CHAPTER 4

General Discussion and Future Prospects

Maize is grown in many regions throughout the world and is considered one of the major staple food sources globally (FAO 2012). The importance of maize production is also highlighted by the alternative uses of maize such as bio-fuel production, livestock feed, and raw material for industrial products. One of the most important diseases influencing maize production is Grey Leaf Spot (GLS) disease. GLS disease has been associated with major maize yield losses in many maize growing regions (DERERA *et al.* 2008). Two sibling fungal species have been identified as the casual agents for GLS disease, namely *Cercospora zeae-maydis* and *Cercospora zeina*. *C. zeina* is associated with GLS affecting African maize production (MEISEL *et al.* 2009). As no effective and sustainable management strategy for GLS disease has been identified (WARD *et al.* 1999), research need to be focussed on the establishment of effective control mechanisms.

On a molecular level, nothing is known about the mechanisms of infection by *C. zeina*. Fungal effectors are a strategic mechanism of the pathogen to prevent detection by its host and to cause successful infection (BOYD *et al.* 2013; JONES and DANGL 2006). Most fungal effectors identified so far have shown no homology to other fungal effectors and are therefore believed to be species specific (STERGIOPOULOS and DE WIT 2009). However, recent findings have shown that some effectors are conserved among fungal species of the same class (e.g. *Cladosporium fulvum Avr4* that is conserved among Dothideomycetes), and some effectors can show an even wider distribution across the fungal kingdom (e.g. *C. fulvum Ecp2* and *Ecp6* that are found in many fungal pathogens) (STERGIOPOULOS *et al.* 2010). Fungal effectors were therefore divided into three classes, namely fungal effectors that are species specific and share no homology to any other identified protein sequences, fungal effectors that have homologs that are conserved in species of the same class, and fungal effectors that are widely conserved across the fungal kingdom (STERGIOPOULOS *et al.* 2010). Based on this knowledge, this study aimed to identify homologs of the *Avr4*, *Ecp2* and *Ecp6* effectors in the draft *C. zeina* (Mkushi) genome sequence.

C. zeina Avr4, *Ecp2*, and *Ecp6* homologs were identified and annotated in the *C. zeina* (Mkushi) genome sequence. Evidence that the above mentioned homologs were present in *C. zeina* included BLAST results, gene structure predictions, identification of conserved domains, confirmation of contig assembly through PCR analysis with gene-specific primers, confirmation of annotations through mapping of *in planta* and *in vitro* RNAseq reads, reciprocal identification of homologs based on the annotated *C. zeina* (Mkushi) sequences, protein alignments and phylogenetic analysis with homologs from closely related species. The *C. zeina* (Mkushi) *Avr4* and *Ecp2* homologs were correctly identified based on the above listed evidence (Chapter 2). Even though *C. zeina* (Mkushi) *Ecp6* was identified, more

evidence is needed to confirm the *Ecp6* gene region as RNAseq reads failed to verify the predicted *Ecp6* intron region. The RNAseq reads were generated from a single time point during the later stages of *C. zeina* infection (see Chapter 2 materials and methods section) that might not have represented high enough transcript levels to confirm the *Ecp6* annotation. Conserved domains identified for the predicted *C. zeina* Avr4, Ecp2, and Ecp6 effectors served as a basis to infer possible functions. Interestingly, homologs of *C. zeina Avr4* and *Ecp2* were detected in *C. zeae-maydis* and *M. fijiensis*. Homologs of *C. zeina Ecp6* were detected in *C. zeae-maydis*, *M. fijiensis*, and *M. graminicola*. Even though the predicted gene regions in these other species could not be verified, putative conserved domains were detected in most homologs. Some of the *Avr4*, *Ecp2*, and *Ecp6* homologs identified in the genomes of *M. fijiensis*, *C. fulvum*, and *M. graminicola* were found to be identical to the effector homologs identified in previous studies (BOLTON *et al.* 2008; JOOSTEN *et al.* 1994; STERGIOPOULOS *et al.* 2010; VAN DEN ACKERVEKEN *et al.* 1993) and served as further evidence that the *Avr4*, *Ecp2*, and *Ecp6* effector homologs were identified in *C. zeina* (Mkushi).

The findings from this study proved that a susceptible line of maize could successfully be inoculated with a virulent strain of *C. zeina* (Mkushi) under phytotron conditions. Phytotron infections can be performed all year round, irrespective of the maize growing seasons, and provide quarantine and controlled conditions for future infections with genetically modified *C. zeina* strains. This research also provided relative expression profiles of *C. zeina* (Mkushi) *Avr4*, *Ecp2*, and *Ecp6* effectors at specific time points throughout infection and correlated these expression levels with fungal quantity at each time point. *C. zeina Avr4* and *Ecp6* relative expression levels had a weak, but positive correlation to fungal quantity. A positive correlation indicated that as fungal quantity increased, the relative expression levels of *Avr4* and *Ecp6* also increased. No correlation was found between *Ecp2* expression and fungal quantity. To obtain a better correlation between fungal quantity and effector gene expression, future experiments might have to include more time points between the chlorotic and necrotic disease symptom development stages. We noted that *C. zeina Avr4*, *Ecp2*, and *Ecp6* are expressed during infection and that all three effectors showed constant levels of expression during infection. Two reference genes namely *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *Cyt III* (cytochrome c oxidase subunit III) were identified and validated for accurate normalisation of *C. zeina in planta* expression levels.

This study provided insight into the molecular mechanisms of infection of *C. zeina* by showing that the *Avr4*, *Ecp2*, and *Ecp6* effectors are present in the *C. zeina* (Mkushi) genome and that these effectors are expressed during infection. Future studies could focus

on determining if *C. zeina* Avr4, Ecp2, and Ecp6 effectors play a role in fungal pathogenesis. This could be achieved by knocking out the respective effector and replacing it with an antibiotic marker. The mutant *C. zeina* strain would then be used to inoculate a susceptible maize line to establish whether the above mentioned effectors are crucial for *C. zeina* pathogenesis. Protocols for *C. zeina* transformation and knocking out of other *C. zeina* pathogenicity factors have been developed and optimised in our research group at the University of Pretoria (J. Liversage- personal communication). If the effectors were to be shown to play an important role in fungal pathogenesis, it would also provide more insight into the possible functional roles that these effectors might have during infection. It has been shown that knocking out *Avr4* in *C. fulvum* causes a decrease in fungal virulence (VAN ESSE *et al.* 2007). Similarly, an *Ecp2* knockout in *C. fulvum* was also associated with reduced fungal pathogenicity (LAUGE *et al.* 1998). The *Ecp6* effector gene from the Dothideomycete *Exserohilum turcicum*, that causes Northern Leaf Blight of maize, was knocked out by using an *Agrobacterium tumefaciens* transformation system (XUE *et al.* 2013). Deletion of *E. turcicum Ecp6* was also associated with significant decreases in virulence to maize.

As *C. zeina* (Mkushi) *Avr4*, *Ecp2*, and *Ecp6* were shown to be expressed during infection, these effectors are possible targets for target-specific control mechanisms for GLS by means of Host Induced Gene Silencing (HIGS). The concept behind HIGS is the target-specific down-regulation of chosen vital pathogen gene(s) within the pathogen through the uptake of plant-derived dsRNA/siRNA molecules specific to the target gene(s) (KHATRI and RAJAM 2007). The target-specific dsRNA molecules are produced by transformation of transgenic plants with a binary vector expressing hair-pin RNA specific to the fungal target sequence. HIGS has been described as a promising method for durable resistance against fungal pathogens (RAJAM 2012). In wheat and barley, the HIGS of transcripts specific to *Blumeria graminis* (powdery mildew fungi) affected the development of the fungi (NOWARA *et al.* 2010).

Important conserved domains were identified for the *C. zeina* Avr4 and Ecp6 effectors, namely the CBM14 domain present in Avr4 and the LysM domains present in Ecp6. Both these domains have previously been shown to be involved in binding to chitin by means of an affinity precipitation assay that was developed for the tomato pathogen *C. fulvum* (VAN DEN BURG *et al.* 2006). The assay involves precipitation with different chitin molecules to be able to determine the binding specificity of the functional domains. A similar approach can be undertaken to determine if the functional domains present in the *C. zeina* Avr4 and Ecp6 effectors binds specifically to the chitin molecules present in the fungal cell wall.

Future experiments would also involve determining if the *C. fulvum* resistance genes *Cf-4* and *Cf-Ecp2* are able to recognise the predicted *C. zeina Avr4* and *Ecp2* effectors. It was shown that *M. fijiensis* and *Dothistroma septosporum Avr4* and *Ecp2* effectors (*MfAvr4*, *MfEcp2*, *DsAvr4*, and *Ds-Ecp2-1*) are able to induce a hypersensitive response in tomato in the presence of the *Cf-4* and *Cf-Ecp2* resistance genes (DE WIT *et al.* 2012; STERGIOPOULOS *et al.* 2010). To achieve this, the *Potato virus* X (PVX)-based expression system (BOLTON *et al.* 2008) can be used to deliver *C. zeina* Avr4 into a tomato line with the *Cf-4* resistance gene present. Similarly, *C. zeina* Ecp2 can also be delivered into a tomato line, containing the *Cf-Ecp2* resistance gene, to determine of the C. zeina Ecp2 effector is recognised by *Cf-Ecp2*. Nonhost-mediated recognition by the cognate *Cf-4* and *Cf-Ecp2* resistance genes would indicate that the *C. zeina Avr4* and *Ecp2* genes are core effectors recognized by single cognate reference genes. Such research would contribute to plant defence gene targets for possible resistance against a broad range of fungal pathogens. It would also be interesting to determine if homologs of the *Cf-4* and *Cf-Ecp2* resistance genes are present in maize by doing BLAST searches against the *Zea mays* genome database.

Disease resistance through the control of a single dominant gene can be lost through mutations in effector genes that cause the effector to be unrecognizable by the resistance gene (JOOSTEN *et al.* 1994). Therefore, a global study on the allelic variation within the *C. zeina Avr4* and *Ecp2* genes and how it affects recognition by the tomato Cf-4 and Cf-Ecp2 resistance proteins would give more insight into the durability of the resistance genes in the field. A similar study has been undertaken in *M. fijiensis* (STERGIOPOULOS et al. 2014). The allelic variation within the *M. fijiensis* effectors *MfAvr4*, *MfEcp2*, *MfEcp2-2* and *MfEcp2-3* and the impact it has on recognition by the tomato Cf-4 and Cf-Ecp2 proteins was analysed in worldwide strains of *M. fijiensis*. It was found that nucleotide substitution patterns, suggesting positive selection and intragenic recombination, shape the evolution of the *M. fijiensis* effectors. It was also shown that effector alleles able to overcome the tomato resistance proteins Cf-4 and Cf-Ecp2 are already present in natural populations of *M. fijiensis* (STERGIOPOULOS *et al.* 2014). Therefore, even if the *Cf-4* and *Cf-Ecp2* resistance genes were functional in the genetic background of banana, it will be improbable that these genes provide resistance.

Future studies could also determine if there is a correlation between the expression of host chitinases and the relative expression of *C. zeina Avr4* and *Ecp6* fungal effectors. As Avr4 and Ecp6 have previously been shown to be involved in defensive roles protecting the fungal cell walls, it is expected that these effectors would be up regulated in the presence of chitinases. Expression analysis of GLS positive plants from experimental field trials have

shown that pathogenesis related genes, especially chitinases, are upregulated in the presence of GLS (Crampton- unpublished data).

An interesting effector *GLS1* (β-1,3-Glucan Synthase) was identified in the maize fungal pathogen *Colletotrichum graminicola* that is responsible for the synthesis of β-1,3-Glucan present in fungal cell walls. Recently it was found that *C. graminicola* down-regulates GLS1 expression during its biotrophic growth stage, as a strategy to evade β-glucan–triggered immunity in maize (OLIVEIRA-GARCIA and DEISING 2013). Research is needed to identify if a similar effect is taking place in *C. zeina*. β-1,3-Glucan is an essential component of fungal cell walls (LATGE 2007) and the *C. zeina GLS1* homolog would therefore be a good target for GLS management.

In conclusion, this study provided more insight into possible gene-specific targets for GLS disease management. It was shown that *C. zeina Avr4*, *Ecp2*, and *Ecp6* are present in *C. zeina* and expressed during infection and therefore may play a role in fungal pathogenesis. However, more insight is needed to determine if these effectors play a role in fungal virulence and if putative maize resistance genes exist. The strategies outlined in this chapter provide possible effective and emerging techniques to improve GLS disease management.

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SUMMARY

Insight into three putative *Cercospora zeina* effector genes and the role they play in virulence

By

Brigitte Lombard

Grey Leaf Spot (GLS) is one of the most important diseases affecting maize production locally and worldwide as it affects the photosynthetic ability of maize leaves, thereby causing major yield losses. *Cercospora zeina* has been identified as the casual agent of GLS in African regions. Generally, no effective control method for GLS management has been established. Fungicide control is environmentally unsustainable and not cost effective for small-holder farmers in Africa. As no information regarding the molecular mechanisms of infection of *C. zeina* exists, more insight on a molecular level is needed to be able to obtain possible fungal targets for GLS management.

Effectors are small pathogenicity factors secreted by the fungus that aim to prevent detection and the subsequent immune response by the host. Most fungal effectors identified have been shown to be species specific, but certain fungal effectors have been shown to be conserved within a specific fungal class or shown to have an even wider distribution across the fungal kingdom. This study aimed to identify *Avr4*, *Ecp2*, and *Ecp6* effector homologs in the *C. zeina* (Mkushi) genome. The *C. zeina Avr4* and *Ecp2* homologs were identified, annotated and the annotation was validated in the *C. zeina* (Mkushi) genome by means of RNAseq data. The *C. zeina Ecp6* homolog was identified and annotated, but the annotation could not be verified by *in planta* RNAseq data. However *in vitro* RNAseq data provided evidence for the *C. zeina Ecp6* gene structure.

Functional domains identified in the *C. zeina* effector homologs included a chitin-binding domain (CBM14) in *C. zeina* Avr4, a putative necrosis-inducing domain (Hce2) in *C. zeina* Ecp2, and three LysM domains (carbohydrate-binding domains) in the *C. zeina* Ecp6 protein. The basic functional roles of these effectors based on these conserved domains could be determined. However more functional analysis through gene knockouts and pathogenicity trials are needed to corroborate these functions. Effector homologs of *C. zeina Avr4*, *Ecp2*, and *Ecp6* were identified in *Cercospora zeae-maydis*, *Mycosphaerella fijiensis*,

Mycosphaerella graminicola, and *Cladosporium fulvum* based on BLASTn and tBLASTx searches against the respective genomes. *Avr4*, *Ecp2*, and *Ecp6* homologs identified in *M. fijiensis* and *C. fulvum* were identical to those found in previous studies giving more confidence in the process applied to predict the *C. zeina Avr4*, *Ecp2*, and *Ecp6* effectors.

In order to determine whether the identified *C. zeina Avr4*, *Ecp2*, and *Ecp6* effectors are expressed during maize infection, expression profiles of the *C. zeina* effectors were determined through RT-qPCR analysis. It was shown that all three *C. zeina* effectors were expressed *in planta* during infection at relatively constant levels. Expression levels of all three effectors were below the detection limit in un-inoculated plants indicating that no background amplification of maize genes or endophytes were present. The relative expression levels at each time point was also correlated with the *in planta* fungal quantity at each specific time point. *C. zeina Avr4* and *Ecp6* relative expression levels had a weak, but positive correlation to fungal quantity, generally meaning that as fungal quantity increased, the relative expression levels of *Avr4* and *Ecp6* also increased. No correlation was found between *Ecp2* expression and fungal quantity. This study also identified two reference genes, namely G*APDH* (glyceraldehyde-3-phosphate dehydrogenase) and *Cyt III* (cytochrome c oxidase subunit III) for accurate normalisation of *C. zeina in planta* expression levels. Importantly, it was also shown that *C. zeina* infection can be achieved under phytotron conditions, making maize infections possible irrespective of the maize growing seasons.

In conclusion, this study provided more insight into the molecular mechanisms of infection of *C. zeina* by showing that the *Avr4*, *Ecp2*, and *Ecp6* effectors are present in the *C. zeina* (Mkushi) genome and that these effectors are expressed during infection. This information is useful for the development of target-specific GLS management.

APPENDIX

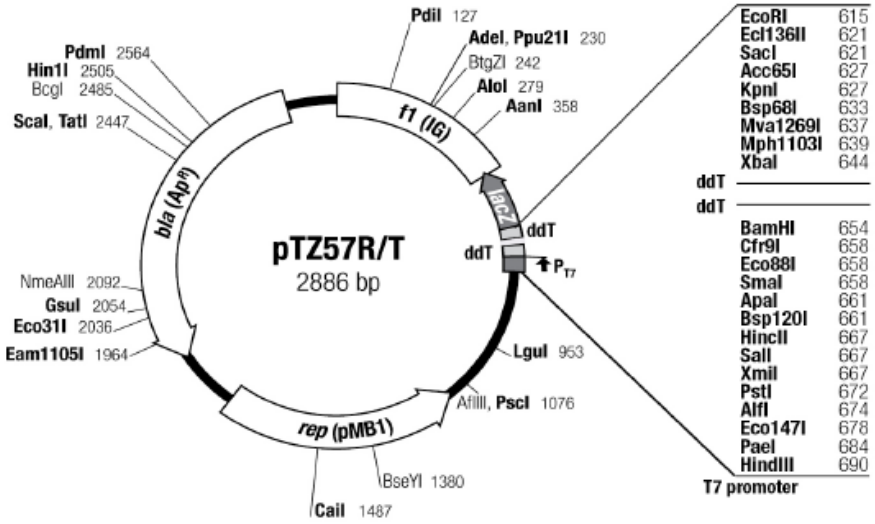

Figure S1. Map of the pTZ57R/T cloning vector, including unique restriction sites, that was used to clone the PCR products (InsTAclone PCR cloning kit, Thermo Scientific, Waltham, Massachusetts). The pTZ57R/T vector sequence can be downloaded from the www.fermentas.com.

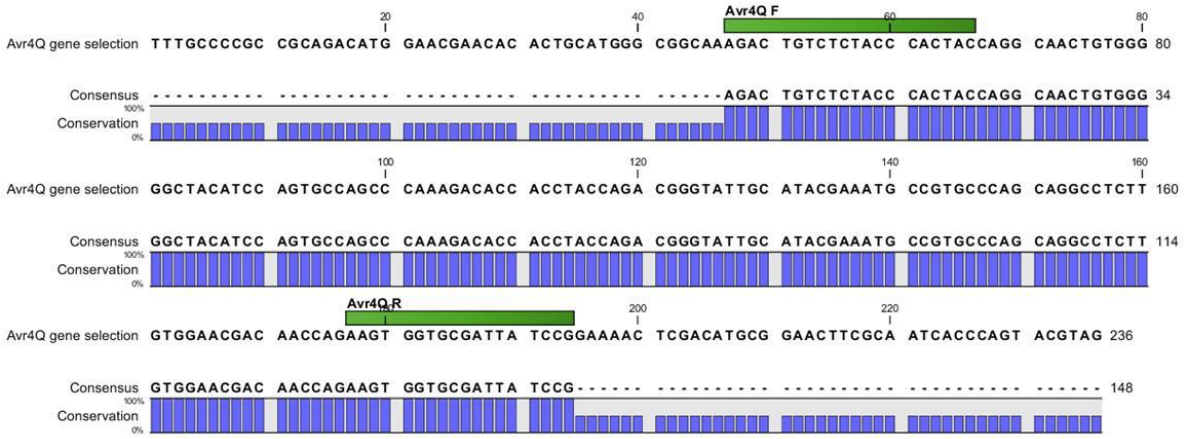

Figure S2. Confirmation of the *Avr4* RT-qPCR product through sequencing.
The RT-qPCR *Avr4* product was cloned and sequenced as confirmation that the amplified product was correct. Forward and reverse sequencing reactions were done from plasmid DNA using standard M13 primers. The forward and reverse sequencing products were combined as a consensus sequence and aligned to the *Avr4* gene region to which the RT-qPCR primers (Avr4Q F and Avr4Q R) (green bars) were designed to. The *Avr4* gene selection included the RT-qPCR *Avr4* gene product (148bp) with up- and downstream regions. The percentage conservation between the aligned regions is indicated by the blue bar graph.

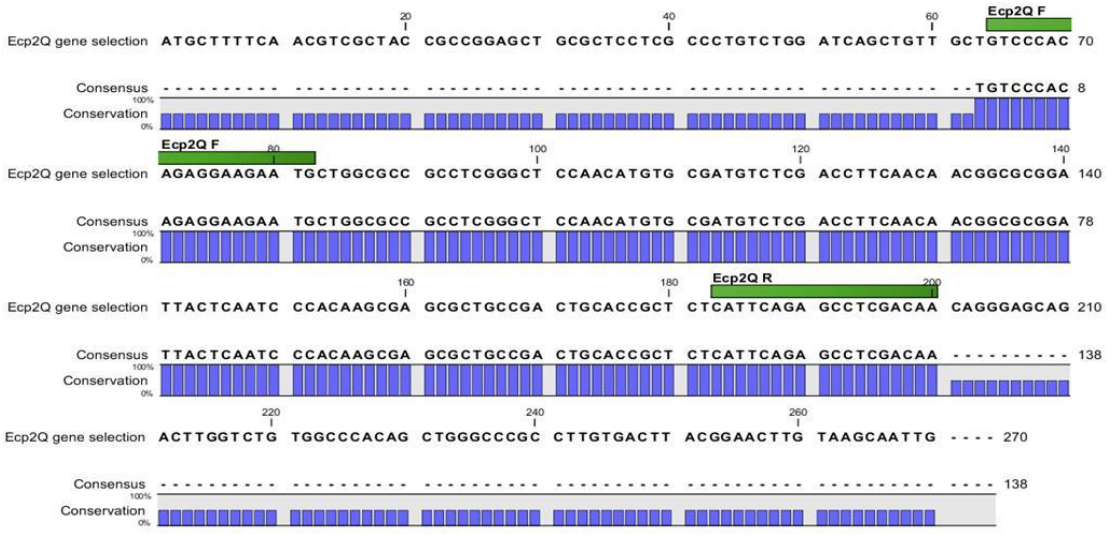

Figure S3. Confirmation of the *Ecp2* RT-qPCR product through sequencing.
The RT-qPCR *Ecp2* product was cloned and sequenced as confirmation that the amplified product was correct. Forward and reverse sequencing reactions were done from plasmid DNA using standard M13 primers. The forward and reverse sequencing products were combined as a consensus sequence and aligned to the *Ecp2* gene region to which the RT-qPCR primers (Ecp2Q F and Ecp2Q R) (green bars) were designed to. The *Ecp2* gene selection included the RT-qPCR *Ecp2* gene product (137bp) with up- and downstream regions. The percentage conservation between the aligned regions is indicated by the blue bar graph.

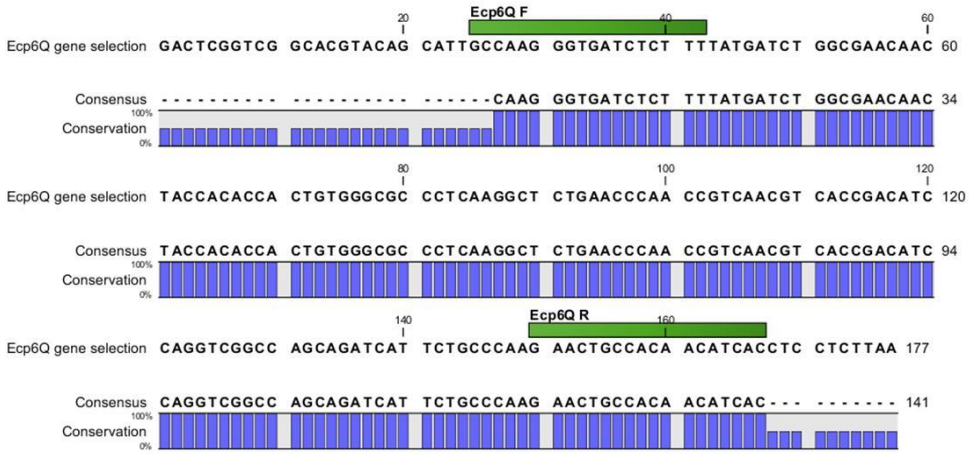

Figure S4. Confirmation of the *Ecp6* RT-qPCR product through sequencing.
The RT-qPCR *Ecp6* product was cloned and sequenced as confirmation that the amplified product was correct. Forward and reverse sequencing reactions were done from plasmid DNA using standard M13 primers. The forward and reverse sequencing products were combined as a consensus sequence and aligned to the *Ecp6* gene region to which the RT-qPCR primers (Ecp6Q F and Ecp6Q R) (green bars) were designed to. The *Ecp6* gene selection included the RT-qPCR *Ecp6* gene product (143bp) with up- and downstream regions. The percentage conservation between the aligned regions is indicated by the blue bar graph.

Figure S5. Confirmation of the *GAPDH* RT-qPCR product through sequencing.
The RT-qPCR *GAPDH* product was cloned and sequenced as confirmation that the amplified product was correct. Forward and reverse sequencing reactions were done from plasmid DNA using standard M13 primers. The forward and reverse sequencing products were combined as a consensus sequence and aligned to the *GAPDH* gene region to which the RT-qPCR primers (GAPDH F and GAPDH R) (green bars) were designed to. The *GAPDH* gene selection included the RT-qPCR *GAPDH* gene product (161bp) with up- and downstream regions. The percentage conservation between the aligned regions is indicated by the blue bar graph.

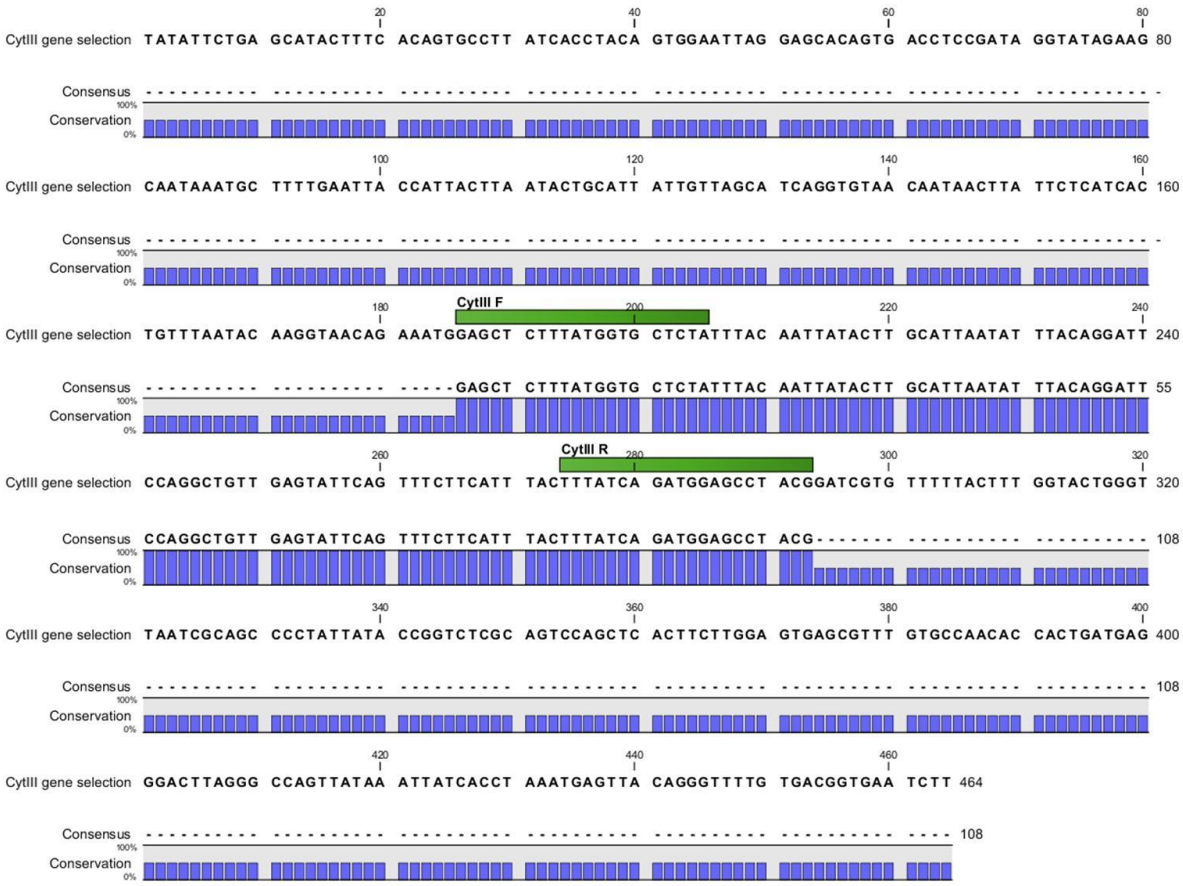

Figure S6. Confirmation of the *Cyt III* RT-qPCR product through sequencing.
The RT-qPCR *Cyt III* product was cloned and sequenced as confirmation that the amplified product was correct. Forward and reverse sequencing reactions were done from plasmid DNA using standard M13 primers. The forward and reverse sequencing products were combined as a consensus sequence and aligned to the *Cyt III* gene region to which the RT-qPCR primers (Cyt III F and Cyt III R) (green bars) were designed to. The *Cyt III* gene selection included the RT-qPCR *Cyt III* gene product (108bp) with up- and downstream regions. The percentage conservation between the aligned regions is indicated by the blue bar graph.

Table S1. *C. zeina* (Mkushi) putative reference gene sequence information. The table includes the six identified *C. zeina* (Mkushi) putative reference genes with the contig numbers, predicted cDNA sequences, and the predicted protein sequence of each gene.

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)

>*C. zeina* (Mkushi) contig NODE_0164488, reverse strand

>Predicted cDNA

ATGGTCGTGAAAGCGGGCATAAATGGCTTCGGGAGAATTGGGAGAATAGTTTTCCGGAATGCAATACAACACGGGGACGTTGAGGTTATTGCCGTAAATGACCCTTTTATAGAGCC
GCACTATGCGGCTTATATGTTAAAGTACGATTCAACTCATGGAATATTCGACGGGAAAATTGAGGTCGACGGCACAAAGGGCTTGATTGTGAATGGCAAAAAGATTCGCTTCTTTAT
GGAAAAAGACCCGGCTGCAATACCCTGGGGTGAAGCAGGTGCAGAGTACATAGTAGAAAGTACTGGGGTCTTTACCACTACTGAGAAGGCTCAGGCCCATATTAAAGGGGGGGC
CAAAAAAGTTGTAATCTCAGCACCAAGCGCAGATGCGCCAATGTTTGTAATGGGCGTTAATAACACTGAATACAAGAGTGACATTCCAGTAATATCGAATGCATCGTGTACAACGAA
CTGCTTGGCACCATTGGCAAAGGTGATCCATAATGAGTTTACAATGATTGAAGGGTTGATGACAACGATACACTCATACACCGCTACACAAAAGACCGTAGATGGACCTTCTGGAAA
AGATTGGAGGGGTGGCCGGACGGCCGCACAAAACATTATTCCATCCAGTACTGGTGCAGCTAAGGCAGTGGGTAAAGTGATCCCAGACTTAAATGGCAAGTTAACGGGAATGTCG
ATGCGCGTCCCTACGGCCAATGTCAGTGTTGTAGATCTAACCTGTCGGATAGAGAAAGGCGCAAGCTACGATGAAATAATTGCAGCGCTTCGAAAGGCGTCCGAGGGCGAATTAA
AAGGTGTCCTCGCTGTGACTGATGACGATGTGGTCAGTTCAGACCTAAACGGCAATATCAACTCGTCCATTGTTGATGTGAAGGCTGGCATTTCGCTAAATAAGAACTTTGTGAAAT
TGGTCTCTTGGTACGACAATGAATGGGGGTACTCTCGCCGGGTAATAGACCTCCTAGCATATATTGCTAAGGTAGATGGCAATGCG

>Predicted protein

MVVKAGINGFGRIGRIVFRNAIQHGDVEVIAVNDPFIEPHYAAYMLKYDSTHGIFDGKIEVDGTKGLIVNGKKIRFFMEKDPAAIPWGEAGAEYIVESTGVFTTTEKAQAHIKGGAKKVVISAPS
ADAPMFVMGVNNTEYKSDIPVISNASCTTNCLAPLAKVIHNEFTMIEGLMTTIHSYTATQKTVDGPSGKDWRGGRTAAQNIIPSSTGAAKAVGKVIPDLNGKLTGMSMRVPTANVSVVDLTC
RIEKGASYDEIIAALRKASEGELKGVLAVTDDDVVSSDLNGNINSSIVDVKAGISLNKNFVKLVSWYDNEWGYSRRVIDLLAYIAKVDGNA

Beta-tubulin (*β-TUB*)

>C. zeina (Mkushi) contig NODE_0145834, forward strand

>Predicted cDNA

ATGCCCACGACAGCGTGGGGCACTCGTCCCCCGTCTAACCGCCCAGTTTGTGCCGCGTTTTGGCAGACCATTAGCGGAGAACATGGTTTGGATGGATCAGGTGTCTATAATGGAA
CGAGCGATCTTCAATTGGAGCGGATGAATGTCTACTTTAACGAGGCATCTGGGAACAAATATGTTCCACGGGCTGTCCTTGTTGATCTTGAACCCGGGACTATGGACGCTGTCCGG
GCTGGACCGTTTGGACAGCTATTTCGGCCCGACAATTTCGTTTTTGGCCAGTCGGGAGCCGGGAACAACTGGGCAAAAGGCCATTACACAGAGGGAGCTGAGCTTGTGGATCAAG
TCCTCGATGTGGTCAGAAGAGAGGCTGAGGGATGTGATTGTCTTCAGGGATTCCAAATTACCCACTCGCTGGGAGGGGGCACAGGCGCAGGTATGGGCACGCTTCTGATTAGTAA
GATCAGGGAGGAATTTCCAGACCGGATGATGGCGACATTTTCCGTAATGCCCTCACCCAAAGTTAGTGATACGGTAGTGGAGCCCTATAATGCCACCCTATCTGTCCACCAGCTCG
TTGAAAATTCGGATGAAACTTTTTGCATCGACAACGAGGCCTTGTATGACATATGTATGCGGACCCTGAAGCTAAATAATCCCTCTTACGGGGACCTCAACCACTTAGTTTCAGCCG
TGATGAGTGGTGTAACGACGTGCTTGCGCTTTCCGGGACAGTTGAATAGTGATCTTAGAAAACTTGCCGTAAATATGGTTCCTTTCCCTAGGCTCCACTTCTTCATGGTAGGTTTTG
CCCCGTTAACATCACGCGGAGCTCACTCTTTTCGCGCGGTGACCGTTCCCGAATTGACCCAACAAATTTTCGATCCAAAAAACATGATGGCGGCTAGTGACTTTCGGAATGGTCGA
TACCTTACGTGTAGTGCCATTTATCGTGGTAAGGTGTCGATGAAGGAGGTCGAGGATCAAATTCGCAACGTGCAAAACAAGAACACAGCCTACTTTGTAGAGTGGATTCCGAATAA
CGTGCAAACTGCGCTTTGCTCGATTCCTCCGCGCGGGCTCAAAATGTCGTCCACCTTCGTAGGGAATTCTACCTCCATTCAGGAACTTTTCAAAAGGGTAGGTGACCAATTCACGG
CAATGTTTCGGCGTAAGGCATTTTTGCACTGGTACACTGGGGAAGGAATGGACGAGATGGAATTTACCGAGGCTGAGTCGAACATGAACGACCTTGTGTCAGAATATCAACAATAC
CAAGAAGCCAGCGTTTCAGAGGGGGAGGAGGAGTATGACGAAGAAGCCCCTTTGGAAGGGGAGGAA

>Predicted protein
MPTTAWGTRPPSNRPVCAAFWQTISGEHGLDGSGVYNGTSDLQLERMNVYFNEASGNKYVPRAVLVDLEPGTMDAVRAGPFGQLFRPDNFVFGQSGAGNNWAKGHYTEGAELVDQVL
DVVRREAEGCDCLQGFQITHSLGGGTGAGMGTLLISKIREEFPDRMMATFSVMPSPKVSDTVVEPYNATLSVHQLVENSDETFCIDNEALYDICMRTLKLNNPSYGDLNHLVSAVMSGVTT
CLRFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGAHSFRAVTVPELTQQIFDPKNMMAASDFRNGRYLTCSAIYRGKVSMKEVEDQIRNVQNKNTAYFVEWIPNNVQTALCSIPPR
GLKMSSTFVGNSTSIQELFKRVGDQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVSEYQQYQEASVSEGEEEYDEEAPLEGEE

Elongation factor (*EF-1*)

>*C. zeina* (Mkushi) contig NODE_2010117, reverse chain

>Predicted cDNA
ATGGGGAAAGAGAAAACCCATATCAACGTCGTTGTTATTGGACACGTGGATAGCGGAAAGTCCACCACCACGGGTCATCTTATTTACAAATGCGGTGGCATCGATAAGAGGACGAT
TGAAAAATTCGAGAAAGAGGCTGCCGAGTTAGGGAAGGGGTCTTTCAAGTACGCATGGGTATTGGATAAGCTAAAGGCGGAAAGGGAAAGAGGCATAACTATCGATATCGCTCTG
TGGAAATTCGAAACCCCGAAGTACTACGTAACCGTGATAGATGCTCCTGGACATCGCGACTTCATCAAGAATATGATTACGGGCACGAGCCAAGCTGACTGTGCGGTTTTGATAAT
CGCAGCCGGCACTGGGGAGTTTGAAGCTGGAATTAGCAAGGATGGCCAGACTCGGGAACATGCCCTGCTAGCTTATACTTTGGGAGTTAAACAGTTAATAGTCGCTATCAATAAGA
TGGATACAACTAAATGGTCTGAGGACCGGTACAACGAAATAATCAAAGAAACTTCATCATTCATTAAGAAGGTGGGCTATAACCCTAAAACTGTACCTTTTGTACCCATTTCAGGTTT
TAACGGTGACAACATGATAGACAATTCTACAAACTGCCCGTGGTACAAGGGGTGGGAGAAGGAGACAAAAACAAAGACCACAGGCAAGACCCTGCTTGAAGCCATTGATGCAATA
GACCCACCCCAACGTCCGACCGAAAAGCCCCTCAGATTGCCCCTACAGGACGTGTACAAGATAGGCGGAATCGGCACTGTGCCAGTCGGTCGAGTAGAAACAGGTATAATAAAAG
CTGGAATGGTCGTAACCTTTGCGCCAGCCGGTGTGACCACCGAAGTGAAATCAGTAGAGATGCATCACGAGCAACTGACCGAAGGGTTGCCCGGTGATAATGTGGGATTTAATGT
CAAAAATGTATCAGTCAAAGAAATACGTAGGGGCAACGTGGCCGGCGACTCTAAAAGTGACCCTCCCAAGGGCTGTGATTCTTTTAACGCGCAAGTGATAGTACTGAATCACCCAG
GACAGGTGGGCGCCGGATATGCCCCGGTCCTCGACTGTCACACAGCGCATATTGCATGTAAGTTTTCCGAGCTATTGGAAAAGATAGATAGACGCTCAGGAAAATCAATAGAAAAT
TCGCCTAAATTTATTAAATCCGGTGATGCTGCGATCGTGAAGATGGTACCTTCTAAACCAATGTGTGTGGAGGCATTCACTGATTACCCACCTTTGGGACGATTCGCAGTACGTGAC
ATGAGGCAGACGGTAGCCGTTGGGGTAATAAAATCTGTCGTGAAAAGTGACAAAGGCGGAGCGGGGAAAGTCACCAAGGCGGCCCAGAAAGCGTCTAAGAAA

>Predicted protein
MGKEKTHINVVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAELGKGSFKYAWVLDKLKAERERGITIDIALWKFETPKYYVTVIDAPGHRDFIKNMITGTSQADCAVLIIAAGTGEFEA
GISKDGQTREHALLAYTLGVKQLIVAINKMDTTKWSEDRYNEIIKETSSFIKKVGYNPKTVPFVPISGFNGDNMIDNSTNCPWYKGWEKETKTKTTGKTLLEAIDAIDPPQRPTEKPLRLPLQD
VYKIGGIGTVPVGRVETGIIKAGMVVTFAPAGVTTEVKSVEMHHEQLTEGLPGDNVGFNVKNVSVKEIRRGNVAGDSKSDPPKGCDSFNAQVIVLNHPGQVGAGYAPVLDCHTAHIACKFS
ELLEKIDRRSGKSIENSPKFIKSGDAAIVKMVPSKPMCVEAFTDYPPLGRFAVRDMRQTVAVGVIKSVVKSDKGGAGKVTKAAQKASKK

Cytochrome c oxidase subunit III (*Cyt III*)

>*C. zeina* (Mkushi) contig NODE_0000096, reverse strand

>Predicted cDNA
ATAGGTATAGAAGCAATAAATGCTTTTGAATTACCATTACTTAATACTGCATTATTGTTAGCATCAGGTGTAACAATAACTTATTCTCATCACTGTTTAATACAAGGTAACAGAAATGGA
GCTCTTTATGGTGCTCTATTTACAATTATACTTGCATTAATATTTACAGGATTCCAGGCTGTTGAGTATTCAGTTTCTTCATTTACTTTATCAGATGGAGCCTACGGATCGTGTTTTTAC
TTTGGTACTGGGTTAATC

>Predicted protein
IGIEAINAFELPLLNTALLLASGVTITYSHHCLIQGNRNGALYGALFTIILALIFTGFQAVEYSVSSFTLSDGAYGSCFYFGTGLI

Cytochrome b (*Cyt b*)

>*C. zeina* (Mkushi) contig_00001, reverse strand

>Predicted cDNA
TTTTTTTTTATGTTTAAATGTAAATCAATTAAACTATTTTCTAATAAACTAAGTAAAGGCACTATAATTAAAAAGTGTGAAAAATATAACACAGTACTAATTTGACCAAACTCTATAAAAG
GCGATTCAACGTGTTTTGCTCCAAGTTGCATTAATACAAGAAAGTTAGCTACAAAAACGTAGAATGCTATTTTACTTAAAGGTCTAAACTGTAACCCTCTGCTTCTACCTAGGTCTGT
GAAGGGCAT

>Predicted protein
MPFTDLGRSRGLQFRPLSKIAFYVFVANFLVLMQLGAKHVESPFIEFGQISTVLYFSHFLIIVPLLSLLENSLIDLHLNIKK

40S ribosomal protein (*40S*)

>*C. zeina* (Mkushi) contig NODE_0145834, forward strand

>Predicted cDNA
ATGTCGCAACAAGCTCTAAACAAGATAGCACCCAATAGTCCATCTAGGCAAAAGCCTAACGAAACTGAGCAACAGATCGCGACTGCCTTATATGAACTAGAGTCAAATATACCAGAT
ATGAAGGCAGCTCTCCGACCGCTACAGTTTGTCAGTGCCCGTGAGGATAAATACATCTTGGACCCTACGAGGCGTGGGGGAGGTCATTGGGCTGCATTTGCCCTGACGCCGGGT
TTCCTCCAGATAGAAGTTGGCCATGGCCGCAAGGCCATAGTGATATTCGTCCCGGTTCCACTACTGCAAGGATGGCACCGTTCACAACAGCGTCTGACGCGTGAGCTAGAGAAGA
AATTTTCGGACCGGCATGTACTTGTAGTTGCGTCCCGTCGAATCTTACCCAGACCCAAAAGGTCCAATAGAAGTAGGACGTCCCAGACCCAGAAGCGTCCCCGAAGTCGAACGCT
TACTGCGGTACATGATGCAATCTTGGCTGATTTAGTATATCCTGTTGAGATCGTGGGCAAGAGACTTCGAACAAAAGAGGACGGATCCAAAGTATTAAAAGTGATACTAGACGAGAA
AGAGCGTGGCGGGGTTGACTATAGGCTGGATACGTATTCCGAAGTATACAAAAGGCTTACAGGTAAGGGTGTGAACTTTGAATTTCCTCAAGCCCCGGCAGAATAT

>Predicted protein
MSQQALNKIAPNSPSRQKPNETEQQIATALYELESNIPDMKAALRPLQFVSAREDKYILDPTRRGGGHWAAFALTPGFLQIEVGHGRKAIVIFVPVPLLQGWHRSQQRLTRELEKKFSDRHV
LVVASRRILPRPKRSNRSRTSQTQKRPRSRTLTAVHDAILADLVYPVEIVGKRLRTKEDGSKVLKVILDEKERGGVDYRLDTYSEVYKRLTGKGVNFEFPQAPAEY

Table S2. *C. zeina* (Mkushi) reference gene homologs identified through tBLASTx analysis. The table includes the fungal organism from which the hit was obtained, the GenBank accession numbers, the significance value of the hit, as well as the authors.

Fungal organism	GenBank Accession	Significance of hit*	Author(s)
GAPDH			
Mycosphaerella graminicola	XM003855615.1	0.0	(GOODWIN *et al.* 2011)
Pseudocercospora fijiensis	XM007925366.1	0.0	(OHM *et al.* 2012)
Neurosporra crassa	XM951884.2	0.0	(GALAGAN *et al.* 2003)
B-TUB			
Mycosphaerella graminicola	XM003856727.1	0.0	(GOODWIN *et al.* 2011)
Pseudocercospora fijiensis	XM007921924.1	0.0	(OHM *et al.* 2012)
Venturia inaequalis	EU853839.1	0.0	(Kucheryava et al unpublished)
EF-1			
Baudoinia compniacensis	XM007673622.1	0.0	(OHM *et al.* 2012)
Pseudocercospora fijiensis	XM007922858.1	0.0	(OHM *et al.* 2012)
Neofusicoccum parvum	XM007579574.1	0.0	(Blanco-Ulate et al unpublished)
Cyt III			
Mycosphaerella graminicola	EU090238.1	1e-35	(TORRIANI *et al.* 2008)
Rhynchosporium secalis	KF650575.1	3e-32	(TORRIANI *et al.* 2014)
Rhynchosporium orthosporum	KF650574.1	3e-32	(TORRIANI *et al.* 2014)
Cyt b			
Cercospora beticola	JQ360628.1	5e-34	(BIRLA *et al.* 2012)
Mycosphaerella graminicola	AY247413.1	2e-32	(FRAAIJE *et al.* 2005)
Mycosphaerella fijiensis	AF343070.1	3e-32	(SIEROTZKI *et al.* 2000)
40S			
Pseudocercospora fijiensis	XM007921573.1	5e-116	(OHM *et al.* 2012)
Mycosphaerella graminicola	XM003853515.1	6e-114	(GOODWIN *et al.* 2011)
Baudoinia compniacensis	XM007673756.1	6e-113	(OHM *et al.* 2012)

***Significance (e-value) of the hit. Values close to zero represent significant hits.

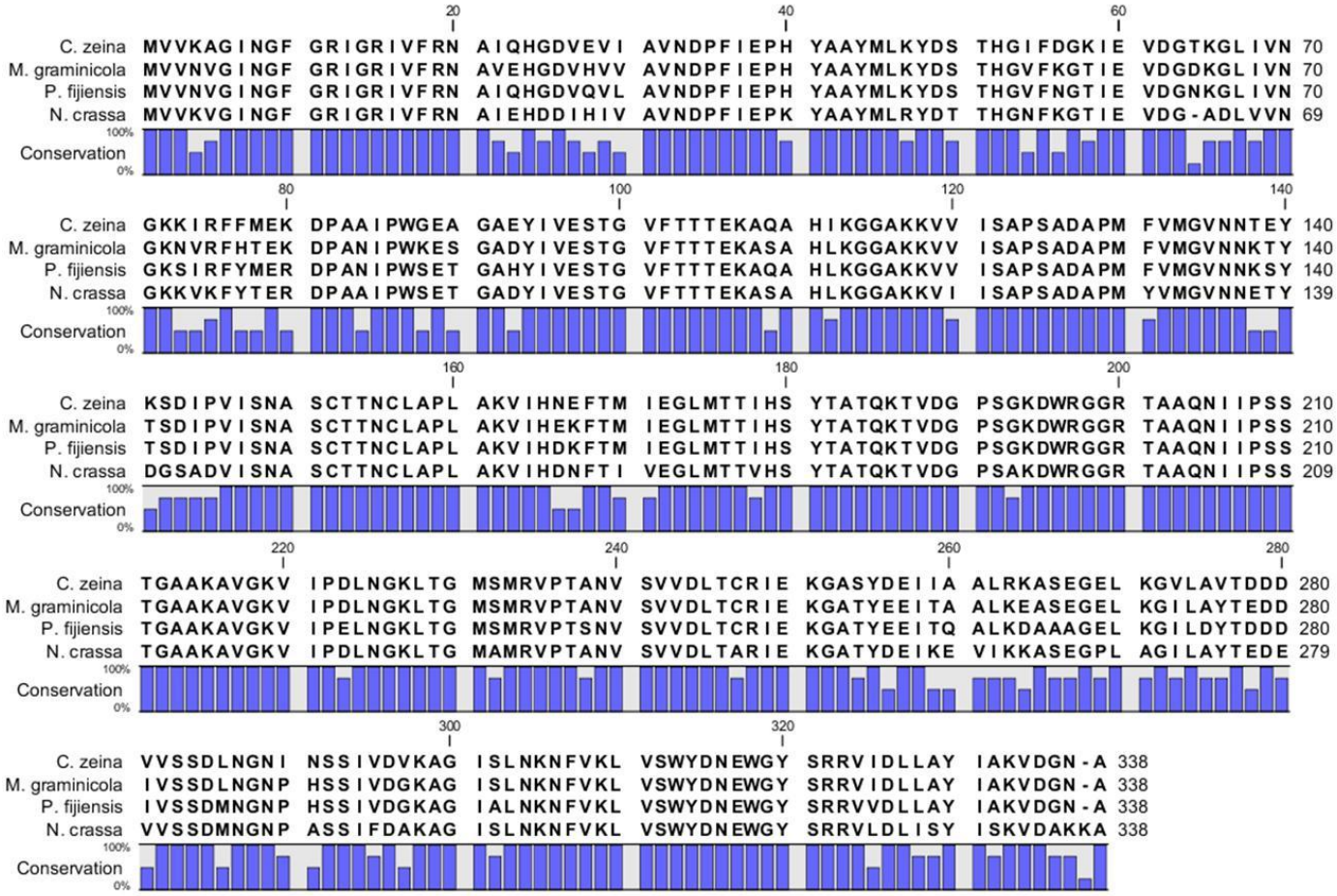

Figure S7. GAPDH protein alignment.
Three protein homologs identified through tBLASTx analysis were aligned with the predicted *C. zeina* (Mkushi) GAPDH protein. Percentage conservation between the sequences was indicated by the blue bar graph. The protein alignment showed high conservation between all four sequences.

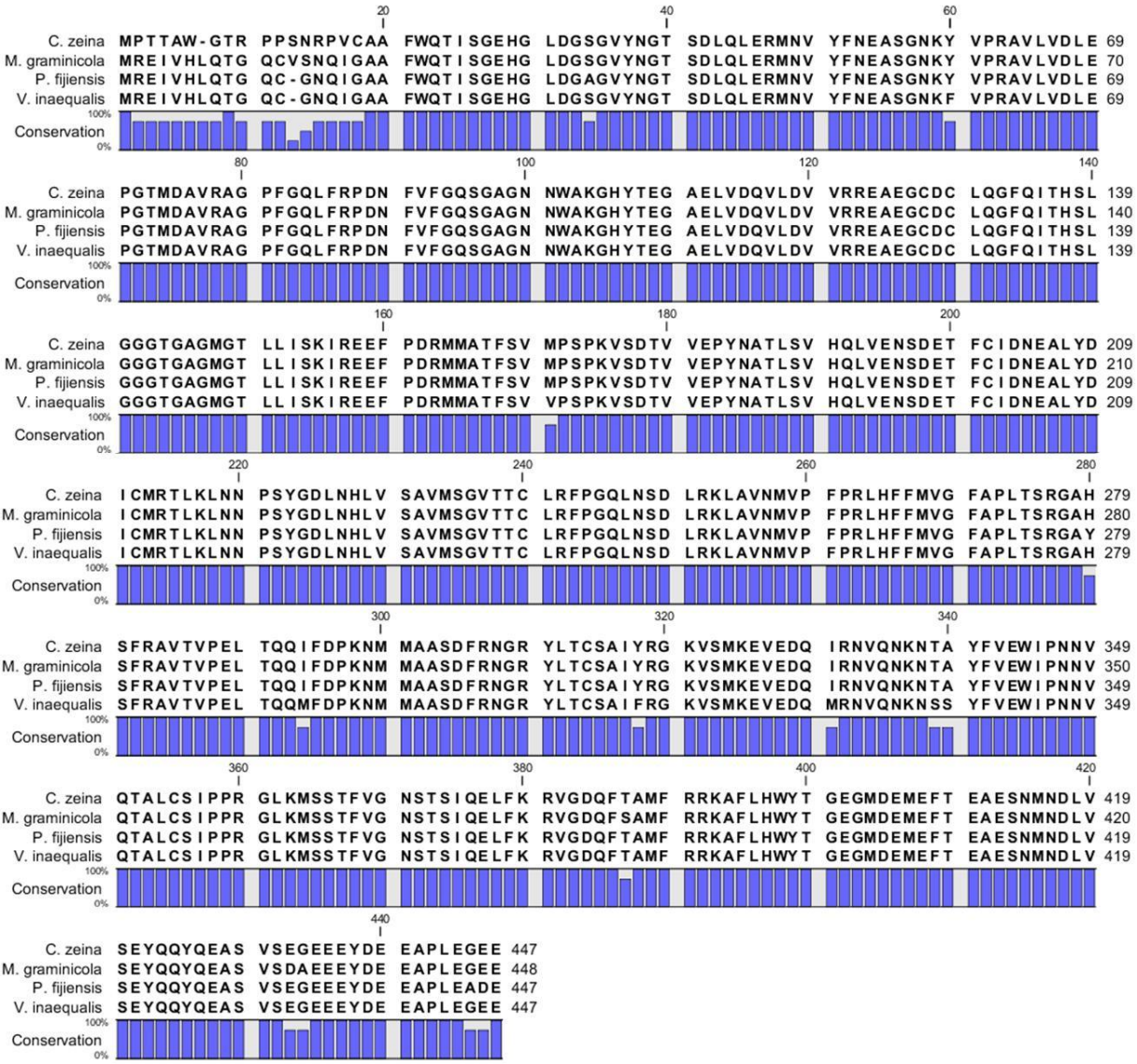

Figure S8. β-TUB protein alignment.

Three protein homologs identified through tBLASTx analysis were aligned with the predicted *C. zeina* (Mkushi) β-TUB protein. Percentage conservation between the sequences was indicated by the blue bar graph. The protein alignment showed high conservation between all four sequences.

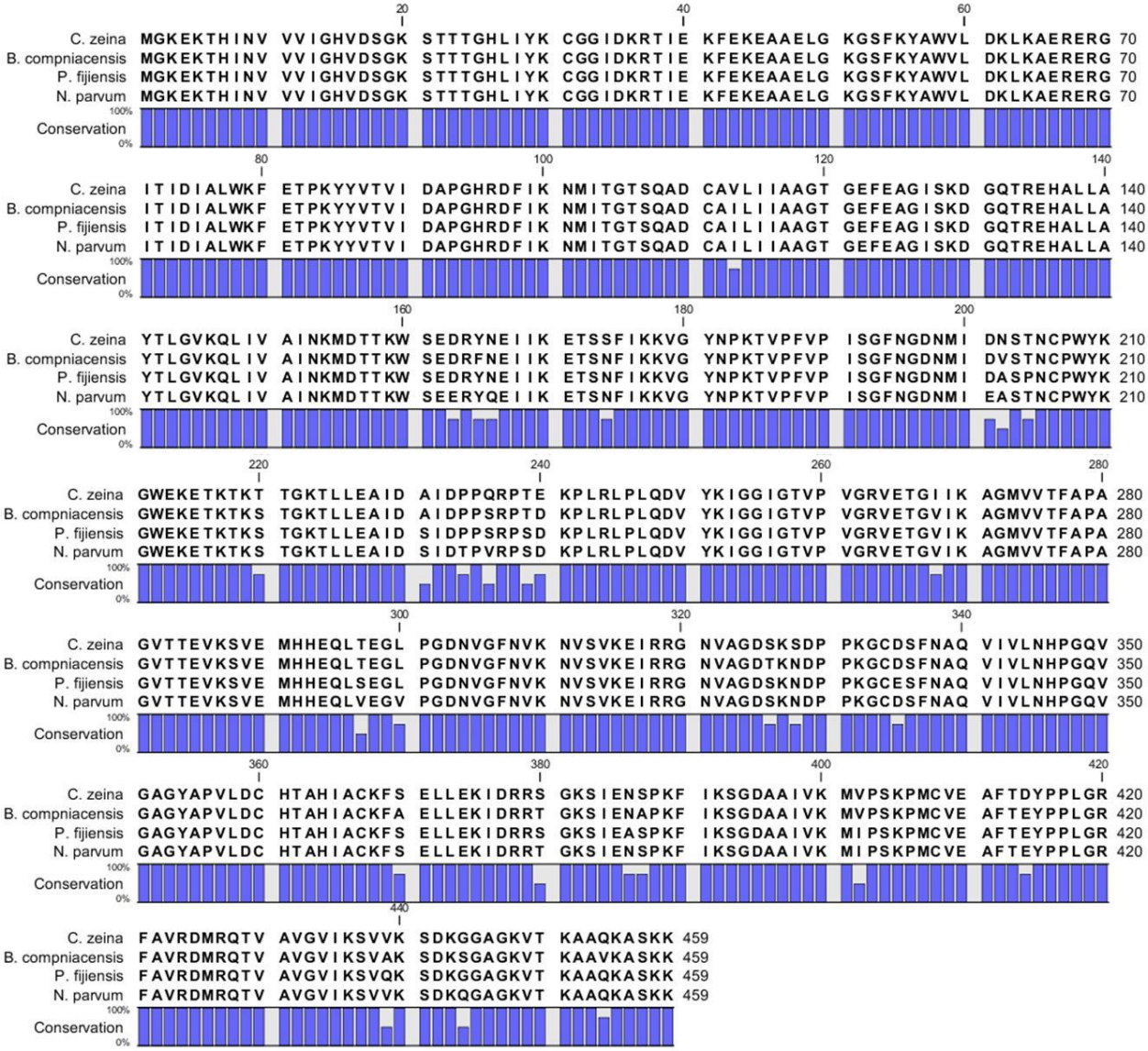

Figure S9. EF-1 protein alignment.

Three protein homologs identified through tBLASTx analysis were aligned with the predicted *C. zeina* (Mkushi) EF-1 protein. Percentage conservation between the sequences was indicated by the blue bar graph. The protein alignment showed high conservation between all four sequences.

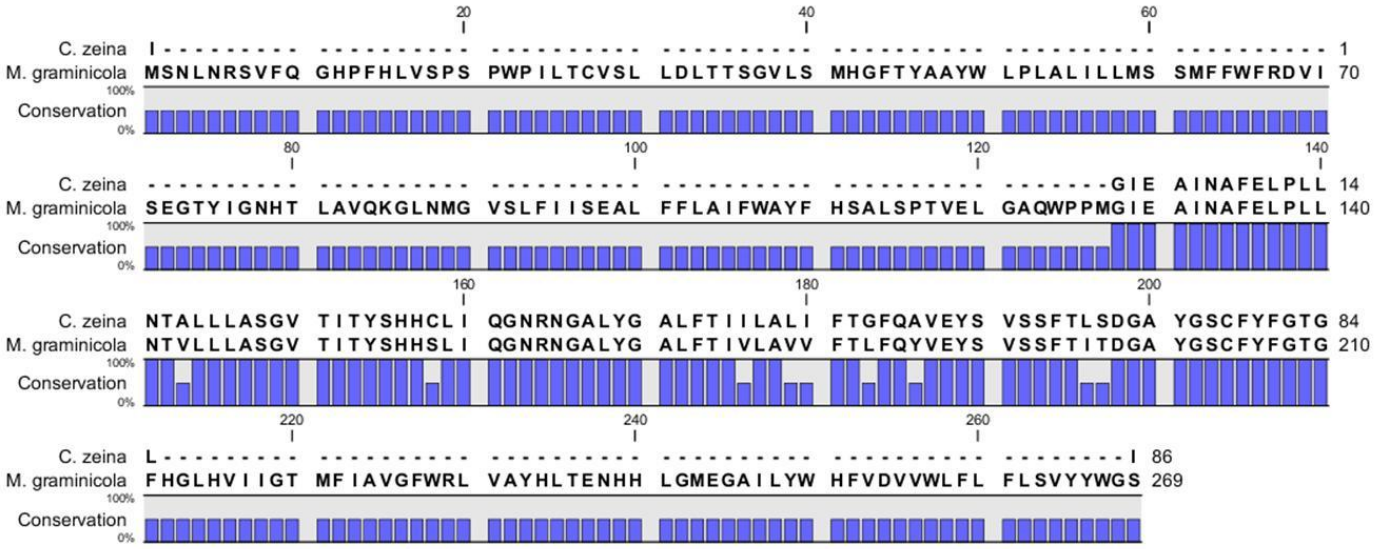

Figure S10. Cyt III protein alignment.

The *M. fijiensis* protein homolog identified through tBLASTx analysis was aligned with the predicted *C. zeina* (Mkushi) Cyt III protein. The predicted C. zeina Cyt III protein was much shorter than the predicted *M. fijiensis* Cyt III. Percentage conservation between the sequences was indicated by the blue bar graph. The aligned region between the two proteins shared high sequence similarity. The predicted *C. zeina* Cyt III protein is most likely longer when compared to the *M. fijiensis* sequence. However, for expression studies the identified *C. zeina* Cyt III partial sequence is sufficient for primer design purposes. primers Only the *M. fijiensis* predicted Cyt III protein sequence was included in the alignment as the other identified homolog sequences were from distantly related fungal organisms and did not share significant sequence similarity to the *C. zeina* Cyt III protein.

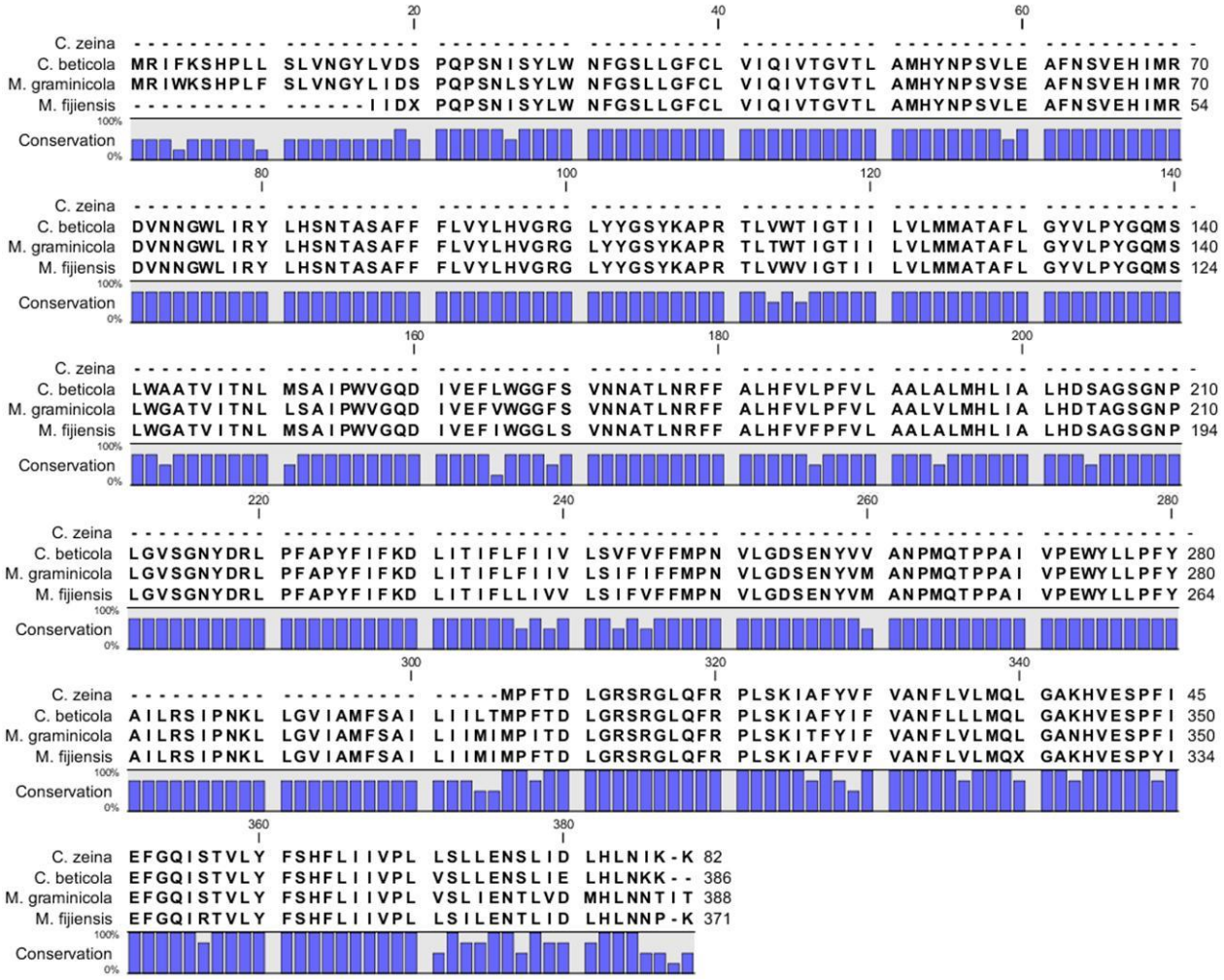

Figure S11. Cyt b protein alignment.

Three protein homologs identified through tBLASTx analysis were aligned with the predicted *C. zeina* (Mkushi) Cyt b protein. Percentage conservation between the sequences was indicated by the blue bar graph. The C. zeina (Mkushi) Cyt b protein was predicted to be much smaller than the other protein homologs; however the aligned region showed high conservation. The predicted *C. zeina* Cyt b protein is most likely longer when compared to the Cyt b homolog sequences. However, for expression studies the identified *C. zeina* Cyt b partial sequence is sufficient for primer design purposes.

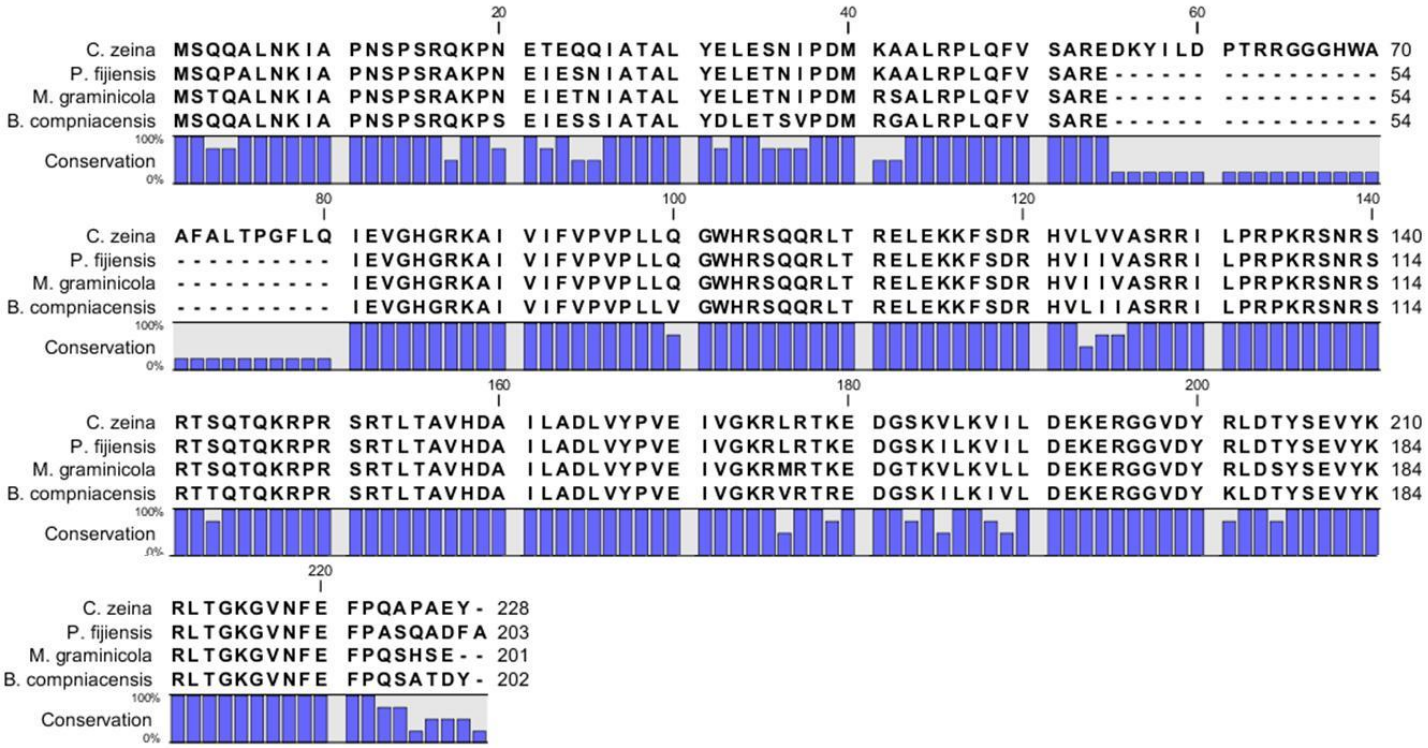

Figure S12. 40S protein alignment.
Three protein homologs identified through tBLASTx analysis were aligned with the predicted *C. zeina* (Mkushi) 40S protein. Percentage conservation between the sequences was indicated by the blue bar graph. The protein alignment showed high conservation between all four sequences.

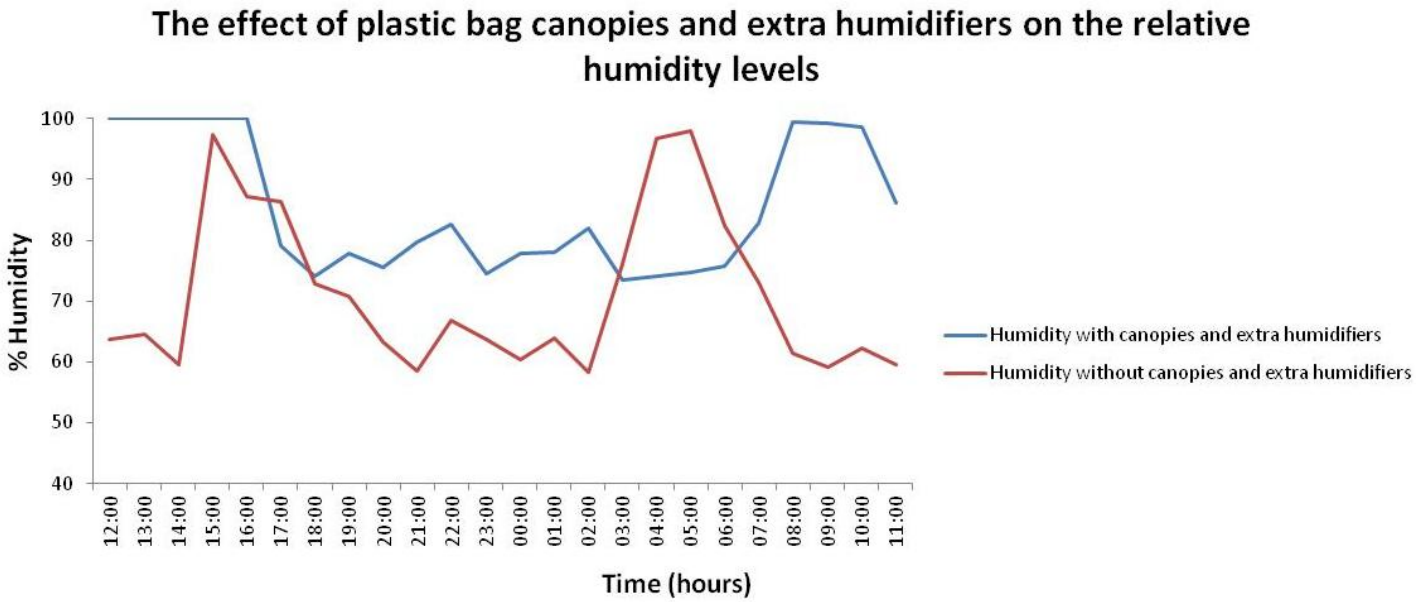

Figure S13. A comparison of the humidity levels in the presence and absence of plastic bag canopies and extra humidifiers. The humidity levels of two separate days (24 hours) were compared to determine the effect of plastic bag canopies and extra humidifiers. Data from the one day represents humidity levels in the presence of plastic bag canopies and extra humidifiers (Blue line). Data from the other day represents humidity levels in the absence of plastic bag canopies and extra humidifiers (Red line). From the graph it is clear that plastic bag canopies and extra humidifiers were needed to maintain humidity levels above 75%. Without the plastic bag canopies and extra humidifiers, the humidity levels fluctuated between 60-100%. Humidity readings were recorded with a HOBO® Pro v2 external temperature and relative humidity data logger.

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