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Running Title: Variation in Fungal Clocks

Natural Variation of the Circadian Clock in *Neurospora*

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ABSTRACT

Most living organisms on earth experience daily and expected changes from the rotation of the earth. For an organism, the ability to predict and prepare for incoming stresses or resources is a very important skill for survival. This cellular process of measuring daily time of the day is collectively called the circadian clock. Because of its fundamental role in survival in nature, there is a great interest in studying the natural variation of the circadian clock. However, characterizing the genetic and molecular mechanisms underlying natural variation of circadian clocks remains a challenging task. In this chapter, we will summarize the progress in studying natural variation of the circadian clock in the successful eukaryotic model *Neurospora*, which led to discovering many

design principles of the molecular mechanisms of the eukaryotic circadian clock. Despite the success of the system in revealing the molecular mechanisms of the circadian clock, *Neurospora* has not been utilized to extensively study natural variation. We will review the challenges that hindered the natural variation studies in *Neurospora*, and how they were overcome. We will also review the advantages of *Neurospora* for natural variation studies. Since *Neurospora* is the model fungal species for circadian study, it represents over 5 million species of fungi on earth. These fungi play important roles in ecosystems on earth, and as such *Neurospora* could serve as an important model for understanding the ecological role of natural variation in fungal circadian clocks.

Key Words: *Neurospora*, circadian clocks, natural variation, ecotypes, adaptation.

1. INTRODUCTION

Natural variation shapes the diversity of life. In nature, variation is selected by ambient environments, such as altitude, depth, seasons, latitude, and longitude, based on their impacts on fitness (Gaston, 2000). Thus, understanding the underlying principles of these variations and their fitness effect is one of the fundamental quests in biology. One main characteristic of all ambient environments to which most living organisms have adapted is daily cycles. Hot daytime follows cold nighttime, and bright daytime follows dark nighttime. Predicting the incoming environmental change is a crucial trait for an organism to escape or prepare for incoming danger, or to take full advantage of a favorable condition. Anticipating to the environmental change is only possible if one has a cellular device capable of measuring time: a biological clock. When the cellular clock

runs with a period of about one day, it is called a circadian clock. Scientists tried to understand clock variation under controlled conditions using different circadian models other than *Neurospora*. In the 1980's, Pittendrigh and Takamura showed how latitudinal changes impact circadian parameters (phase, period and amplitude) using *Drosophila* (Pittendrigh, Kyner, & Takamura, 1991; Pittendrigh & Takamura, 1989). Later, several studies showed that naturally occurring polymorphs in the *Drosophila* period gene (*dper*) impact phenotypic variation in locomotion and temperature compensation (Low, Chen, Yildirim, & Edery, 2012; Low, Lim, Ko, & Edery, 2008; Rosato & Kyriacou, 2011; Sawyer et al., 1997). Additionally, the geographical variation of eclosion rhythms and adult diapause was reported (Lankinen & Forsman, 2006). A recent study has also reported latitude specific sleep variation in the *Drosophila* natural population (Svetec, Zhao, Saelao, Chiu, & Begun, 2015). Latitude-specific circadian variation has also been observed in other eukaryotes like plants, birds, and mammals. In *Arabidopsis*, natural allelic variations are associated with several circadian phenotypes like hypocotyl growth (Coluccio, Sanchez, Kasulin, Yanovsky, & Botto, 2011; Raschke et al., 2015), flowering time (Lempe et al., 2005; Press, Lanctot, & Queitsch, 2016), periodic leaf movements (Michael et al., 2003; Swarup et al., 1999) and temperature compensation (Edwards, Lynn, Gyula, Nagy, & Millar, 2005). These same allelic variations are also associated with the phenotypes for breeding time (Hau, 2001) and diurnal and seasonal variation of hormone secretion (Dawson, King, Bentley, & Ball, 2001; Reierth, Van't Hof, & Stokkan, 1999) in birds, and seasonal variation of melatonin circadian rhythms (Adamsson, Laike, & Morita, 2016) and latitudinal and seasonal specific qualitative and quantitative variations of sleep (Friborg, Bjorvatn, Amponsah, & Pallesen, 2012) in

humans. Based on the population genetic studies, it has also been reported that human clock gene variants are associated with time of day preferences (Allebrandt & Roenneberg, 2008). Genome analysis of human populations at different latitudes revealed a correlation between different day-length (photoperiod) variations of clock genes and certain psychiatric disorders (Forni et al., 2014). Thus, research into natural variation of circadian clocks is no longer limited to model organisms.

In this chapter, we reviewed *Neurospora* as a potential model system for understanding the molecular mechanisms of clock variation and its consequences. Although *Neurospora* is a successful circadian model in eukaryotes (Dunlap & Loros, 2017). the molecular mechanisms of natural variation have not been fully explored. We hope this review will guide researchers to understand the importance of *Neurospora* in investigating the ecological importance of circadian clocks in fungi.

2. *NEUROSPORA* IN NATURE

2.1. The Genus of *Neurospora*

Red bread mold was first reported by Léveillé in France in the 1840s. This fungal species was later incorporated into the genus *Monilia* as *Monilia sitophila*. By the early 20th century, this fungus had been identified across Europe and the USA. Experimental reports showed that this fungus could grow in artificial cultures. In the late 1920s, based on the morphological (conidia and perithecia) similarities, some of the *Monilia sitophila* group species were transferred into the new genus *Neurospora* and named as *N. sitophila, N. crassa*, *N. tetrasperma* and *N. erythraea* (Shear & Dodge, 1927). Over the next few decades, several fungal species across the globe were studied and classified

under the genus *Neurospora*. In the 1970s, Perkins and his colleagues collected over 4,500 *Neurospora* isolates from nature, analyzed their physiological characteristics, and classified them into groups: heterothallic (*N. crassa, N. discrete, N. intermedia and N. sitophila*), pseudohomothallic (*N. tetrasperma*) and homothallic (*N. africana*, *N. dodgei, N. galapagosensis, N. lineolate, N. terricola* and *N. pannonica*) (Perkins & Turner, 1988). *Neurospora* has three different types of spores; a sexual spore called an ascospore, and two asexual spores (also called conidia) called macroconidia and microconidia (Springer, 1993). Almost all non-homothallic species of *Neurospora* are conidiating species. (Turner, Perkins, & Fairfield, 2001). *Neurospora* species are also widely distributed geographically. Initially, *Neurospora* species were found only in tropical and sub-tropical regions, but were later identified across the globe. In the laboratory, one can produce hybrid populations by crossing *N. crassa* and *N. intermedia*, although the reproductive success rate is lower than that of a cross within species. This hybrid population has been a useful tool in testing ecological factors including reproductive isolation and speciation (J. R. Dettman, Anderson, & Kohn, 2008, 2010; J. R. Dettman, Jacobson, Turner, Pringle, & Taylor, 2003). Additionally, biological and phylogenetic species of the *Neurospora* genus have been well characterized by Dettman and his colleagues (J. R. Dettman, Harbinski, & Taylor, 2001; J. R. Dettman, Jacobson, & Taylor, 2003; Jeremy R. Dettman, Jacobson, & Taylor, 2006; J. R. Dettman, Jacobson, Turner, et al., 2003), which helps to understand clock variation within different ecotypes.

2.2. Natural Habitats

Ironically, the natural habitats of *Neurospora* are not well understood. Although there is vast amount of knowledge in *Neurospora* biology, most of the studies have been done in a controlled laboratory setting. There are few well-established ecological observations of *Neurospora's* natural habitat. *Neurospora* is a fire-adapted fungus. The sexual spore is resistant to most environmental stresses and requires a heat shock or a furfural gas that is generated during the fire to germinate (Emerson, 1948; Goddard, 1935). This provides an advantage for local colonization of *Neurospora* after wildfires, as it has been shown that most of the natural strains of *Neurospora* are collected after wildfires (D. J. Jacobson et al., 2006). This suggests that *Neurospora* is not a competitive organism in the natural environment unless a condition is right; it cannot colonize on natural substrates unless other natural microbes are absent in a given experimental condition (Lee, 2012). The preferred natural habitat of *Neurospora* has also been shown to vary regionally. In North America, *Neurospora* prefer to colonize beneath tree bark, whereas in Europe they prefer to colonize above tree bark (D. J. Jacobson et al., 2006; David J. Jacobson et al., 2004).

2.3. Collection of *Neurospora* Ecotypes

There are more than 6,000 natural ecotypes that have been collected and deposited in the FGSC (Fungal Genetics Stock Center), including an informative record of the collection site of each species deposited. Among the strains deposited in the FGSC, there were unique geographical collection sites for each of the three-major *Neurospora* species; *N. intermedia* (253 sites), *N. crassa* (114 sites), and *N. discreta* (85 sites) (Figure 1). The *N. discreta* species complex is a large clade of species that is different from the clade that includes *N. crassa*. Unlike other species of *Neurospora* whose habitat is mostly in

temperate regions (Turner et al., 2001), *N. discreta* has successfully adapted to habitats in diverse latitudinal environments, and thus will likely be an ideal species for studying the local adaptation of circadian clocks (Figure 1).

[place Figure 1 here]

2.4. *Neurospora* as a Model for Clock Variation Studies

Among all filamentous fungi, *Neurospora* has been widely used to understand several aspects of biochemistry, ecology, evolution, genetics, and circadian biology. *Neurospora* is a haploid filamentous fungus, which has a larger evolutionary timescale compared to other model systems (Baker, Loros, & Dunlap, 2012; Dunlap & Loros, 2017; Turner et al., 2001). *Neurospora* has been well adapted to different latitudes and longitudes across the globe. Therefore, it can potentially serve as a model for understanding several ecological and evolutionary aspects including natural variation. Around the 1970s, Perkins and his colleagues started collecting different species of *Neurospora* across the globe. Most of the collections were submitted to FGSC (Perkins & Turner, 1988; Perkins, Turner, & Barry, 1976; Turner et al., 2001). As mentioned in section 2.3, there are more than 6,000 *Neurospora* ecotypes deposited in FGSC. Among those collected by the FGSC, the most well-characterized reference species is *N. crassa*, the model species that has been widely used to understand the biological aspects of *Neurospora*. The genome of *Neurospora* is relatively small compared to other eukaryotes, and there is a strong synteny of genome among species, especially in *N. crassa*. Additionally, more than 11,000 knockout mutant strains were generated for understanding the gene functions and

associated morphology (Colot et al., 2006; McCluskey, Wiest, & Plamann, 2010). In the laboratory, the maintenance of *Neurospora* is easy and economical. Since it has a short life cycle and can produce a high number of progeny by both asexual and sexual reproduction, it is a good model system for studying population genetics. The most documented circadian-regulated phenotype in *Neurospora* is asexual development, which is a fitness trait (Brandt, 1953; Pittendrigh, Bruce, Rosensweig, & Rubin, 1959). Based on multiple advantages, *Neurospora* can serve as a potential model to understand natural variations of the circadian clock and its fitness.

3. THE CIRCADIAN CLOCK OF *NEUROSPORA*

Our intention in this section is not an in-depth review of all known molecular mechanisms of the *Neurospora* circadian clock, but rather a brief review to understand studies in natural variation of the *Neurospora* clock. We recommend to readers other excellent reviews on *Neurospora* circadian clocks for a more comprehensive description (Dunlap & Loros, 2017; C. Heintzen & Liu, 2007; J. Hurley, Loros, & Dunlap, 2015; Lakin-Thomas, Bell-Pedersen, & Brody, 2011).

3.1. FRQ Dependent Oscillators

Although *Neurospora* is a simple eukaryotic organism in comparison to higher eukaryotes, the molecular structure of the *Neurospora* circadian clock shows significant complexity, thus providing an opportunity to dissect network motifs for more complex eukaryotic circadian systems (Bell-Pedersen, Crosthwaite, Lakin-Thomas, Merrow, & Okland, 2001). This molecular structure is composed of the negative components

FREQUENCY (FRQ) and VIVID (VVD), and the positive components WHITE COLLAR-1 (WC-1), and WHITE COLLAR-2 (WC-2). (Baker et al., 2012; P. Cheng, Y. Yang, K. H. Gardner, & Y. Liu, 2002; Dunlap & Loros, 2017; J. Hurley et al., 2015). Traditionally, clock studies were aimed at studying three aspects of the clock: input, the oscillator, and output. In *Neurospora*, FRQ is a key component of the oscillator, WC-1 and WC-2 are part of both the input and the oscillator, and VIVID (VVD) is part of both the input and the output (Q. He & Liu, 2005a; Schneider et al., 2009; Zoltowski & Crane, 2008). It has been shown that WC-1 is a blue light receptor for the circadian oscillator (Allan C. Froehlich, Liu, Loros, & Dunlap, 2002; Qiyang He et al., 2002), and FRQ-interacting RNA Helicase (FRH) is a protein which interacts with the clock component FRQ (Ping Cheng, Qun He, Qiyang He, Lixin Wang, & Yi Liu, 2005). Several studies have reported the role of casein kinases 1 and 2 (CK1 and CK2) in the negative feedback loop (Q. He, Cha, He, et al., 2006; Mehra et al., 2009; Querfurth et al., 2011). The key positive elements in the feedback loop contain PAS domain(s), through which they form homo- or hetero-dimers. This includes VVD, another PAS protein and a blue light receptor, which was shown to form a second connected negative feedback loop through WC-1 (Christian Heintzen, Loros, & Dunlap, 2001; Schwerdtfeger & Linden, 2003). The emerging picture is that the clock is composed of multiple regulatory loops.

3.1.1. Simple and Coupled Loops

Positive regulation of WC-1: WC-1 is a blue light receptor and a transcription binding factor (Allan C. Froehlich et al., 2002; Qiyang He et al., 2002), and regulates its own expression (Ballario et al., 1996; P. Cheng, Yang, Wang, He, & Liu, 2003) When either

of the functional WC-1 or WC-2 proteins are absent in a cell, *wc-1* transcription is not light induced (Ballario et al., 1996). Later, it was shown that WC-1 and WC-2 work as a heterodimer activator complex (White Collar Complex, WCC)(Ping Cheng, He, Yang, Wang, & Liu, 2003; Ping Cheng, Yuhong Yang, Kevin H. Gardner, & Yi Liu, 2002; Allan C. Froehlich et al., 2002; Qiyang He et al., 2002; Talora, Franchi, Linden, Ballario, & Macino, 1999). Although at the surface WCC seems to have a simple genetic regulation with the forward regulation feedback loop, there is more complex regulation at the molecular level._

Coupled loops in WCC: WC-1 negatively regulates WC-2 expression (P. Cheng et al., 2003), and thus, forms a coupled loop within WCC regulation. WC-1 is a multi-functional protein that contains motifs for sensing the environment and protein-protein interactions, and activation domains with specialized roles in the light and darkness (P. Cheng et al., 2003; Lee, Dunlap, & Loros, 2003; Toyota, Onai, & Nakashima, 2002). Expression of *wc-1* appears to be regulated for its multiple functions, with three promoters identified as necessary for the transcription of *wc-1*(Kaldi, Gonzalez, & Brunner, 2006). One of the promoters, P_{dist}, is responsible for the expression of WC-1 in darkness, and its transcription activity is dependent on WC-1 function. The transcriptional activity of another promoter, P_{prox}, is independent of WC-1 in the dark. The last promoter, P_{int}, is located in the open reading frame of the *wc-1* gene. The transcription activity of the promoter P_{int} is insensitive to a light signal. The alternative translational start site in the P_{int}-originated transcript produces a truncated WC-1 protein that is lacking the amino-terminal activation domain, NpolyQ, whose activation function influences the circadian period of an organism (Lee et al., 2003; Michael et al., 2007;

Toyota et al., 2002). The altered transcriptional activity of *wc-1* also leads to an altered phase of an organism (Kaldi et al., 2006). It appears that WCC can regulate WC-1 expression by choosing different regulatory sites depending on ambient light conditions.

Negative regulation of FRQ: One of the major conceptual breakthroughs in circadian clock studies was the demonstration of the negative feedback loop of the negative regulator, FRQ (Aronson, Johnson, Loros, & Dunlap, 1994). In this classical study, the authors used an overexpression construct of FRQ. When FRQ was overexpressed in a cell by an inducer, its own transcription was down-regulated. The detailed mechanisms of the negative feedback mechanisms, however, have been shown to be more than simple negative regulation. Constitutive expression of *qrf* (*frequency antisense transcript*) negatively regulated the level of *frq*, and the absence of *qrf* compromises the light dependent resetting of clock (Li, Joska, Ruesch, Coster, & Belden, 2015; Xue et al., 2014).

3.1.2. Interlocked Loops in WCC/FRQ

WCC and FRQ control each other's expression through coupled positive and negative regulatory loops.

Negative feedback loop: The WCC binds to the proximal light regulatory element (PLRE) and clock-responsive element (C-box) in the *frq* promoter. Binding of the WCC to the PLRE leads to acute *frq* induction upon light exposure, which resets the circadian clock. Binding of the WCC to the C-box activates the transcription of *frq*, which is involved in the transcriptional/translational feedback loop of the core circadian oscillator. However, binding of the WCC to the C-box can be interrupted by nucleosomes. SWI-

SNF chromatin remodeling complex clears nucleosomes and promotes the binding of the WCC to the C-box and induces the expression of *frq* (Dunlap & Loros, 2017; Wang, Kettenbach, Gerber, Loros, & Dunlap, 2014). After the *frq* gene is translated, FRQ homodimers bind to FRH (FRQ-associated RNA helicase) in the cytoplasm and form the FFC (Negative elements), which enhances the stability of FRQ (P. Cheng, Q. He, Q. He, L. Wang, & Y. Liu, 2005; Guo, Cheng, & Liu, 2010). Upon localization of FFC into the nucleus, several kinases interact with the FFC complex to phosphorylate FRQ (Baker et al., 2012; J. Hurley et al., 2015). Of these kinases, casein kinases (CK1a and CK2) are particularly associated with clock dependent phosphorylation (Q. He, Cha, He, et al., 2006; Mehra et al., 2009; Querfurth et al., 2011). CK1a interacts with FFC through the FRQ/CK1a interaction domain (FCD), which is located near the three phosphorylation sites of the *frq* gene. The interaction of CK1a with the FFC plays a crucial role in the negative feedback mechanism. CK1a also phosphorylates and inactivates the WCC. This process is mediated by the physical interaction between the FFC and the WCC (Q. He, Cha, He, et al., 2006). As the activator function of the WCC diminishes, the level of *frq* transcription also diminishes. After the rate of *frq* transcription and translation slows down, the level of FRQ in the nucleus diminishes (Aronson et al., 1994; Q. He, Cheng, Yang, Yu, & Liu, 2003). The low level of FRQ in the nucleus allows WCC in the nucleus to resume transcription of *frq*, which closes the negative feedback loop. The period of circadian oscillation of the clock gene FRQ is about 22 h, which includes the time for synthesis, phosphorylation, and degradation of the FRQ protein (Dunlap & Loros, 2017; J. Hurley et al., 2015; M. W. Merrow, Garceau, & Dunlap, 1997).

Positive feedback loop: FRQ is involved not only in negative regulation of its activators (WCC), but also in the positive regulation of WCC. FRQ is involved in the up-regulation of WC-1 at the post-transcriptional level and in the up-regulation of WC-2 at the transcriptional level (P. Cheng, Yang, & Liu, 2001; Lee, Loros, & Dunlap, 2000). Thus, FRQ plays a role in coupling the negative and positive loops. This inter-connected loop has also been reported in *Drosophila* and mammals (Glossop, Lyons, & Hardin, 1999; Shearman et al., 2000). In all cases, the inter-connected loop plays a crucial role in maintaining the robustness of the circadian oscillation (P. Cheng et al., 2001).

3.1.3. Post-Translational Modification of WCC and FFC

In addition to transcriptional/translational feedback regulation, post-translational regulation plays a role in circadian regulation (Brunner & Schafmeier, 2006). The phosphorylation status of the WCC is important for its activation and functionality. Phosphorylation of the WCC is FRQ-dependent, and hyper-phosphorylated WCC is an inactive form of WCC (Q. He & Liu, 2005b; Schafmeier et al., 2005). Upon exposure to light, the WCC forms a complex that is much larger than the WCC in darkness (Allan C. Froehlich et al., 2002; Q. He & Liu, 2005b). Because the number of FFCs in a cell is lower than the number of WCCs, it was speculated that the negative function of FRQ-FRH could be achieved by recruiting kinases for phosphorylation of the WCC. There are several kinases that are known to phosphorylate clock proteins, (Ping Cheng et al., 2005; A. C. Froehlich, Noh, Vierstra, Loros, & Dunlap, 2005; Görl et al., 2001; Q. He, Cha, Lee, Yang, & Liu, 2006) including casein kinases, which phosphorylate the WCC and FRQ. To finish the negative loop, the negative elements are degraded. This is achieved

through the ubiquitin-proteasome system much like in mammals and *Drosophila* (Q. He & Liu, 2005a). There are four kinases and two phosphatases involved in FRQ phosphorylation (Garceau, Liu, Loros, & Dunlap, 1997; Görl et al., 2001; Liu, Loros, & Dunlap, 2000; Pregueiro, Liu, Baker, Dunlap, & Loros, 2006; Yang, Cheng, & Liu, 2002; Yang, Cheng, Zhi, & Liu, 2001; Yang et al., 2004). Eventually, hyper-phosphorylated FRQ is recruited by FWD-1, an F-box/WD-40 repeat-containing protein. FWD-1, a substrate-recruiting subunit of an SCF (SKP/Cullin/F-box)-type ubiquitin ligase, physically interacts with hyper-phosphorylated FRQ, which leads to the degradation of the FRQ protein (Q. He et al., 2003; Q. He & Liu, 2005a). For the simplicity of the model, we did not include the degradation of FRQ by ubiquitination in our model. In the absence of FRQ, FRH can interact with WCC (Guo et al., 2010), however, the role of FRH in the circadian clock was unclear. It has been shown that FRH also plays a vital role in cell viability (J. M. Hurley, Larrondo, Loros, & Dunlap, 2013; Lauinger, Diernfellner, Falk, & Brunner, 2014; Shi, Collett, Loros, & Dunlap, 2010), though a recent study has shown that FRH may not play an essential role in the circadian clock (Conrad et al., 2016).

3.1.4. Negative Feedback Loop in WCC/FRQ/VVD

The gene VVD also forms negative feedback loops. VVD is another blue light receptor that has two major roles: sensing the change of light intensities and photoadaptation (Q. He & Liu, 2005b; Christian Heintzen et al., 2001; Schwerdtfeger & Linden, 2003; Shrode, Lewis, White, Bell-Pedersen, & Ebbole, 2001). The role of VVD in endogenous clock regulation is that of a clock-controlled output gene, and also a modulator of the

light input pathway to the clock (Chen, DeMay, Gladfelter, Dunlap, & Loros, 2010; Christian Heintzen et al., 2001; Hunt, Thompson, Elvin, & Heintzen, 2010). VVD is one of the early light-response genes that are activated by WCC (Q. He & Liu, 2005b; Christian Heintzen et al., 2001; Schwerdtfeger & Linden, 2003). Once VVD is expressed, it negatively regulates its own expression by blocking the activator function of the WCC by directly binding to the WCC (Chen et al., 2010; Hunt et al., 2010). This photo-adaptation function ensures the circadian oscillator runs during the daytime (Elvin, Loros, Dunlap, & Heintzen, 2005). VVD also plays a key role in resetting circadian rhythm at dusk (light to dark transition) by enhancing *frq* degradation, and thus, *de novo* synthesis occurs, causing a new cycle to begin (Elvin et al., 2005).

3.2. FRQ-Independent Oscillators

In the 1980s, Feldman's lab first reported FLO (FRQ less oscillator) rhythms using a *frq* mutant (Loros, Richman, & Feldman, 1986). Over the years, several studies were done to understand the mechanism underlying the developmental rhythms by FLO. In the late 90s, a report was published that frq^9 mutants can be entrained by temperature, but not with light (Chang & Nakashima, 1997; M. Merrow, Brunner, & Roenneberg, 1999). Furthermore, Lakin-Thomas and Brody published that deficiency of lipids can restore the developmental rhythms in absence of a core circadian oscillator, which includes photoreceptors like *wc-1* and *wc-2* (Lakin-Thomas & Brody, 2000). These observations support that temperature and metabolism can play an essential role in generating developmental rhythms in addition to the core circadian oscillator. Based on these studies, a two-oscillator model has been proposed; the core circadian oscillator responds

to light and FLO responds to non-photic signals such as temperature. These two oscillators may or may not be coupled to generate developmental rhythms (Bell-Pedersen et al., 2001). In addition to a Choline-Deficient Oscillator, CDO (Lakin-Thomas, 1998; Lakin-Thomas & Brody, 2000), a Farnesol or Geraniol based Oscillator, FGO (Granshaw, Tsukamoto, & Brody, 2003), and a Cryptochrome-dependent Oscillator, CRO (Nsa et al., 2015) have been identified in *N. crassa*. Additionally, microarray studies have shown that several clock-controlled genes are rhythmic in the absence of *frq* (Correa et al., 2003). This suggests that different FLOs can take over the developmental rhythms. The relationships between the FRQ-based oscillator and FLOs have yet to be characterized.

[Place Table 1 here]

In the context of natural variation, metabolic enzymes and metabolic components, e.g. mitochondria, have been reported (Table 1). Interestingly, allelic variations of *nit-2* for nitrate reductase and *nit-4* for nitrogen regulation have been observed in *N. sitophila* and *N. intermedia* respectively. Recently, a study has been done on the *frq*-less Nitrate Reductase Oscillator in *N. crassa*, NRO (Christensen et al., 2004), in which NIT-2 and NIT4 are the positive elements of the feedback loop.

4. NATURAL VARIATION AND THE CIRCADIAN CLOCK

There is a large volume of literature on natural variation studies using *Neurospora*. In section 4.1., we will highlight a few selected studies that are worth revisiting in the study of natural variation of the *Neurospora* circadian clock. Considering the recent advancements in our understanding of the existence of metabolic oscillations and their

cross-talk with the genetic oscillator, we believe that old literature on natural variation studies in metabolites and enzymes can provide potentially new insights for studying natural variation in *Neurospora* circadian clocks.

4.1. Natural Variation in *Neurospora* Ecotypes

From the 1920s, *Neurospora* wild isolates from different natural habitats were grown in synthetic media to study physiological variation of ascospores, conidia, and perithecia (female sexual organ). The ability to grow *Neurospora* in a defined artificial media made *Neurospora* an attractive model organism. Different species of *Neurospora* have been characterized based on physiological variations such as number of asci, ascospore size, pigmentation, the size of perithecia, mating behavior, etc. (Perkins et al., 1976; Shear & Dodge, 1927; Tai, 1935). Several early studies have shown phenotypic variation in different environmental conditions: speed of conidial germination was influenced by the ambient temperature (Shear & Dodge, 1927; Thom & Ayers, 1916), a higher ascus abortion rate in the Texas strain of *N. tetrasperma* has been reported on different media (Dodge, 1939), and mycelial growth and sexual development of *N. lineolate* varied in different media conditions (Frederick, Uecker, & Benjamin, 1969). Other investigations on nutritional factors suggest that variations in perithecia formation occur due to the concentrations of glucose and potassium nitrate of a synthetic medium, which favors sexual reproduction in *Neurospora* (Westergaard & Mitchell, 1947). Around the 1950s, scientists started to understand the biochemistry of conidia and mycelia using *Neurospora*. A 1954 study reported the importance of light and oxygen in the synthesis of carotenoids of *N. crassa* (Zalokar, 1954). Furthermore, variations in respiratory

enzymes such as NAD nucleotidase, β-galactosidase and β-glucosidase have been reported in conidia compared to the mycelia (Eberhart, 1961; Zalokar, 1959; Zalokar & Cochrane, 1956). The activity of the citric acid cycle was shown to decrease in conidia. Therefore, Weiss and his colleagues to begin to understand the variation in activity of mitochondrial enzymes such as oxidative and glycolytic enzymes. Among them, variation of glycolytic enzymes (ethanol dehydrogenase and pyruvate carboxylase) has been reported in conidial and mycelial cultures (Weiss & Turian, 1966).

Several studies have also investigated the genetic control associated with phenotypic variations of enzymes in wild isolates of *Neurospora*. *ars* for aryl-sulfatase (Metzenberg & Ahlgren, 1971); *arg-12* for ornithine transcarbamylase (Grindle & Davis, 1970); *leu-5* for leucyl-tRNA synthetase (Beauchamp, Horn, & Gross, 1977); *inv* for invertase (Yu, Garrett, & Sussman, 1971); *nit-4* for nitrate reductase (Perkins & Turner, 1988); *pts-1* for protease (Hanson & Marzluf, 1975); *nit-2* , *nmr* for nitrogen regulation (Grove & Marzluf, 1981; Young & Marzluf, 1991) and *T* locus for tyrosinase (Horowitz, Fling, Macleod, & Sueoka, 1961; Ruegg, Ammer, & Lerch, 1982). Several other studies have investigated the variation in other enzymes such as amylases, allozymes, β-glucosidases, cellulases, and esterases, etc (Perkins & Turner, 1988; Perkins et al., 1976; Reddy & Threlkeld, 1971a, 1971b, 1972; Spieth, 1975; Turner et al., 2001). Interestingly, most of the enzymatic variations have been associated with enzymes involved in metabolic pathways. In addition to enzymatic variations, mitochondrial variants have been reported in natural populations of *Neurospora*.

In the late 1970s, mitochondrial DNA variants were reported in *N. crassa* (Mannella, Pittenger, & Lambowitz, 1979). Later, mitochondrial DNA variants were

identified in inter and intraspecific populations of *Neurospora* (Collins & Lambowitz, 1983; Griffiths, 1995; Perkins & Turner, 1988; Taylor, 1986; Taylor, Koupel, & Whitmer, 1987; Turner et al., 2001). Additionally, natural variation in the shape and size of mitochondrial plasmids was found in different species of *Neurospora.* Integration of circular plasmids into the mitochondrial genome can interfere with mitochondrial functions, subsequently causing death of cultures (Senescence), which has been observed mainly in *N. intermedia* (Griffiths, 1992, 1995; Maheshwari & Navaraj, 2008; Perkins & Turner, 1988; Turner et al., 2001). Table 1 summarizes more natural variation studies, which might be useful for characterizing the natural variation in circadian clock. For more information on natural variations and Neurospora natural population, there are excellent reviews available (Perkins & Turner, 1988; Perkins et al., 1976; Turner et al., 2001).

4.2. Natural Variation of Core Clock Genes

Using inverted race tube assays (Park & Lee, 2004), 143 ecotypes of *N. crassa* have been characterized. There was significant variation in circadian period, phase, and temperature compensation among the ecotypes (Michael et al., 2007). In addition to the phenotypic variation of the clock, genetic variation has been studied in four key clock genes, FRQ, WC-1, and WC-2, and VVD. A subsequent study tested the hypothesis that the copy number variation of Simple Sequence Repeats (SSR) in the clock genes could serve as a way of creating phenotypic variation in *Neurospora*. This hypothesis was based on one of the conclusions from a parallel genome-wide study of the SSR distribution in *Neurospora* genomes that tri-nucleotide SSRs in the exon might play a role in the

variation of protein functions in *N. crassa* (Kim et al., 2008). All core clock genes contain SSR repeats. Most interestingly, a photoreceptor and transcription factor WC-1 has two large SSR repeats: one in the amino-terminal, NpolyQ, and one in the carboxyl-terminal, CpolyQH. In the *wc-1* alleles that lack NpolyQ, $wc\text{-}1^{MK1}$ and $wc\text{-}1^{rhy\text{-}2}$, there is no functional clock running, whereas, *frq* is light induced (P. Cheng et al., 2003; Lee et al., 2003; Toyota et al., 2002). This suggests that WC-1 activation domains may function in a modular fashion, and NpolyQ could be a main activation domain for the clock function. The estimated repeat size of NpolyQ was between 17-80 base pairs among ecotypes, and the laboratory strain 87-3 had 39 repeats. There was a significant correlation between the size of NpolyQ repeats and the circadian period among 143 natural ecotypes. To test if the size of NpolyQ repeats explains the period variation, two ecotypes with similar structural features except the size of NpolyQ repeats were crossed: FGSC#3223 (29 repeats) and FGSC#4724 (57 repeats). The period of F1 progeny segregated with the size of NpolyQ alleles. The authors concluded that the natural variation in WC-1 NpolyQ of ecotypes may have served for fine tuning their circadian clock to a local habitat (Michael et al., 2007). This suggests that SSR variation could be the answer to how new gene function could arise in a genome with several strong genome defense mechanisms in which any duplicated genome sequences more than 500 nt would be mutated (Aramayo & Selker, 2013).

The conservation of FRQ function in the family Sordariomycetes has been demonstrated by complementing the *Neurospora frq* mutant by expressing the FRQ homolog from *Sordaria fimicola*. The resulting fusion protein had 11 amino acids from *N. crassa* in the amino-terminal with the rest of protein sequence from *S. fimicola* (M. W.

Merrow & Dunlap, 1994). Encouraged by this success, the sequence of the *frq* gene has been used to establish a phylogenetic relationship among species in the genus of *Neurospora* and in Ascomycete fungi with a limited success (Lewis & Feldman, 1996; Skupski, Jackson, & Natvig, 1997). A more comprehensive sequence analysis has been done with five clock genes (FRQ, WC-1, WC-2, FRH-1, and FWD-1) in 64 sequenced fungal genomes (Salichos & Rokas, 2010). FRH-1 and FWD-1 appeared to be the most ancient proteins, followed by WC-1 and WC-2. Interestingly, FRQ appeared to be the most recent protein and appears in only three families: Sordariomycetes, Leotiomycetes, and Dothideomycetes in the Ascomycetes (Salichos & Rokas, 2010).

4.3. Technical Challenges for Studying Clock Variation in *Neurospora* Natural Populations

As is true for other biological research, having an easily assayable phenotype is important for a successful outcome. For *Neurospora* circadian studies, the rhythmic asexual development (conidiation) was a blessing. In the 1950s, the rhythmic pattern of conidiation in *Neurospora* was reported (Brandt, 1953; Pittendrigh et al., 1959). Unlike the unicellular yeast, filamentous fungi like *Neurospora* will grow continuously away from the center of inoculation. Spending the majority of time in the vegetative stage, *Neurospora* will grow as mycelia (transparent or white color). Once approximately 24 hr have passed, *Neurospora* will stop growing as mycelia and start producing conidia (orange color). The conidia are produced from a specialized aerial hyphae cell type called a conidiophore (Springer & Yanofsky, 1989). In a macroscopic view, the area of *Neurospora* that produces conidia is seen as denser in comparison to that of mycelia. This

dense area is called a ‘band’. When *Neurospora* is grown in a glass tube with a medium on the bottom (race tube), the developmental pattern can be easily visualized. To measure the circadian period (in constant darkness condition), or phase (in a cyclic light/dark condition), the growing front of the mycelia is marked on the glass tube under the red light, which does not interfere the *Neurospora* circadian rhythm. Period is measured by the distance from one reference point to the next. Phase is measured as the distance from a reference point of environmental cycle, e.g. dark to light transition, or to a reference point of a development. Traditionally, the reference point has been the peak of a conidiation band. Recently, the photographic image is read by software such as Chrono, developed by Till Roenneberg, to calculate the clock phenotypes of the sample using different reference points, e.g. trough, onset or offset (Roenneberg & Taylor, 2000). However, visualizing the developmental bands in the natural strains was not a trivial matter. Conidiation is repressed by the accumulated CO_2 produced by the respiration of the cells, which is trapped inside of the race tube (Sargent & Kaltenborn, 1972). Therefore, virtually all circadian studies in *Neurospora* have been done in the band (*bd*) mutant background to visualize the banding phenotype (Sargent & Woodward, 1969). The *bd* mutation enabled the fungus to overcome the adverse effect of CO_2 accumulation on conidiation. Four decades after the discovery of the band mutation, it was shown to be located in the *ras-1* gene, which is involved in producing reactive oxygen species known to stimulate conidiation (Belden et al., 2007). Unfortunately, one cannot use this mutant background to study circadian rhythms of ecotypes. To create the *bd* mutant background, one has to cross an ecotype with a *bd* mutant. In this process, the unique genotype adapted to one local habitat will be lost. the Alternative methods to study the ecotypes

without crossing them to a *bd* mutant strain were proposed, such as blowing fresh air to remove CO_2 or adding Rubidium-chloride to the medium (Morgan & Feldman, 1998). These methods were not ideal, however, as blowing air into a race tube tends to spread the hydrophobic conidia into other parts of the media, and adding RuCl causes additional variables in an asexual development of wild ecotypes. One successful assay method to visualize the overt developmental rhythm of natural ecotypes was developed by simply inverting the conventional race tubes. This is known as an inverted race tube assay (Park & Lee, 2004). This modified race tube assay allowed the accumulated CO_2 in the race tube to be ventilated out without blowing fresh air into the race tube, as CO_2 is heavier than air. Because the natural strains grows faster than the band mutants, as the *bd* mutation reduces growth rate, it was necessary for the modified race tube to be longer than the conventional race tube designed for the *bd* mutants (Park & Lee, 2004).

4.4. Methods for Clock Variation Studies

4.4.1. QTL Analysis on Clock Phenotypes

To elucidate the mysteries of gene function caused by genetic variations in the natural population, quantitative genetic techniques have been successfully utilized over the past decades (Alonso-Blanco & Koornneef, 2000). These methods describe how known mutant loci can interact with one another, as well as to isolate new loci in the same pathway (Shimomura et al., 2001). Over the past century, the field of quantitative genetics has grown out of the desire to exploit natural variation and to describe traits with a continuous distribution of phenotypes. An extension of Mendelian principles of polygenic trait is quantitative genetics (phenotypes encoded by multiple loci). Most of the

phenotypic variation identified in natural populations is due to quantitative trait loci (QTL), in which multiple loci contribute small effects on phenotypes, as opposed to a single major-effect locus (Alonso-Blanco & Koornneef, 2000).

Haploid organisms have multiple advantages for studying quantitative traits: 1) For assays, a large number of individuals can be maintained clonally, which may reduce the error from environment; 2) they have a single allele at any given locus, so dominance does not contribute to the genetic variation; 3) QTL can be detected after the first generation; and, as in the case of *Neurospora*, 4) they are fast and easy to work with. For these reasons, *Neurospora* can serve as a potential model to elucidate fundamental questions of quantitative genetics for complex behaviors including circadian clocks. As our understanding of the molecular structure of circadian clocks advances, it becomes apparent that the circadian clock is more tightly linked with other cellular machinery than we imagined (Bell-Pedersen et al., 2001). Based on this, one cannot dissociate the circadian clock from basic cellular metabolism. In previous efforts to find clock genes, all morphological mutants and any mutants with abnormal metabolisms other than the period phenotypes were excluded from analysis. As we have learned more about the importance of the circadian clock in cellular activities, we have realized that there could be clock genes involved in these basic metabolic activities as well. These genetic loci with subtle clock phenotypes or with essential cellular functions could not be studied using traditional genetic screening methods. QTL analysis could reveal novel clock genes and clock mechanisms which would be overlooked by the traditional forward genetics approaches. QTL analysis is a systematic way of mapping all the multiple genetic loci for subtle quantitative traits and essential cellular functions.

To study the natural allelic variants, three mapping populations were prepared using six *N. crassa* ecotypes from distant geographic locations (N2; FGSC3223 from USA x FGSC4724 Malaysia, N4; FGSC4720 from India x FGSC4715 Haiti, N6; FGSC4825 Ivory Coast x FGSC2223 USA). QTL analysis on circadian period and entrained phase phenotype identified 43 QTL (Kim, Logsdon, Park, Mezey, & Lee, 2007). Thirteen of 43 QTL co-localized to known clock genes, which supports that QTL analyses can identify clock genes. More surprising was that 30 of 43 QTL (70%) did not co-localized to known clock genes. This suggests that there are many uncharacterized genetic elements whose variation contributed to the variation of the circadian clock for a specific habitat. Identifying QTL (a chromosomal region which could contain multiple genetic elements) is relatively easy in comparison to identifying causative genes and the nature of variation that caused the phenotypic variation. Since *Neurospora* has a relatively small haploid genome (about 40 Mb), it is a promising system to characterize the causative natural allelic variation for clock variation.

4.4.2. Circadian Clocks in Natural Substrates and Natural Environment

Unlike animals, a filamentous fungus cannot move to other locations when one place does not provide a favorable environment. However, a filamentous fungus can grow indefinitely to a new place that might provide a better living condition. This is why filamentous fungi are the largest living organisms on earth. In one recorded case, one genetically identical fungus *Armillaria bulbosa* grew over 1,500 years covering over 15 hectares with an estimated weigh over 10 tons (M. L. Smith, Bruhn, & Anderson, 1992). One might have a hard time justifying ecological studies on circadian rhythms of *A.*

bulbosa in a 27-inch race tube under the laboratory conditions. For the reasons that we argued throughout this review, *Neurospora* is the best model system for ecological and evolutionary studies of circadian rhythms. The current challenge is developing an experimental system that utilizes natural substrates in a natural environment, and generates reproducible experimental data for characterizing circadian rhythms of a fungal strain. As the first step toward this goal, *Neurospora* has been grown on different natural substrata (Lee, 2012). Utilizing new functional genomics tools, *Neurospora* could serve as a model system for addressing circadian-clock associated ecological questions (Ellison et al., 2011; Lee, 2012; Lee & Dighton, 2013; Romero-Olivares, Taylor, & Treseder, 2015)

5. Future Challenges

Considering the important ecological role of the fungal kingdom in the ecosystem on earth, we have no doubt that understanding the natural variation of the fungal circadian clock is both interesting and an important area of research. As discussed earlier, we are hopeful that more researchers will invest time in the study of natural variation of fungal circadian clocks due to the many advantages that the system offers. However, we also expect a few tough challenges that require much deliberation and a consensus from the research community. These challenges are present not only in the study of fungal clocks, but also in natural variation studies in general.

There is a great advantage in working with a model organism like *Neurospora*, especially when it is a reference strain with a great deal of resources. Because of the large amount of

resources invested on a few select model organisms, we now enjoy a detailed understanding of clock mechanisms. In choosing a model reference organism, it was necessary that the three minimum characteristics required to be called an 'authentic' circadian clock be present: an approximate 24 hr period in constant conditions, the oscillator is entrainable by the local environment, and period is temperature compensated within a physiological range of temperatures. However, as we study the ecotypes (or accessions) of the model organisms that are adapted to a diverse habitat, we find significant deviations in these clocks from that of the reference strain. Circadian clocks have been observed in fungi other than *N. crassa*, however, these clocks are not satisfying the community-agreed criteria to be called an authentic circadian clock (Austin, 1968; Bluhm, Burnham, & Dunkle, 2010; Greene, Keller, Haas, & Bell-Pedersen, 2003; Hevia, Canessa, & Larrondo, 2016; Hevia, Canessa, Muller-Esparza, & Larrondo, 2015; Oliveira et al., 2015; Traeger & Nowrousian, 2015). We believe it is more interesting to find different kinds of clocks with different properties, which reflect the life history of the organism (or species) and speak volumes about the real biology of the clock in nature. As we classify circadian clocks into 'authentic' and 'not-so-authentic', we may somehow be de-emphasizing the studies of those clocks that are not satisfying the minimum criteria of the authentic clock.

Most of the known clock properties in a model organism have been characterized in a controlled laboratory environment using laboratory strains, which served its purpose well to generate reproducible data. However, to study the ecological roles of the circadian clocks, we need to perform experiments in their natural habitat. In a pleasant spring day, the surface temperature of the ground (where many fungi live) could fluctuate from 0 -

40° (Celsius) in a day. The physiological temperature range that we know for temperature compensation spans 20 - 30° for a typical laboratory *Neurospora* strain. We envision a need to develop criteria to characterize non-model fungal clocks (or ecotypes of model species) in natural habitats.

Several studies on eukaryotes shown the role of circadian clock in maintaining fitness (Beaver et al., 2002; Gill & Panda, 2011; Green, Tingay, Wang, & Tobin, 2002; Miller et al., 2004; Xu, DiAngelo, Hughes, Hogenesch, & Sehgal, 2011). Even though, asexual reproduction is a fitness trait of filamentous fungi like *Neurospora*, natural variations of the circadian clock and associated fitness trait is not well understood. There are different ways of measuring 'fitness' in filamentous fungi, but there is no clear consensus in the fungal community. One could measure the growth rate, estimating the amount of nuclei by PCR, number of conidia, germination rate of conidia, number of perithecia (fertilized female organ), number of ascospores (sexual spore), germination of ascospores, etc (Pringle & Taylor, 2002). According to a classical definition of fitness, one might measure the number of progeny (conidia or ascospore). However, there are many aconidial species that do not produce conidia. Even in conidial species with a known sexual cycle, the number of progeny is greatly changed depending on the media and environment. Many fungal species also have different developmental pathways. For example, *Neurospora* has two different asexual progeny (macroconidia and macroconidia) and one sexual progeny (ascospore). Macroconidia contain 3-5 nuclei, and microconidia contain one nucleus. Depending on the natural habitat a *Neurospora* strain lands on, it will produce different ratios of macroconidia to microconidia (Lee, 2012). It

is a challenging task to measure fitness in an unbiased way. We hope that it will prove possible to develop a consensus on what is an appropriate measure of fitness.

Technical Challenges: In an ideal situation, one would like to study a natural strain in a natural habitat. Unlike animals or plants, it is difficult to study fungi in their natural environment. A luciferase reporter is a great way of monitoring the molecular clock, assuming that the fungus of interest can be transformed with the reporter construct and have its expression measured by a luminometer. One possibility to remedy this difficulty is to create a laboratory setting that closely mimics the natural environment.

[Figure 2 here]

CONCLUDING REMARKS

Even though *Neurospora* is a classical model for studying the circadian clock, it has not been widely used for understanding the ecological and evolutionary aspects of clocks. In this chapter, we reviewed the challenges, advantages, and promising future of the system to study natural variation of circadian clocks. We proposed that *N. discreta* be used for studying effects on fitness, as it has greater adaptability to different latitudinal environments compared to other *Neurospora* species (Figure 1). Since asexual development is one of the most well documented clock-regulated phenotypes, *Neurospora* is an excellent system to study the evolutionary and ecological roles of natural variation of the circadian clock (Figure 2).

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Table 1. Natural variant studies in *Neurospora*.

Species	Category	Traits	Reference
	Chromosomal modifications	Satellite	(Perkins, Metzenberg, Raju, Selker, & Barry, 1986)
		Tandom duplications	(Selker & Stevens, 1987)
		Translocations	(Perkins & Turner, 1988)
	Enzymes	b-Glucosidase	(Mahadevan & Eberhart, 1964)
		Cellulase	(Eberhart, Beck, & Goolsby, 1977)
		Fatty acid synthesis	(Goodrich-Tanrikulu, Stafford, & Jacobson, 1999)
		Invertase	(Yu et al., 1971)
		Leucyl-tRNA synthetase	(Beauchamp et al., 1977)
		Nitrogen regulation	(Young & Marzluf, 1991)
		Ornithine transcarbamylase	(Grindle & Davis, 1970)
		Protease	(Hanson & Marzluf, 1975)
		Tyrosinase	(Horowitz et al., 1961; Ruegg et al., 1982)
	Cell cycle	Meiosis impaired	(D. A. Smith, 1975)
		Recombination repression	(D. G. Catcheside, 1975)
		Sexual phase recessives	(Leslie & Raju, 1985; Raju & Leslie, 1992)
		Synaptic sequence	(D. E. Catcheside, 1981)
	Mitochondria	Mitochondrial DNA variants	(Collins & Lambowitz, 1983; Mannella et al., 1979; Taylor, Smolich, & May, 1986)
		Mitochondrial plasmids	(Griffiths, 1995; Taylor, Smolich, & May, 1985 1985)

		Senescence	(Navaraj, Pandit, & Maheshwari, 2000)
	Nutrients		(Leslie & Raju, 1985; Perkins et al., 1976)
	Reproductive Success		(J. R. Dettman, Jacobson, Turner, et al., 2003)
	Resistance to *Sk*		(Campbell & Turner, 1987)
	Resistance to toxic agents	Canavanine	(Perkins, Radford, Newmeyer, & Bjorkman, 1982)
		Cadmium resistance	(Levine & Marzluf, 1989)
		Surfactant resistant	(Al-Saqur & Smith, 1980)
		Triphenyl tetrazolium chloride	(Perkins et al., 1982)
	Vegetative incompatibility	*het* loci	(Mylyk, 1975, 1976; Perkins, 1975)
		Suppressor	(Newmeyer, 1970)
N. discreta	**Enzymes**	Fatty acid synthesis	(Goodrich-Tanrikulu et al., 1999)
	Mitochondria	Mitochondrial Plasmids	(C. He, Nastasja de, Bok, & Griffiths, 2000)
	Speciation	Phylogenetic variation	(Jeremy R. Dettman et al., 2006)
N. intermedia	**Chromosomal modifications**	Tandom duplication	(Selker & Stevens, 1987)
		Translocations	(Perkins & Turner, 1988)
	Enzymes	Allozymes	(Spieth, 1975)
		b-Glucosidases	(Perkins & Turner, 1988)
		Cellulase	(Perkins & Turner, 1988)
		Estreases	(Reddy & Threlkeld, 1972)
		Nitrate reductase	(Blakely & Srb, 1962)
		Tyrosinase	(Perkins & Turner, 1988)
		Microcycle conidiation	(Turner et al., 2001)
		Nitrogen regulation	(Young & Marzluf, 1991)
		Fatty acid synthesis	(Goodrich-Tanrikulu et al., 1999)
	Mitochondria	Mitochondrial DNA variants	(Collins & Lambowitz, 1983)
		Mitochondrial Plasmids	(Griffiths, 1995; Natvig, May, & Taylor, 1984)

		Senescence	(Bertrand, Chan, & Griffiths, 1985; Bertrand, Griffiths, Court, & Cheng, 1986; Griffiths & Bertrand, 1984; Rieck, Griffiths, & Bertrand, 1982)
	Resistance to toxic agents	Surfactant resistant	(Al-Saqur & Smith, 1980)
	Reproductive Success		(J. R. Dettman, Jacobson, Turner, et al., 2003)
	Spore killer	Resistance to *Sk*	(Campbell & Turner, 1987)
	Virus-like particles		(Perkins & Turner, 1988; Tuveson & Peterson, 1972)
N. sitophilia	**Chromosomal modifications**	Tandom duplication	(Selker & Stevens, 1987)
	Enzymes	Amylases	(Reddy & Threlkeld, 1971a, 1971b, 1972)
		Nitrogen regulation	(Grove & Marzluf, 1981; Perkins & Turner, 1988; Young & Marzluf, 1991)
		Fatty acid synthesis	(Goodrich-Tanrikulu et al., 1999)
		Tyrosinase	(Perkins & Turner, 1988)
	Mitochondria	Mitochondrial Plasmids	(Griffiths, 1995)
		Mitochondrial DNA variants	(Collins & Lambowitz, 1983)
N. tetrasperma	**Chromosomal modifications**	Tandom duplication	(Selker & Stevens, 1987)
	Enzymes	Aryl-sulfatase	(Metzenberg & Ahlgren, 1971)
		Estreases	(Reddy & Threlkeld, 1971a)
		Fatty acid synthesis	(Goodrich-Tanrikulu et al., 1999)
	Mitochondria	Mitochondrial Plasmids	(Griffiths, 1995; Natvig et al., 1984)

Figure Legend:

Figure 1. Distribution of natural ecotypes of *N. crassa* (A) and *N. discreta* (B). Among the natural ecotypes deposited in Fungal Genetics Stock Center, we chose 114 *N. crassa*

and 85 *N. discreta* strains that have a complete collection record without redundancy. The distributions of these representative strains are plotted by the collection sites (C). Error bars represent one standard deviation of the mean. The distribution between two species in the Northern hemisphere had a statistically significant difference (*t-test*, $p < 0.0001$).

Figure 2. Overview of natural variation studies in fungal circadian clocks. Each natural strain has been successfully adapted to the local environment. Many layers of ambient environmental factors will function as selection forces. Thus, the variation observed in the circadian rhythm reflects the natural history of the organism. Among the circadian controlled phenotypes, asexual and sexual development in *Neurospora* have been extensively studied. Cellular oscillators: blue lines for expressed gene products, red lines for up-regulation (arrow) or down-regulation (line) of a gene, dotted line for unknown relationships. Images for a conidial development are modified from (Lee & Dighton, 2010) and from (Lee, 2012). Images of sexual development are modified from (Lee, 2012).