FRQ in constant light (LL) and over the course of 48h in the dark. *N. crassa* samples are included for comparison.

Protein	Identities	Positives	Gaps
FRQ	91	94% (929/980)	2/980
FRH	96	96% (1075/1109)	8/1109
WC-1	94	95% (1067/1114)	19/1114
WC-2	95% (505/531)	96% (511/531)	6/531

Table I. Conservation of the core clock proteins between *N. crassa* and *N. discreta* 8579.

Experiment		N. crassa bd		N. discreta sensu stricto		<i>N. discreta</i> PS4b (8579)	
Light	Temp	Period	Phas	Period	Phase	Period	Phase
	(°C)		e				
DD	20	21.5 ± 0.3	n/a	21.0 ± 0.8	n/a	NR	NR
		(n=6)		(n=8)			
DD	25	23.2 ± 0.6	n/a	21.2 ± 0.6	n/a	21.4 ± 0.9	12.02 ∓10.4
		(n=7)		(n=12)		(n=12)	(n=30)
DD	20	20.0 + 0.6	1	20 () 0 0	1	ND	ND
DD	30	20.9 ± 0.6	n/a	20.6 ± 0.8	n/a	NK	NK
****		(n=6)		(n=11)	10.0 + 0.0	24.0 + 0.0	10.00
White	25	-	-	24.3 ± 0.9	19.3 ± 0.3	24.0 ± 0.9	12.02
LD				(n=5)	(n=6)	(n=30)	± 10.40
12:12							(n=30)
Blue LD	25	-	-	24.3 ± 1.2	20.0 ± 0.5	-	-
12:12				(n=7)	(n=7)		
Red LD	25	21.2 ± 0.4	n/a	22.0 ± 0.8	n/a	21.8*	n/a
12:12		(n=5)		(n=5)		(n=6)	
Red LL	25	22.2 ± 0.4	n/a	21.6 ± 0.1	n/a	NR	n/a
		(n=6)		(n=5)			
White	25	NR	n/a	NR	n/a	NR	n/a
LL							
Blue LL	25	NR	n/a	NR	n/a	NR	n/a
DD	23 to 25	23.8 ± 0.3		23.4 ± 0.3		24.5 ± 1.0	
		(n=5)		(n=6)		(n=6)	

Table II. Summary of period and phase determinations. No rhythm (NR), not applicable (n/a), a dash indicates the experiment was not performed.

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