IDENTIFYING THE MOLECULAR MECHANISM AND GENETIC REGION FOR HABITAT

SPECIFIC CLOCK VARIATION IN N.DISCRETA PS4B

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THESIS ABSTRACT

Identifying the molecular mechanism and genetic region for Habitat Specific Clock Variation in \textit{N.discreta PS4B}

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The 24-hour biological rhythm, or circadian rhythm, has been attributed as a fitness trait in multiple organisms. [1][2][3] To identify how organisms adapted their circadian rhythms to increase their fitness, we used a global population from the \textit{Neurospora discreta} complex. Under cycling light conditions, North American strains in \textit{N. discreta PS4B} exhibit no rhythms in their sporulation output. To understand the molecular variation of the oscillator underlying these divergent phenotypes, we analyzed the expression of the key clock protein FREQUENCY and found FRQ levels have a rhythm in constant conditions. Based on our findings, we concluded that the North American strains in the \textit{N. discreta PS4B} population have decoupled their developmental rhythm from their molecular oscillator to enhance their fitness. Our mathematical model supports the hypothesis that North American strains have increased their fitness by decreasing their coupling coefficient and threshold. In addition, we found the candidate region for habitat specific clock variation (HSCV) occurs on chromosome 3. In an intercontinental F1 population, we
observed an opposite allele effect, resulting in strains with the North American phenotype having the African parent allele. To understand the mechanism and components of another oscillator, we used the strain PRD-1, which is hypothesized in the literature to be a key component of the metabolic oscillator. We have observed arrhythmic ATP oscillation in PRD-1 compared to WT strains.
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INTRODUCTION

Circadian Rhythms

A circadian clock is a diurnal cellular oscillator controlling the timing of numerous biological activities from mitochondrial metabolism to mitosis [4][5]. Circadian rhythm is the result of interconnected positive and negative feedback loops within the cell [6]. The molecular mechanism behind these feedback loops increases in complexity in higher-level eukaryotic organisms [7].

To have an authentic circadian clock, an organism must meet three conditions. First, a rhythm must be present in constant conditions. These experimental conditions are usually in constant dark and at a constant temperature. Under these conditions, an authentic circadian rhythm will have a period of approximately 24 hours. Periods longer than 24 hours could be the result of a mutation, such as in the strain PRD-1, FGSC4902 [8][9][10]. Moreover, periods longer than 24 hours could be the results of some organisms lacking a circadian rhythm; rather, they have a circa-annual rhythm as seen in reindeer [11]. Second, the circadian period should be temperature compensated. As temperature increases 10°C, enzymatic activity normally increases two-fold. However, an authentic circadian rhythm can compensate for the increase in enzymatic activity and still produce a period of approximately 24 hours. Third, the organism must have the ability to entrain to cycling conditions. Currently, the molecular mechanism for entrainment in *Neurospora crassa* has been linked to the proximal promoter of *frequency* [12].
A common simple eukaryotic organism to use as a model organism for studying circadian rhythms is the filamentous fungus *Neurospora crassa*. There are three main components of circadian rhythms: an input, a molecular oscillator, and an output. A circadian rhythm can be influenced by several inputs such as light, temperature, and nutrients. Light input is the primary focus of this study, and it directly affects the positive elements of the circadian clock. In *Neurospora crassa* circadian clock, positive elements are comprised of two main proteins: White Collar-1 (WC-1) and White Collar-2 (WC-2). WC-1 and WC-2 interact through their PAS domains to form the heterodimer protein complex called the White Collar complex (WCC). In light conditions, the WCC undergoes conformational changes, and binds to the proximal promoter of the primary negative element of the clock frequency (FRQ). In dark conditions, the WCC complex binds to distal responsive elements [12]. Proper *frq* transcription is dependent on a protein called CLOCKSWITCH, which is important for DNA structure [13]. Upon translation, FRQ proteins form dimers, are progressively phosphorylated, and form a complex with FRQ-associated RNA helicase (FRH) to form the FRQ-FRH complex (FFC)[14]. The FFC enters the nucleus and physically interacts with the WCC, promoting its phosphorylation, which inhibits the DNA binding capacity of the complex.

Current research shows the transcriptional and translational oscillator (TTO) controls the circadian output and asexual spores production [15]. However, there is evidence suggesting that the molecular oscillator is not necessary for a developmental rhythm. For example, microarray data in an FRQ-less oscillator (FLO) strain shows there is still rhythmic expression of certain clock-controlled
genes. As a result, this work showed evidence of multiple oscillators controlling asexual spore production [16]. This decoupling also occurs in the opposite direction, where strains with no apparent developmental rhythm have an authentic molecular oscillator[17][18]. All these results were determined by using lab strains of *N. crassa*. To date, there has been no report of natural strains exhibiting this decoupling between the molecular oscillator and developmental rhythm. This study examines strains within the *N. discreta* species complex as an alternative of using *N. crassa*.

**Neurospora discreta**

*Neurospora discreta* is a useful model organism because it can be found in more diverse climates compared to the temperate climate *N. crassa* strains. As a result, a more in-depth global adaptation and genetic study can be completed using *Neurospora discreta*. Interestingly, a subgroup species in the *N. discreta* complex, *N. discreta PS4b*, has a significant ecological difference in their local habitat. North American strains within the *N. discreta PS4B* population were only found only living underneath the bark of burnt trees. Conversely, PS4B strains from other continents were found living on top the bark of burnt trees [19]. We concluded previously, in the PS4b population, that strains differed in their sporulation pattern and FRQ protein levels. We measured their sporulation pattern using an inverted racetube assay. This assay uses a glass tube filled with minimal nutrients and agar and the fungus is inoculated at one end. The fungus then grows in tube and conidiates rhythmically with a period of 22 hours. Traditionally, this represents the output of the circadian clock [20].
The North American strain had no banding in racetubes and had a low rhythmicity index (R.I.) score. Rhythmicity index is calculated by two parameters: an R-value and amplitude. The R-value is defined by how consistent the rhythm is during an experiment. For example, a rhythm with sporadic amplitudes will have an R-value below one, whereas a strain with a rhythm peaking consistently at a given time will have an R-value close to one. The strength of the rhythm is also determined by the average amplitudes of the peaks in the circadian trace. These two values, amplitude and R, are multiplied to give the rhythmicity index (R.I.) value.

We also concluded that FRQ levels are lower in North American strains compared to other continental strains (O.C.). It has been demonstrated that higher FRQ levels correspond to a stronger circadian output [21]. We questioned what the molecular mechanism underlying this Habitat Specific Clock Variation (HSCV) was. The two main hypotheses being tested were: (1) do North American strains have a weakened oscillator, or (2) is the molecular clock decoupled from the developmental rhythm?

Besides identifying the molecular mechanism, we wanted to identify the gene(s) responsible for this HSCV. We decided to use a genome wide association to identify candidate genes by collaborating with Dr. Shende and Dr. Scheinfeldt. We found a region on chromosome 3, which had an inverse relationship between the R.I. score and the parental allele. This has been reported in similar studies, where a phenotype was associated with the opposite parent allele [22].
PRD-1

Our results in *N. discreta* have shown a decoupling between the molecular oscillator and developmental rhythm in North American strains. To elucidate the connection between the TTO and conidiation, we chose to study a classical mutant in *N. crassa* called period-1 (PRD-1 previously called frq-5) FGSC4902, which is hypothesized to be part of the metabolic oscillator [9]. This strain has been studied extensively; however, the molecular mechanism has not been fully described. PRD-1 is known to have a period approximately 26 hours in racetubes, grow at 60% of the rate of WT on minimal or complete media, and have an altered fatty acid composition in its membrane [8][23][24]. In double mutants with FLO and PRD-1, there has been developmental rhythm when exposed to high temperature pulses or when media has been supplemented with farenesol. Farenesol is hypothesized to activate the mevalonate pathway, which is an important pathway for cellular respiration and ATP production [25]. Unpublished results from Nick Starkey concluded that PRD-1 has lost its ability to compensate its period in different glucose conditions. In higher glucose conditions, PRD-1 has a period of approximately 22 hours. As a result, we hypothesized that PRD-1 was a key component of the metabolic oscillator. Our focus was on the key metabolic compound in the cell, ATP. There are several studies demonstrating that ATP release in media is controlled by the circadian clock and is important for growth. The molecular mechanism of ATP release is hypothesized to be dependent on Ca$^{2+}$ levels in the cell and IP3 signaling [26][27][28][29]. We hypothesized that PRD-1
would have an altered extracellular ATP rhythm because of its altered period and possible connection to the metabolic oscillator.

Currently the evidence of the metabolic oscillator has been from FLO and the present of a rhythm in AMP in *Neurospora crassa* and oscillation in oxygen consumption is mitochondria respiration. Our results will elucidate on the underlying mechanism of the metabolic oscillator [30] [31].
MATERIALS AND METHODS

Culture conditions and western analysis

Liquid cultures were made by inoculating mycelia tissue into 100 ml flasks. Shaking liquid cultures (100 RPM) in high glucose liquid medium incubated in constant darkness at 25°C (DD) for ~2 hours for the experiment in figure 1. An eight-hour light exposure was given to the samples. The samples were frozen in liquid nitrogen, and stored at -80°C. Using a mortar and pestle, the tissue was ground to fine powder under liquid nitrogen and the tissue suspended in a non-reducing/denaturing extraction buffer (50 mM HEPES pH 7.4, 137 mM NaCl, 10% glycerol, pepstatin A 1 μg/ml, leupeptin 1 μg/ml, and phenylmethanesulfonylfluoride 1 mM). Protein concentration for each sample was determined in triplicate via the Bradford Assay. Fifty to eighty micrograms (μg) of total protein was loaded into each lane. Prior to SDS-PAGE, the samples were heated to 95°C in a protein loading buffer containing 50 mM Tris pH 6.8, 0.1 M DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol. Samples were electrophoresed at 150V for 65 minutes on a 7.5% polyacrylamide gel and transferred to a PVDF membrane with a BIO RAD Trans-Blot Turbo Transfer System (1.0 amps, 25 volts, for 30 minutes). For immunodetection, FRQ antiserum was used (1:40 dilution) in a 5% milk and phosphate buffered solution containing 0.3% Tween 20 (PBS-T) incubated for one hour. The membrane was washed every 20 minutes for an hour. A stabilized goat anti-rabbit IgG (H+L) peroxidase conjugated secondary antibody (Thermo Fischer Scientific Product ID 32460) was used at a 1:250 dilution in 5% milk PBS-T solution and incubated with
the blot for 30 minutes. The membrane was washed 12 times every 10 minutes. Chemiluminescence detection was performed using ECL plus and blots were imaged using a Storm Phosphorimager (Amersham)

**Realtime PCR**

The primers had a concentration of 100 uM. The reaction was 10 ul of SYBR Green master mix from Applied Bio Systems, 2ul of Reverse and Forward Primer, 1ul cDNA (treated with DNase prior and cDNA was diluted 1:100 from the original cDNA reaction) 100 ng/ul, 5ul Dnase/Rnase free water. The reaction required a 10 minute activation at 95C. The PCR reaction followed a two-step reaction: Denaturing at 95C for 15 seconds, and Annealing/Extension step at 60C for 1 minute.

**Gene Ontology Terms**

Candidate Genes on chromosome 3 were based on the common region between both association studies (1634000-2685000). Genes in this region were sorted by their Gene Ontology (GO) term based on their molecular function using FungiDB. Parameters were set to molecular function, p value cut off 0.05 and using InterPro predictions for the GO Association Source. The P-Value Cutoff is the value that a GO term must meet before it is considered enriched in the gene result. The P-value is a statistical measure of the likelihood that a certain GO term appears among the genes in particular results more often than it appears in the set of all genes for that organism (background). GO terms in FungiDB are associated with genes by mapping gene products to the InterPro domain database resulting in 100% electronically transferred GO associations.
**Racetube Assay**

Strains used in study were obtained from the Fungal Genetic Stock Center. Strains were grown in minimal media, which contained 1X Vogel’s Salts, 2% sucrose, pH 5.8. Strains were inoculated in high glucose liquid media, and then inoculated into inverted racetubes. Racetube media contained 1X Vogel’s Salts, 0.17% L-arginine, 0.1% D-glucose, pH 5.8, 2% agar.

**Racetube Analysis**

RI scores was obtained using ChronOSX 3 by using the CompAnalysis and the data were smoothed by four hours.

**DNA Extraction**

Petri dishes (100 mm x 15 mm) containing 20 ml of high glucose liquid medium (2% glucose, 0.5% L-Arginine, 1X Vogel’s salt, pH 5.8) were inoculated with conidia and incubated for 36-48 h at 25ºC. Mycelia tissues were washed with water, vacuum filtrated, frozen in liquid nitrogen, and stored at -80º C. Tissue disruption was achieved using the QIAGEN TissueLyser bead-beater system, and genomic DNA (gDNA) was extracted using QIAGEN DNeasy Plant Mini Kits.

**Luciferase Assay**

The media used was HGLM. HGLM was mixed with D-luciferin with a ratio of 0.6 ul of 200x luciferin to 25 ml HGLM. Then, 180uL of the HGLM with luciferin was pipetted into each well of a 96 well white plate. Then, 20uL of 1:1 conidial suspension (in autoclaved water) was added to the well and mixed by pipetting. The wells are then covered with the breathable film, and all excess film was removed using a razor blade.
**Genome Wide Association Study**

Our first approach was using Python and the Fisher Exact Test to determine the most significant single nucleotide polymorphisms (SNPs), separating North American strains’ genotype from other continental strains’ genotype. Our second approach was through the R program using linear regression.

**Mathematical Modeling**

We used the classical Kuramoto model for coupled oscillators:

$$\dot{\theta}_i = \omega_i + \sum_{j=1}^{n} K_{ij} (\sin \theta_j - \sin \theta_i)$$

In this model, $\theta_i$ represents the phase of the i-th oscillator, and $K_{ij}$ represents the coupling coefficient between the i-th and the j-th oscillator. It is known that high values of coupling induces the oscillator to be synchronous (i.e., same frequencies and close phases) [32]. We assumed the first oscillator to be the molecular circadian clock with varying coupling level to the other oscillators (which, in turn, are weakly coupled among themselves).

**Cell counting**

Strains were grown in minimal media, and equal amounts of conidia were inoculated into tubes containing an equal amount of *Acer saccharum* (Sugar Maple). Strains were placed in a growth chamber in either 12-light and 12-dark cycle, or in constant darkness. All experiments were completed at 25°C. Conidia were collected and stained with Acridine orange (Becton Dickinson Company(Cat261182) using 20 ul of dye and 20 ul of conidia. Imaging and quantification were completed using
Cellometer X2 (Nexcelom). Statistical tests were completed using the Wilcoxon rank sum test in R computational program.

**Strain and tissue collection for PRD-1**

Strains N334-6, N334-8, N334-9 (PRD-1 Strains), and N334-14, N334-21, N334-23 (WT strains) were used from a cross between strains lab strain N308-54 to FGSC4720. Strains were grown in complete media slants. Strains were inoculated in petri dishes containing LGLM (0.1% glucose) media. This was done because racetube assays show PRD-1 has a longer period in low glucose racetube media, and a shorter period, ~22, in high glucose racetube media. Strains were grown until a mycelia pad was present (~3 days). Holes in the tissue were punched, and the punched tissue was placed in a 125ml flask containing 50ml of LGLM pH 5.8. The strains were placed in a chamber for 12 hours LL 25C. After the 12 hour incubation, the strains were moved every four hours to DD 25C for 24 hours. Strains were harvested, and the media was filtered and kept in a 15ml eppendorf tube by placing the 15ml tube underneath the vacuum funnel and in the vacuum flask. This prevented any cross contamination. The funnel was washed with ethanol and water after every strain. Strain tissue was kept and frozen in liquid nitrogen. 100ul of the media for each strains and time point was placed in a 96 well plate to ensure quick use for the ATP assay. The plate was frozen using liquid nitrogen and stored at -80C.

**Extracellular ATP measurements**

ATP was measured using the StayBrite Highly Stable ATP Bioluminescence Assay Kit (Catalog #791-100). This kit provides a long-lasting signal, and is ideal for our assay. The enzyme mix was reconstituted using 1.1ml reconstitution buffer
(provided in the kit). An ATP standard was created by reconstituting the ATP vial with 100 ul of dH2O (this gave a 10mM ATP stock). Serial dilution and the rest of procedures were completed as described in the Bio vision protocol.

**Intracellular ATP measurement**

Tissue was ground using a mortal and pestle, and 10mg portions were added to a prechilled 1.5ml tube. The samples were kept frozen until 100 ul of 1x reaction buffer was added to the samples (provided in the biovision kit). The samples were spun down at 14,000 rpm at 4°C for one minute. The supernatant (10 ul) was added to the enzyme and buffer mix in a white 96 well plate. The plate was read using a luciferase plate reader. The plate reader setting were: dark-adapt for five minutes and 10 second read per well. It was important to make sure the plate and enzyme is ready for use before extracting ATP. The longer one takes in this process, the more ATP degradation.
RESULTS

N. discreeta PS4B

Previous work has shown that North American strains within the *N. discreeta* PS4B population had lower amount of FRQ protein compared to other continental strains. We wanted to identify the level of regulation of FRQ levels. We chose to form quantitative PCR on a F1 population (N309, a cross between FGSC8831 from Golikro, Ivory Coast, Africa and FGSC8578 from Belen, New Mexico, United States of America) to test the levels of *frq* messenger RNA. This population was chosen because of its isogenic background, and our genome wide association study would focus on identifying genes responsible for the HSCV in N309. The data demonstrate there are no differences between *frq* mRNA levels between low R.I. and high R.I. strains. Our results indicate FRQ regulation occurs at the posttranscriptional level (Figure 1). Our assumption was low R.I. strains in N309 would have lower FRQ protein compared to high R.I. strains.

To test our hypothesis that the HSCV was due to FRQ protein levels we formed Western analysis on the N309 population. To determine if FRQ levels in the F1 population correlate and co-segregate with the R.I. values. The data showed that there was no statistical significance between high R.I. strains and low R.I. strains when comparing FRQ protein (Figure 2). As a result, we rejected our hypothesis that N.A. strains have a weakened rhythm in racetubes due to a weakened molecular oscillator.

To test our second hypothesis, we used a luciferase reporter strain in a North American strain from Tok, Alaska D199(FGSC9980). The results showed that the
North American strain had a free running period in constant conditions. In addition, the strain had a period approximately 24 hours (23.94±0.52). Racetube data showed no visible banding. This demonstrated that there is a decoupling between the molecular oscillator and developmental rhythm (figure 3).

To understand the adaption for the decoupling, we formed a fitness test and compared the amount of asexual spores produced between FGSC8831 and FGSC8578, the parents of the N309 population. Traditionally, the amount of conidia produced has been a way to measure fitness [33]. We hypothesized that the African strain would have a greater fitness in cycling conditions compared to constant conditions. We also expected that the North American strains, which are adapted for growth under the tree bark, would have a lower fitness in cycling conditions and a higher fitness in constant conditions compared to the African strain. Strains were grown on sawdust of *Acer saccharum* (Sugar Maple) to mimic natural conditions. As expected, the Africa strain had a nominally statistically significant higher fitness in cycling conditions compared to constant conditions, $p$ value = 0.08084. The North American strain did not have a statistically higher fitness in constant conditions compared to cycling conditions ($p$ value = 0.6787; Figure 4). The North American strains did have a higher fitness than African strains ($p$ value = 0.001088).

To investigate how North American strains increase their fitness, we collaborated with Dr. Benedetto Piccoli to create a mathematical model to demonstrate the relationship between coupling and fitness. We used the established mathematical model for multiple oscillators, the Kuramoto model [32]. The results demonstrate the coupling coefficient, $K$, and threshold are important parameters for
sporulation and fitness. A higher K represents a stronger coupling of oscillators within a system. If within in the cell the threshold is crossed then the fungus will produce its asexual offspring. If the threshold and K are high then fitness will be lower because of the amount of stress needed to induce sporulation. However, if the threshold and K are low, then sporulation can occur with low stimulation or stress. As a result, North American strains more closely resemble the model conditions when K is low and the threshold is low (Figure 5).

To identify the genes and pathways responsible for this HSCV and decoupling, we used a genome wide association study to find candidate genes. We hypothesized that the natural population would have too many significant SNPs separating their genomes because of their HSCV. This hypothesis was not rejected based on the natural association study (Figure 6). The N309 population was used instead to identify the candidate region for the HSCV. We used the Fisher Exact test and linear regression to confirm our findings. In both methods, we saw that there was a significant region on chromosome 3, which appeared to be the major genetic difference between N.A. and O.C. strains. This region was about 1.2 Mb long with approximately 300 genes (Figure 4). We compared both association studies, and found the common region between both (Figure 7).

Even though the SNPs on chromosome 3 had a correlation with R.I. values, there was an opposite effect between strains (Figure 8). Strains with the North American allele on chromosome 3 had high R.I. in F1. We rejected this due to contamination in racetubes labeling (Figure 9). When new R.I. values were calculated, the linear regression in R was used to determine the candidate region in
the current racetube data set (Figure 9). Once again, chromosome 3 was the candidate region for the HSCV. To reject the hypothesis, there was DNA contamination. We re-extracted DNA and used Sanger sequencing for identifying SNPS for multiple strains. We used the gene RIA-1, which was the top candidate gene on chromosome 3. Once again, N309-10 (high R.I.) had the same SNPs as the North American parents (Figure 10). A candidate gene list for chromosome 3 was compiled using the common region in both F1 association studies (Figures 11). The new candidate gene list showed that 45% of the candidate genes were important for nucleic acid binding based on GO terms.

**PRD-1**

To identify any possible connection between the TTO and metabolic oscillators, we used the strains PRD-1 in *Neurospora crassa*. Our results show ATP oscillation in the third backcross population from the original cross FGSC4902 X WT FGSC4720. This population was named N334. We used N334 because this population was more isogenic than other populations, and would eliminate any phenotypes not associated with the longer period phenotype. Our data shows that PRD-1 has an arrhythmic release of extracellular ATP compared to the strains with a WT period (Figures 12-14). PRD-1 had a 0.116 R-value, while WT had a 0.962 R-value in chronosX3. R.I. scores were 170044 in WT compared to PRD-1 with a R.I. of 1853.831 when computing the average trace of the ATP levels. In addition, PRD-1 has an arrhythmic intracellular ATP compared to strains with a WT period (Figure 15). There appears to be no correlation with overall ATP levels, extracellular or intracellular, when comparing between PRD and WT strains.
DISCUSSION

This study observed that the circadian clock in the cell is not the only determining factor for circadian output. There was no correlation between the strength of the oscillator and final output. In addition, there is an authentic circadian TTO clock running in a representative North American strain. Therefore, we observed a decoupling of the TTO from the developmental conidiation in *Neurospora discreta*. This is the first decoupling reported in an entire organism in nature. We concluded that N.A. strain’s circadian clock has lost the ability to control asexual developmental to enhance fitness in their local environment. The mathematical model led us to hypothesize that N.A. strains have no selective fitness in cycling or constant conditions by lowering their coupling effect and lowering threshold.

This study has also demonstrated that PRD-1 has an arrhythmic release of extracellular ATP. It is unclear if differences in PRD-1 are due to a deficit in FRQ rhythmicity or calcium level changes in PRD-1, as suggested by the literature. Interestingly, there was a rhythmic expression of ATP within the cell, which has not been reported in Neurospora. Future directions include identifying the full molecular mechanisms behind any connection between the altered period and arrhythmic ATP rhythm seen in the PRD-1 strain.
Figure 1. *frq* mRNA is consistent despite differing in R.I.

This is real-time PCR data showing no differences in frequency RNA expression between two strains in the N309 population, N309-10 (Africa-like), and N309-30 (NA-like). Actin expression was used to normalize *frq* expression. P values (T-Test) ZT 8 = 0.53 ZT 20 = 0.97
Figure 2. FRQ protein does not co-segregate with R.I. score.
FRQ protein levels were measured after being exposed to light for 8 hours. Error bars represent standard error from three biological replicates. R.I. score was determined by racetube assay. There was a statistically significant difference between FRQ levels between low R.I. strains and high R.I. strains in the F1 population. There was a statistically significant difference between the F1 parents when comparing FRQ levels. Levels were normalized using a non-specific band. Negative control (-) was the protein extract from FGSC11554 (the frq null strain). The positive control (+) was from the ras band strain 328-4 which is known to have a robust circadian rhythm in racetubes.
Figure 3. A molecular oscillator is running in a North American in N.discreta PS4B. Luciferase reporter strain from Tok Alaska FGSC was used to demonstrate decoupling between the molecular oscillator and sporulation pattern. The average period was 23.94±0.52. The racetube shows the same strain with no clear banding.

Figure 4. North America strains exhibit a fitness advantage in both cycling and constant conditions. The number of conidia is counted in two strains (FGSC8831 from Golikro, Ivory Coast, Africa, and FGSC8578 from Belen, NM USA, North America from constant dark conditions (DD). Cells counted represent the number of cells per 20ul. Four biological replicates for FGSC8831 and FGSC8578 were grown and error bars represent standard error.
Figure 5. Mathematical model demonstrates fitness is determined by threshold and coupling strength.

(a) Time evolution (T=10) for n=5 coupled oscillators with strongly coupling (K=10) between first oscillator (Circadian) and the others and initial frequencies are distributed randomly. Very rapidly (before time t=2) all oscillators are coupled to the first ones keeping a small phase shift.

(b) Evolution (T=10) of the total signal (sum of 1+sin of oscillators phases) for the time evolution (a). The total signal rapidly (before time t=2) align its frequency to the first oscillator (Circadian).

(c) Time evolution (T=10) for n=5 coupled oscillators with weakly coupling (K=1) between first oscillator (Circadian) and the others and initial frequencies are distributed randomly. Oscillators keep uncoupled both in frequency and phase.

(d) Evolution (T=10) of the total signal (sum of 1+sin of oscillators phases) for the time evolution (c). The total signal does not show a clear rhythm.

(e) Plot of fitness (total number of conidia) as function of the oscillators’ coupling (K=1 to 10) and the threshold (2 to 8). Data correspond to 1000 simulations with random initial frequencies for each value of coupling and
threshold. Conidiation is assumed to happen when the total signal exceed the threshold.
Figure 6. Chromosome 3 is the candidate region for the PS4b phenotype. The genome wide association study demonstrated the most significant SNPs separating high R.I. strains from low R.I. stains was on chromosome 3. There were no significant SNPs on the clock genes WC-1 and FRQ on chromosome 7. P values were determined by Fisher Exact Test and the –log10 was taken for each P value.

Figure 7 SNPS in common in Natural and N309 Association study. This data demonstrates the overlap between the raw data on chromosome 3 for the Natural and N309 association study. The blue squares represent the SNPs found on chromosome 3 from the N309 population. The grey diamonds demonstrate the
significant SNPs positions

Figure 8. An opposite allele is effect is present in the F1 population.

On chromosome 3 there was a significant correlation between R.I. score and SNPs. However, the strains in N309 with a low R.I. had the Africa parent allele for all the candidate genes on chromosome 3. Error bars represent standard deviation of the R.I. values with strains containing the African or North American Allele.
Figure 9 Racetube in F1 population is not contaminated and gives an accurate candidate region for the PS4b phenotype.
Racetube assay was preformed again and analyzed again to identify if there was any mislabeling in the original racetube data. The same candidate region on chromosome 3 was found when running the analysis in R.

<table>
<thead>
<tr>
<th>Race and Parent</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
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<tbody>
<tr>
<td>8831-1-f2_AFRICA_Parent/25-667</td>
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<td>8578-f2 NSW_DNA_SA_Parent/26-663</td>
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</table>

Figure 10. There was no DNA contamination in N309 or F1 parents.
DNA was sequenced using Sanger sequencing for a candidate gene on chromosome 3, RIA-1. Previous SNP data showed high R.I. strains had the North American allele for the entire candidate region on chromosome 3. New sequence data was collected by extracting DNA again for the F1 parents and selective N309 strain.

Figure 11. Candidate Genes on chromosome 3 based on the common region between both association studies (1634000-2685000). Genes in region were sorted based on their Gene Ontology (GO) term based on their molecular function using FungiDB. Parameters were set to molecular function, p value cut off 0.05 and using InterPro predictions for the GO Association Source. The P-Value Cutoff that a GO term must meet before it is considered enriched in your gene result.
Figure 12. ATP oscillates rhythmically in WT. ATP oscillation in the media from WT stain N334-21 in DD conditions. The release of ATP from cells in rhythmic in the WT strain. N=1. Three technical replicates were used. Standard deviation was used to determine error bars. R value 0.962 in chronosX3.

Figure 13 ATP is arrhythmic in PRD-1. ATP oscillation in the media from PRD-1 strain N334-9 in DD conditions. The release of ATP from cells is arrhythmic in the PRD-1 strain. N=1. Three technical replicates were used. Standard deviation was used to determine error bars. R value 0.116 in chronosX3.
Figure 14. PRD-1 does not have a rhythmic release of extracellular ATP compared to WT. Each trace represents 6 time points in DD. ATP was measured using a plate reader and the biovision Bioluminescence Assay Kit. Left strains are WT with a normal period. Right strains are PRD-1 strains with a longer period observed in racetubes (data not shown).
Figure 15. Intracellular ATP levels change over time in constant dark. Intracellular ATP was extracted from WT and PRD-1 strains. There appears to be more of a pattern of intracellular in WT compared to PRD-1.
REFERENCES


