chi3 Gene Encodes a Chitinase, CHIT30: An Attempt To Locate chi3 in Entomopathogenic Fungi

Pamela Greengarten
Department of Biological Sciences
Fordham University

Abstract

Entomopathogenic fungi are naturally occurring pathogens of some insects that produce enzymes capable of penetrating into an insect's cuticle. Chitinases are thought to be responsible for entomopathogenic fungi's ability to degrade cuticle. Primers were designed for the chi3 gene which encodes a chitinase CHIT30, from the mRNA transcript present in *Metarhizium anisopliae*. These primers were unable to amplify the gene in *Beauveria bassiana, Paecilomyces fumosoroseus* and *Paecilomyces farinosus*.

Introduction

Entomopathogenic fungi are naturally occurring fungi that act as pathogens towards insects, ticks and other arthropods. Traditionally, tick populations have been regulated through the use of acaricides but due to the evolution of resistance, (Ostfeld et al. 2006, Roush 1993), biological control (biocontrol) is being looked at as a way of controlling pest populations. Entomopathogenic fungi have been demonstrated to kill various insects including ticks (Ostfeld et al. 2006, Samish and Rehacek 1999), beetles (Hluchy and Samseinakova 1989, Lord 2001), locusts (Hernandez-Crespo and Santiago-Alvarez 1997) and *Triatoma infestans* (Luz et al. 2004). Entomopathogenic fungi have also been demonstrated to kill anopheline mosquitoes which are the vector for malaria parasites. (Kanzok and Jacobs-Lorena 2006). The fungi have proteases, chitinases, and toxins which allow the fungi effectively attack various insects. Since entomopathogenic

fungi have many means of attacking insects, it is unlikely that insects will develop anti-fungal resistance (Scholte et al. 2004).

Fungal chitinases are enzymes which are required for hyphal growth and are thought to be partially responsible for host penetration (Takaya et al. 1998, Tikhanov et al. 2002). Additionally, chitinases break down chitin microfibrils which make up approximately 30 % of a ticks cuticle. (Silva et al 2005, Tikhanov 2002). In this study, I sought to locate a particular chitinase gene in four entomopathogenic fungi. The chi3 gene encodes the CHIT30 chitinase that has been sequenced and is present in the NCBI database for the species *Metarhizium anisopliae (M. anisopliae)*. This gene has not been characterized in three common entomopathogenic fungi, namely *Beauveria bassiana (B. bassiana), Paecilomyces fumosoroseus (P. fumosoroseus) and Paecilomyces farinosus (P. farinosus)*. I sought to characterize the chi3 gene sequence of these species.

Materials and Methods

Primer Design

Primers were designed for the chi3 gene from sequencing data obtained from the NCBI database for *M. anisopliae*. Primers were designed with approximately 50 % G-C content and were 20 base pairs long. Primers were designed to amplify between 170 and 209 base pairs. Forward and reverse primers were designed to overlap one another so no area of the gene was left uncovered. Primers were blasted using the NCBI database.

A pair of primers termed Ba02 were used as a positive control. They amplify a microsatellite marker in *B. bassiana* which is expected to be common to all species used (Rehner and Buckley 2003).

Primer Pairs #1 and #2 that generated product from the chi3 gene are shown in Table 1. Primer PairBa02 was used to amplify the microsatellite loci designed for *B. bassiana* and is also shown in Table 1.

Table 1. Primers used to amplify the chi3 gene and the microsatellite loci.

Primer Name	Amplification Size	Forward Primer	Reverse Primer
Primer Pair #1	170 bp	5' GGACACAAGC TCACCGTCTA 3'	5' GCTTTCTTCTG AGCCTGCGA 3'
Primer Pair #2	209 bp	5' CCAAGATGAG TATCGGCAAC 3'	5' CAGTGTTGGC AATCTGTTGG 3'
Primer Pair Ba02	140 bp	5' AACGCTATGCCTTGACGAC 3'	5' GACGCCGAGCAATGTAACA 3'

Fungi Inoculation and Storage

Four species of fungi were inoculated from samples previously found at the Louis Calder Center in Armonk, NY. Dr. Amy Tuininga graciously donated purified samples of *M. anisopliae, B. bassiana, P. fumosoroseus*, and *P. farinosus*. Inoculated samples were grown on Potato Dextrose Agar (PDA) medium and used for the purposes of this study. SAMPLES were kept at room temperature to allow growth for one week. DNA was then extracted before the plates were completely grown with fungus at which point fungi were transferred to the refrigerator for storage.

DNA Extraction and Purification

A sterile scalpel was used to scrape the fungus off of the PDA medium paying careful attention to scrape the conidia and not only spores. The fungal material was transferred to a 2 ml microcentrifuge tube and submerged in liquid nitrogen. The frozen

fungus was thoroughly crushed with a sterile glass rod until ground to a fine powder. The cells were lysed by adding 400 µl Buffer AP1 and 4 µl RNase A stock solution provided by Qiagen to the powder and incubating at room temperature for 10 minutes. The total DNA was purified from the fungal tissue using DNeasy Plant Mini Kit (Qiagen) according to protocol. DNA was eluted twice with 50 µl Buffer AE incubated at room temperature for five minutes before centrifuging at 8,000 rpm for one minute. The DNA was quantified using a spectrophotometer and the aliquot with the higher DNA concentration was used in subsequent experiments.

PCR

PCR was performed following the Promega protocol for 25 µl reactions. Each reaction contained 12.5 µl Go Taq Green Master Mix 2X, 0.5 µl Forward Primer (10 pmol), 0.5 µl Reverse Primer (10 pmol), 6.5 µl Nuclease-Free Water and 5 µl DNA template (2.0 ng). In negative controls, the DNA template was replaced with an equal amount of Nuclease-Free Water.

Cycles of PCR were as follows: Initial denaturation was for 5 minutes at 94° C for 1 cycle, denaturation at 94° C for 30 seconds, annealing at 58° C for 30 seconds, extension at 72° C for 30 seconds for 50 cycles. Final extension was at 72° C for 7 minutes.

6 µl of each PCR reaction were run on a 1.5% agarose gel containing ethidium bromide in order to separate nucleic acids based on size. The gel was visualized under UV light and a photograph was taken.

PCR Product Purification

The Marligen Rapid PCR Purification protocol was followed to purify PCR products. DNA was eluted with 50 µl of 70° C Nuclease-Free Water instead of TE Buffer. Concentrations of DNA were determined using a spectrophotometer.

Sequencing

Samples prepared for DNA Sequencing were sent out to GENEWIZ for sequence analysis. Samples consisted of 8 µl of PCR product and 0.8 µl of either Forward or Reverse Primer. Sequences were blasted in the NCBI database and compared to known sequences.

Results

The primers designed to amplify a region of the chi3 gene generated the expected 170 and 209 base pair products for *M. anisopliae*. No PCR products were generated for the other three species (Figure 1).

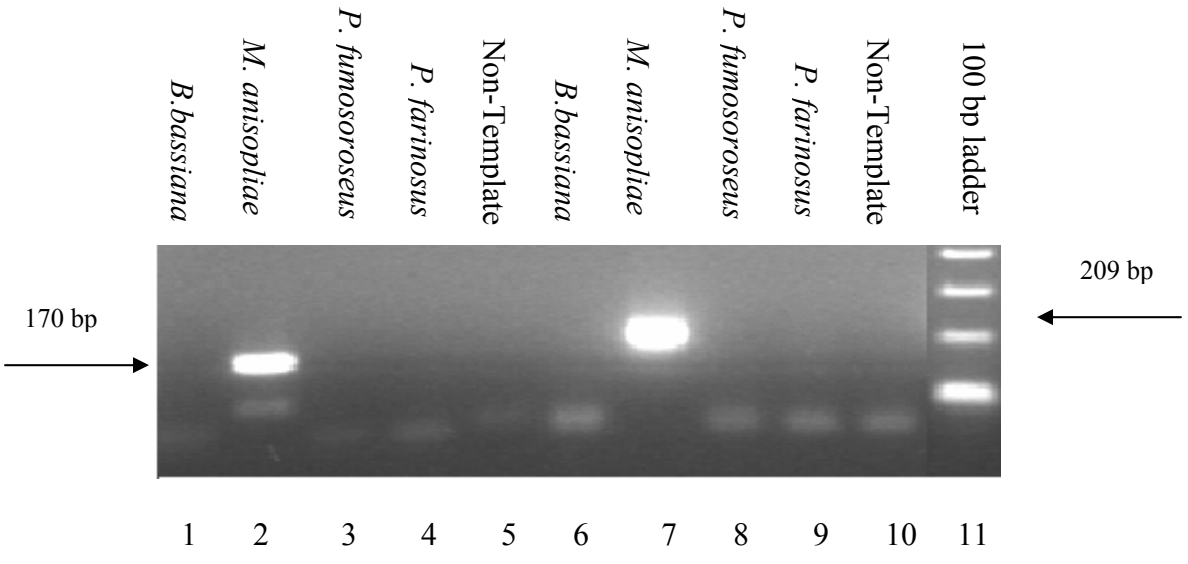

Figure 1. PCR products using Primer Pair #1 , Lanes 1-5 and Primer Pair # 2, Lanes 6-10. Sizes of the PCR products were determined by using the 100 bp ladder as a comparison. The 170 and 209 bp products were of the expected size. *M. anisopliae* was the only species that generated product.

When the primers designed for a microsatellite marker for *B. bassiana* were used as the positive control, all 4 species generated strong, clear bands of the expected 140 bp size (Figure 2).

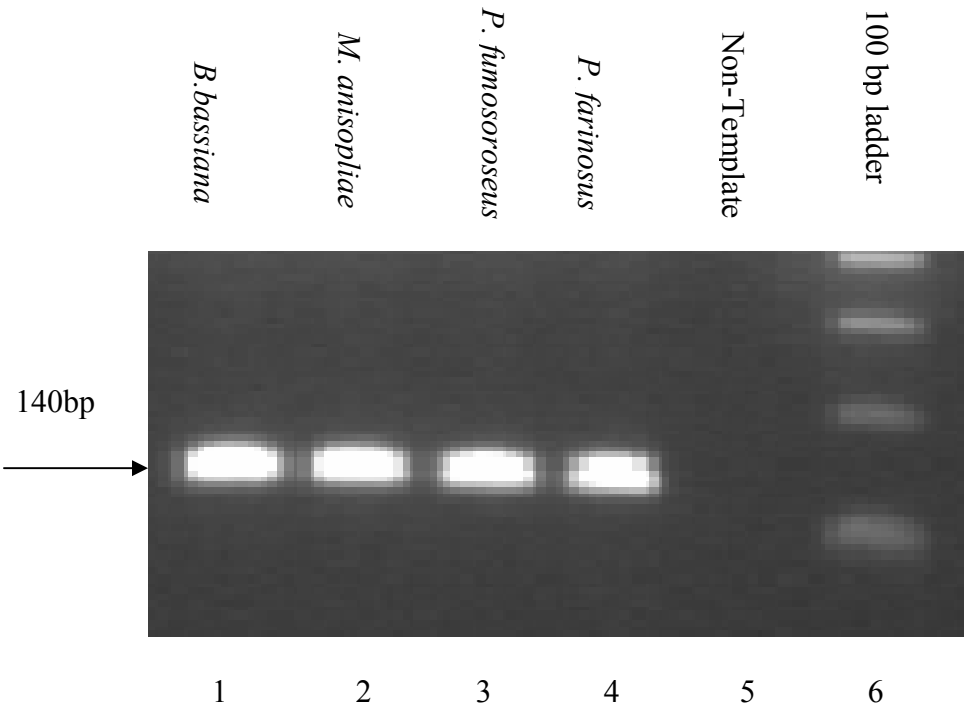

Figure 2. PCR products using Primer Pair Ba02. All lanes generated the expected 140 bp size for the microsatellite loci designed for *B. bassiana*. A 100bp ladder was used to assess the size of the products.

DNA sequences were aligned for product generated with Primer Pair #1 for *M. anisopliae*, Primer Pair #2 for *M. anisopliae*, and for Primer Pair Ba02 for *B.bassiana* (Figure 3).

Figure 3. Sequence alignments

A) Sequence of 170bp products amplified using Primer Pair #1

```
Query  22   CTGGATGACGTTATGCAACGACGCTTCTTATGATGTTGTCAACTTGGCTTTTCTGAGCCA  81
            |||||||||||| ||||||||| |||| ||||||||||||||||||||||||||||||||
AY545982 79   CTGGATGACGTT-TGCAACGACTCTTCCTATGATGTTGTCAACTTGGCTTTTCTGAGCCA  137

Query  82   CTTCTTCTCCGGCGGAGGCTACCCACGGATGAGTATCGGCAACCTCGCCGGCCCCTCGCA  141
            |||||||||||| ||||||||||   |||||||||||||||||||| ||||||||||||
AY545982 138  CTTCTTCTCCGCCGGAGGCTACCCCAAGATGAGTATCGGCAACCTCGACGGCCCCTCGCA  197

Query  142  GGCATCAGAAGAAAG  156
            ||| ||||||||||||
AY545982  198  GGC-TCAGAAGAAAG  211
```

B) Sequence of 209 bp products amplified using Primer Pair #2

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Query  48   GGACGGTAGCTCCCTCGTCAAGTCCATCAAGAACTTGCCAGAGCAAGGGCAAGCCAGTCA  107
            |||||||||||||||||||||||||||||||||| |||||||||||||||||| || |
AY545982 231  GGACGGTAGCTCCCTCGTCAAGTCCATCAAGAACT-GCCAGAGCAAGGGCAAGCCTGTGA  289

Query  108  TGCTCAGCATGGGCGGAGCGACAGATTACTCCGATGTCCAGCTACACTCAGATGCTCAGG  167
            | |||||||||||||||| |||||||||||||| ||||| ||| |||| || |||||||
AY545982 290  TTCTCAGCATGGGCGGAGCCACAGATTACTCCGACGTCCAACTAAACTCGGACGCTCAGG  349

Query  168  GCCAACAGATTGGCCAACACTG  189
            |||||||||||| ||||||||||
AY545982 350  GCCAACAGATT-GCCAACACTG  370
```

C) Sequence of 140 bp products amplified using Primer Pair Ba02

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Query  72   TGAAAAAGAACCTCCTTCGCCGTCGTCAAGGCATAGCG  109
            |||||||||||||||||||||||||||||||||||||
AY545982 190  TGAAAAAGAACCTCCTTCGCCGTCGTCAAGGCATAGCG  153
```

Figure 3. Aligned sequences from NCBI database. **A)** Sequence alignment for the 170 b.p. product using Primer Pair #1. The alignment shows 93% identity in the sequence for *M.anisopliae* for the chi3 gene. **B)** Sequence alignment for the 210 b.p. product using Primer Pair #2. The alignment shows 92% identity in the sequence. **C)** Sequence alignment for the 140 b.p. product using Primer Pair Ba02. The alignment shows 100% identity to the *B. bassiana* clone Ba02 microsatellite sequence.

Discussion

Primers were designed for the chi3 gene from sequence data available on NCBI for *M. anisopliae*. Since all four species used in this study are entomopathogenic fungi currently researched for potential biological control (biocontrol) of ticks and other arthropod pests, it was assumed that this chitinase gene would be present in all species. The primers designed for use in this experiment were found to be unable to amplify the gene in *B. bassiana, P. fumosoroseus*, and *P. farinosus*. The only amplified DNA fragment came from *M. anisopliae,* which was the species whose DNA sequence was used to design the primers.

Since the designed primers were unable to amplify the gene in three of the species used for this study, I can not make a definitive conclusion as to the presence or absence of the chi3 gene in these organisms. However, I am able to conclude that either the gene does not exist in *B. bassiana, P. fumosoroseus* and *P. farinosus,* or they are not homologous enough to *M. anisopliae* to enable PCR amplification using the primers generated.

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