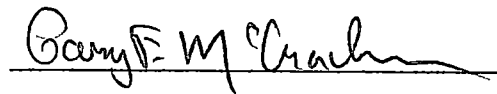


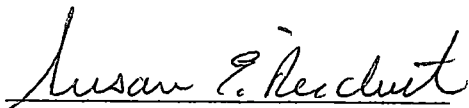
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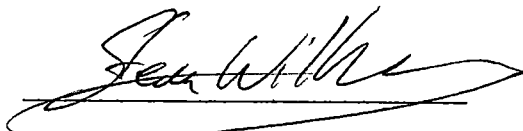


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Interim Vice Provost and
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Molecular Analysis
of Big Brown Bat (*Eptesicus fuscus*) Feces:
Genotyping and Diet

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Sunitha Vege
December, 2000

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ABSTRACT

Primers for PCR (Polymerase Chain Reaction) amplification of targeted DNA sequences were developed for the corn earworm (*Helicoverpa zea*) and tobacco budworm (*Heliothis virescens*), both major agricultural insect pests that are eaten by bats. Two *H. zea* primer sets from an intron region of the nuclear PBAN locus (Davis *et al.*, 1992) showed species-specific amplification when tested against genomic DNA of *H. zea*, *H. virescens*, *Spodoptera exigua*, and *S. frugiperda*. *H. virescens* mitochondrial (Miller *et al.*, 1990) and nuclear (Logsdon *et al.*, 1995) primers were species-specific when tested against genomic DNA of *H. virescens*, *H. zea*, *S. exigua*, and *S. frugiperda*.

Molecular techniques were applied to fecal material for identifying these moths in DNA extracted from feces of bats. Feces were obtained through controlled feedings of target moth species to big brown bats, *Eptesicus fuscus*. Species-specific DNA markers for *H. zea* and *H. virescens* were tested against fecal DNA of known insect content. Four fecal extraction methods, modified DNeasy, N-phenacylthiazolium bromide (PTB), guanidium thiocyanate (GuSCN/silica), and modified sodium lysis were assessed in their abilities to yield good quality DNA from bat feces.

The modified DNeasy protocol yielded the most consistent PCR amplification results with the moth DNA markers. Although the markers were species-specific for genomic moth DNA, the markers were not species-specific for moth DNA extracted from fecal material, with PCR products of the expected sizes amplifying against fecal DNA for the target and non-target moth species. Fecal DNA isolated using the PTB, GuSCN/silica, and modified sodium lysis protocols gave poor PCR amplification results. Contaminants and inhibitors present in the fecal DNA were suspected to interfere with PCR.

PCR-RFLP (PCR-Restriction Fragment Length Polymorphism) was tested to differentiate between specific and non-specific PCR products that amplified from fecal DNA. Fecal PCR products for the target and non-target moth species were all cut with the same restriction enzymes. Therefore, PCR-RFLP was not useful for distinguishing between moth species in bat fecal DNA.

In a separate experiment, big brown bats were genotyped from their feces using three microsatellite primers (EF1, EF6, and MM5). Genotypes obtained from bat fecal DNA consistently matched the genotypes obtained from DNA extracted from wing membrane tissue of the same bats. Genotypes obtained from multiple fecal DNA samples from the same bat were identical. Fecal studies present research opportunities for addressing ecological and behavioral questions for animals that are difficult to capture, rare, or endangered. Unlike tissue material, feces can be collected without the need of CITES permits.

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OVERVIEW

The objectives of this project were to use molecular techniques 1) to identify Lepidopteran prey species in the diet of big brown bats (*Eptesicus fuscus*), and 2) to directly genotype *E. fuscus* individuals through their feces. The thesis is divided into three chapters.

In Chapter 1, I describe the development and testing of species-specific DNA primers for the target moth species, the corn earworm (*Helicoverpa zea*) and the tobacco budworm (*Heliothis virescens*). This chapter is formatted as a primer note for submission to Molecular Ecology.

In Chapter 2, I describe experiments to determine the consistency of PCR amplification of the markers in DNA isolated from *E. fuscus* feces. The markers were tested against DNA isolated from feces obtained from controlled feedings of the target moths to *E. fuscus*. Four fecal extraction protocols were tested: modified sodium lysis, GuSCN/silica, PTB, and modified DNeasy tissue. The quality of the DNA yielded by the different protocols was assessed visually on a 2% agarose gel. In addition, PCR-RFLP was tested as a method for differentiating specific and non-specific PCR products that amplified from fecal DNA isolated using the DNeasy tissue protocol.

In Chapter 3, I tested the reliability of genotyping bats through fecal DNA extracted using the modified DNeasy tissue protocol. I compare the genotypes obtained from bat wing membrane tissue DNA with genotypes obtained from the same bat's fecal DNA. The reproducibility of this protocol was tested by comparing the genotypes obtained from multiple fecal extractions from the same bat.

CHAPTER 1

Identification of species-specific DNA moth markers

Lepidopterans of the Family Noctuidae are economically important pest species in the United States and Canada, where outbreaks cause immense damage to a variety of agricultural crops. In particular, species in the genera *Helicoverpa*, *Heliothis*, and *Spodoptera* are among the world's most harmful crop pests (McCaffery, 1998). Efforts to control insect pests involve developing host plant resistance, cultural control through timing of crop harvest to reduce insect attacks, conservation of natural enemies, and the use of insecticides. Among these management strategies, pesticide applications are by far the most common practice for control of pest species (Allen and Hoelscher, 1997). With increasing concern regarding extensive insecticide use and growing resistance to insecticides by many pests (McCaffery, 1998), integrated pest management (IPM) strategies that target specific pest species have been advocated.

The larvae of both corn earworms (*Helicoverpa zea*) and tobacco budworms (*Heliothis virescens*) forage on and cause damage to similar host plants including cotton, corn, tomatoes, and soybeans. This polyphagous behavior (Fitt, 1989) complicates management efforts. Since it is difficult to distinguish between the two species during their larval stage, it is essential to have a simple and reliable technique for differentiating them (Roehrdanz, 1997). In addition to differentiating the two moths during their larval stages, there is considerable interest in identifying insect pests that are eaten by bats. Recent fecal analyses of insectivorous bats suggest that bats consume enormous numbers of insects nightly (Whitaker *et al* , 1996, Lee, 1999). In particular, Mexican free-tails (*Tadarida brasiliensis*) forage on millions of agricultural moth pests (Kunz *et al* , 1995, Whitaker *et al* , 1996, Lee, 1999). However, due to the degradation of the insect remains in feces, it is not possible to document the predation on particular moth species by bats. Here, I present species-specific DNA markers that allow for identification of moths, *H. zea* and *H. virescens*.

Adults and pupae of corn earworms, tobacco budworms, beet armyworms (*Spodoptera exigua*), and fall armyworms (*S. frugiperda*) were obtained from USDA insect breeding facilities. Adult moths and pupae were finely chopped and digested at 55°C overnight in a standard DNA extraction buffer (100mM NaCl, 10mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 0.5% SDS) with 20µL of 20mg/ml Proteinase K (Promega). DNA was extracted using a standard phenol/chloroform, ethanol precipitation method (Sambrook *et al.*, 1989). DNA was eluted in 1/10 TE, quantified using a Hoeffer DynaQuant© 200 fluorometer, and stored at 4°C until analysis.

Primers were designed from intron sequences that amplified a region of DNA that is likely to be conserved within species but not across species. GenBank (Benson *et al.*, 2000) was the primary source for sequence information. Primers were designed using the GeneFisher (F Meyer, C Schleiermacher, and R Giegerich) software program and were optimized on a gradient thermocycler (Eppendorf Mastercycler Gradient). PCR products were visualized on 2% agarose gels by ethidium bromide staining.

Two primer sets (Table 1-1) were designed for *H. zea* from a genomic DNA sequence of a neuropeptide gene (PBAN) which controls sex pheromone biosynthesis in female moth species (Davis *et al.*, 1992). PCR reactions for the two primer sets were carried out in 12µL volumes, each containing 10ng of genomic DNA, 1X Promega PCR buffer, 1.04mM MgCl₂, 0.10mM dNTP's, 100ng of each primer, 1 unit of *Taq* DNA polymerase, and sterile distilled H₂O to 12µL. The PCR amplification profile consisted of initial denaturation for 2 min at 94°C, followed by 30 cycles of 94°C for 1 min, 55°C for 1 ½ min, and 72°C for 1 min. Both primer sets successfully amplified the expected fragment from twelve *H. zea* DNA individuals (Fig. 1-1). Neither primer set amplified products for twelve *H. virescens*, ten *S. exigua*, or seven *S. frugiperda* individuals, suggesting that both primer sets are specific for *H. zea* (Figure 1-1 and 1-2).

Table 1-1 Species-specific moth primers for *H. zea* and *H. virescens*. For each primer, the locus, primer codes and sequences, annealing temperature for PCR amplification, and PCR product size are included

Moth species	Locus	Primer Code	Primers 5' ~ 3'	Annealing temp. (°C)	Product size (bp)
<i>Helicoverpa zea</i> , corn earworm	PBAN _a , nuclear	Hzban1/2	F GGTA GTGCTCACATTGCA R CCAGGGTAACTGAATCCA	55	146
<i>Helicoverpa zea</i> , corn earworm	PBAN _a , nuclear	Hzea2/3	F GTACCGCCCAAGACCCCTGAAC R GTAGTTCTTCGGGACGCGGG	55	161
<i>Heliothis virescens</i> , tobacco budworm	p63 isoform _b , mitochondrial	Hvp63-1/2	F CCGCGAAAAGGCTTATTGA R TGCCTTCTTCAACTGCTGC	58	148
<i>Heliothis virescens</i> , tobacco budworm	TP1 _c , nuclear	Hvir1/2	F GATTTCATACACAAGACAAAGCAG R GGACATCTTGAGCCCTTAAAC	48	196

^a - Davis *et al* , 1992

^b - Miller *et al* , 1990

^c - Logsdon *et al* , 1995

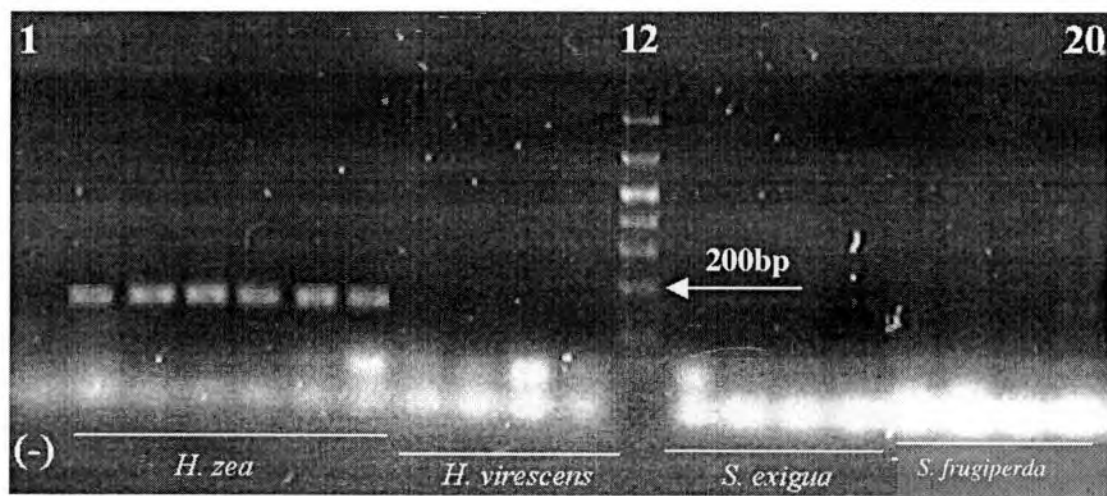


Figure 1-1: PCR amplification of the *H. zea* PBAN primer set (Hzban1/2) from genomic DNA of four moth species. Lane 12, 1kb, 50bp size marker; lane 1, negative control of water; lanes 2-7, genomic *H. zea* DNA, PCR product of 150bp amplified; lanes 8-11, genomic *H. virescens* DNA; lanes 13-16, genomic *S. exigua* DNA, lanes 17-20, genomic *S. frugiperda* DNA; no PCR products amplified from genomic DNA of *H. virescens*, *S. exigua*, or *S. frugiperda*.

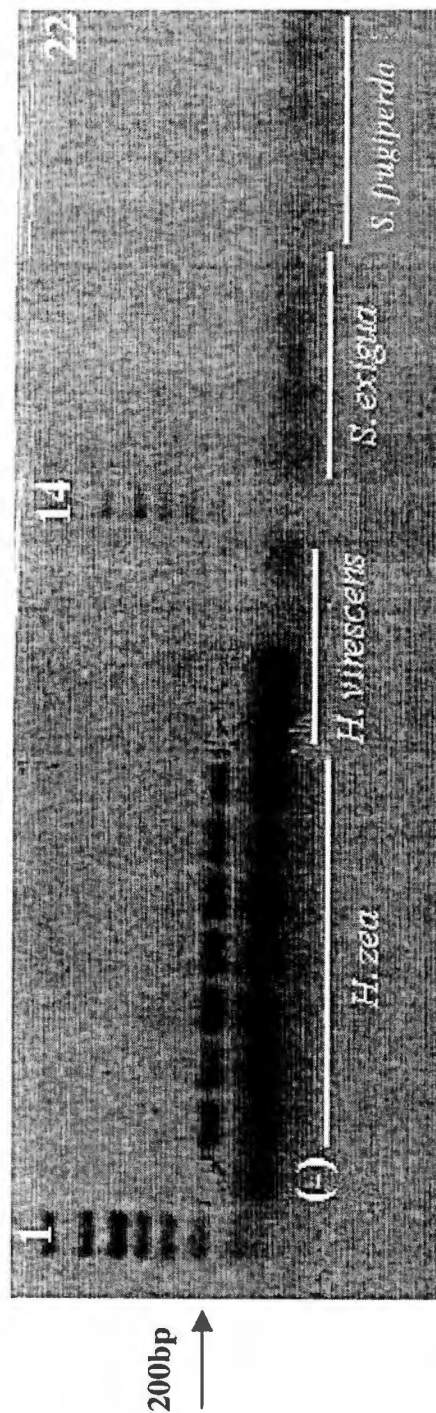


Figure 1-2: PCR amplification of genomic DNA of four moth species with the *H. zea* primer set, Hzea2/3. Lanes 1 and 14, 1 Kb, 50bp size marker; lane 2, negative control of water; lanes 3-9, genomic *H. zea* DNA, expected PCR product of 160bp amplified; lanes 10-13, genomic *H. virescens* DNA; lanes 15-18, genomic *S. exigua* DNA, lanes 19-22, genomic *S. frugiperda* DNA; no PCR products amplified from genomic DNA of *H. virescens*, *S. exigua*, or *S. frugiperda*.

A primer set was designed for *H virescens* from the mitochondrial p63 gene (Miller *et al* , 1990) (Table 1-1) The PCR conditions and amplification profile were the same as above, except that the annealing temperature was increased to 58°C The *H virescens* primers were tested against twelve *H virescens* individuals, twelve *H zea*, ten *S exigua*, and seven *S frugiperda* The expected product amplified only for *H virescens* (Figure 1-3)

Another primer set was designed from a sequence containing introns and exons from a triose-phosphate isomerase gene of *H virescens* (Logsdon *et al* , 1995) The PCR reaction and amplification profile was identical to that used for the *H zea* PBAN primer sets with the only variation that the annealing temperature was 48°C (Table 1-1) The *H. virescens* nuclear primer set consistently amplified a product against twelve individuals of *H virescens* Products did not amplify for twelve *H zea*, ten *S exigua*, or seven *S frugiperda* individuals (Figure 1-4)

In total, four species-specific primer sets were identified two specific to *H zea* and two specific to *H virescens* These moth markers could be useful for potentially discriminating between *H zea* and *H virescens* that are infesting agricultural crops and for verifying the consumption of specific moth species by bats

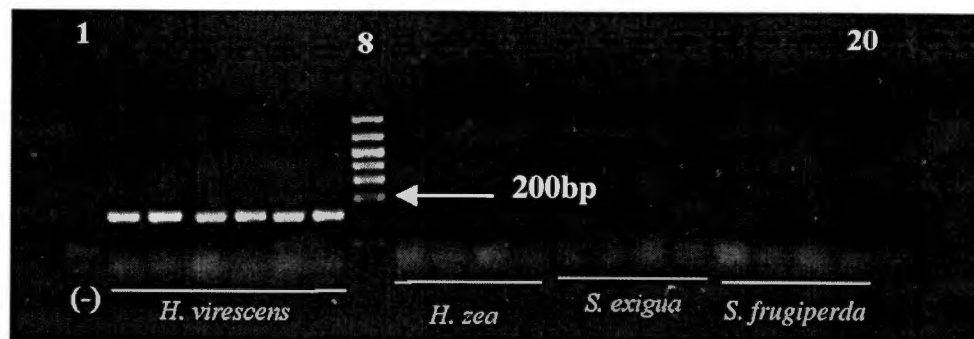


Figure 1-3: PCR amplification of genomic DNA of four moth species with the *H. virescens* mitochondrial primer set. Lane 8, 1kb, 50bp size marker; lane 1, negative control of water; lanes 2-7, genomic *H. virescens* DNA, expected PCR product of 150bp amplified; lanes 9-12, genomic *H. zea* DNA; lanes 13-16, genomic *S. exigua* DNA, lanes 17-20, genomic *S. frugiperda* DNA; no PCR products amplified for *H. zea*, *S. exigua*, or *S. frugiperda*.

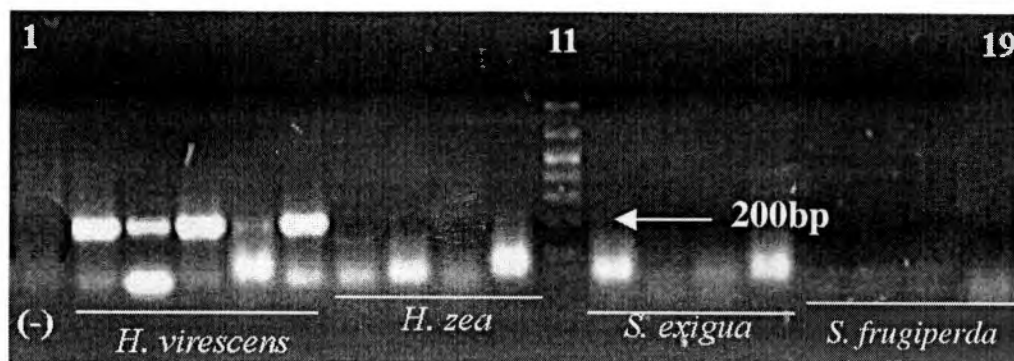


Figure 1-4: PCR amplification of genomic DNA of four moth species with the *H. virescens* nuclear primer set. Lane 11, 1kb, 50bp size marker; lane 2, negative control of water; lanes 2-6, genomic *H. virescens* DNA, expected PCR product of 200bp amplified; lanes 7-10, genomic *H. zea* DNA; lanes 12-15, genomic *S. exigua* DNA, lanes 16-19, genomic *S. frugiperda* DNA; no PCR products amplified for *H. zea*, *S. exigua*, or *S. frugiperda*.

CHAPTER 2

Use of molecular techniques for the identification of Lepidopteran prey items in bat guano

INTRODUCTION

Fecal material is often abundant and can be collected easily, non-invasively, without trauma, and in many cases, without even the handling of animals (Putman, 1984, Morin and Woodruff, 1996, Kohn and Wayne, 1997; Schlotterer, 1998). Therefore, it is no surprise that fecal analysis has contributed enormously to our information on the diets of many animals (McInnis *et al* , 1982, Putman, 1984, Norbury, 1988, Greene, 1989, Farrell *et al* , 2000). Information on prey composition has been obtained from visual examination of insect remains in feces of many insectivorous bats (e g , Anthony and Kunz, 1977, Whitaker *et al* , 1981, Kunz and Whitaker, 1983, Brack and LaVal, 1985, Griffith and Gates, 1985, Swift *et al* , 1985, Whitaker, 1988, Barclay, 1991, Lee, 1993, Rolseth *et al* , 1994, Lee, 1999). For example, Anthony and Kunz (1977) found that though little brown bats (*Myotis lucifugus*) have a diverse diet, pregnant and lactating females are more selective in their diet, preferring to forage on beetles and mayflies when these insects are relatively abundant. Similarly fecal analysis showed that to meet high energetic needs reproductive females of *M. evotis* tend to forage in forest areas where insects, often moths, are prevalent (Barclay, 1991).

Insectivorous Mexican free-tailed bats (*Tadarida brasiliensis*) are widely distributed throughout the southern regions of the United States with some populations in caves numbering in the millions of individuals (Davis *et al* , 1962, Cockrum, 1969, McCracken and Gustin, 1991). The population in Bracken Cave outside of San Antonio, Texas, is estimated at 20 million bats and is thought to be the largest congregation of any mammal species in the world (Davis *et al* , 1962). Information on the diet of these bats is important in determining the impact they have on insect populations, especially insects that are major agricultural pests. Mexican free-tailed bats

often forage at altitudes of 1,200 meter or more above the ground (Davis et al , 1962, Williams et al , 1973, McCracken, 1996, McCracken *et al.*, 1996, McCracken *et al* , 1997) and recent information has documented the existence of enormous populations of moths at these altitudes (Wolf et al , 1990, Wolf *et al* , 1994, Westbrook et al , 1995) Dietary studies (Kunz et al , 1995, Whitaker *et al* , 1996, Lee, 1999) have demonstrated that the 100 million free-tailed bats residing in Texas each summer forage on a variety of insects with a significant portion of their diet consisting of moth species that do major damage to agricultural crops Kunz et al (1995) estimated that Mexican free-tailed bats (*T. brasiliensis*) in central Texas consume approximately one thousand tons of insects nightly to satisfy their energetic demands Even if only 10% of the bats' diet were moth, the Mexican free-tailed bats are capable of devouring 340 million moths each night (10% equal to approximately 34 moths, each moth weighing 250mg) Over a two-week period, these figures translate into the estimated total population of 100 million Mexican Free-tailed bats in Texas consuming approximately 7 billion moths (McCracken and Westbrook, 1999)!

In a recent work, Lee (1999) examined fecal pellets to determine the major insect prey of Mexican free-tailed bats (*T. brasiliensis*) Lee's study is one of the most complete and thorough dietary analyses for any mammal population, identifying the prey in the bats' diet to 12 orders and 38 families During much of the summer, moths constituted a small portion of the diet of the bats However, in June, during the times of corn earworm (*Helicoverpa zea*) migration into Texas and the emergence of local moth species, the bats' diet was dominated by moths (Lee, 1999) Lee's results demonstrate that free-tailed bats forage heavily on moths when they are abundant in the Texas region Although Lee's study offers extraordinary detail on the diet of Mexican free-tailed bats, identification of the prey species consumed was not possible due to the degradation of the insect remains in the feces. Although some moth fragments could be categorized as belonging to the Order Lepidoptera, identification of moth species was impossible

Molecular techniques offer the potential for verifying the consumption of specific moth species by Mexican free-tailed bats

Fecal analysis, when coupled with molecular techniques such as PCR (Polymerase Chain Reaction), provides a sensitive tool for studying an animal's diet (Morin and Woodruff, 1996, Kohn and Wayne, 1997, Schlotterer, 1998). From an initial sample containing only a few copies of template DNA, PCR is capable of generating hundreds of thousands of copies of an amplified DNA sequence (Arnheim *et al* , 1990). However, the extraction of DNA from feces and its PCR amplification are difficult. Fecal DNA is often unsuitable for genetic analyses because of the presence of PCR inhibitors such as bile salts and complex polysaccharides (Deuter *et al* , 1995, Monteiro *et al* , 1997). Nonetheless, extraction protocols have been developed for the isolation of DNA from fecal material (Hoss *et al* , 1992, Gerloff *et al* , 1995, Kohn *et al* , 1995, Taberlet *et al* , 1996, Reed *et al* , 1997, Poinar *et al* , 1998, Gerloff *et al* , 1999, Whittier *et al* , 1999, Ernest *et al* , 2000, Farrell *et al* , 2000).

While molecular analysis of feces have been applied to questions on parasitic loads (Bretagne *et al* , 1993, Van der Maarel *et al* , 1998), population structuring (Hoss *et al* , 1992, Kohn *et al* , 1995, Van der Kuyl *et al* , 1996, Reed *et al* , 1997, Poinar *et al* , 1998, Whittier *et al* , 1999, Ernest *et al* , 2000, Farrell *et al* , 2000), and social behavior (Gerloff *et al* , 1995, Taberlet *et al* , 1996, Gerloff *et al* , 1999) for many mammals, they have been used much less frequently to address dietary questions (Hoss *et al* , 1992, Poinar *et al* , 1998). Two dietary studies from fecal remains of brown bears (*Ursus arctos*, Hoss *et al* , 1992) and the extinct ground sloth (*Nothrotheriops shastensis*, Poinar *et al* , 1998) have employed molecular assays to determine the major plant taxa in the diet of these organisms. In the brown bear study, primers specific for the *rbcL* chloroplast gene were amplified from feces. Sequencing of PCR products has shown that plants in the genus *Phontinia* are a primary component of the bears' diet (Hoss *et al* , 1992).

Cloned PCR products of the *rbcL* chloroplast gene in the ground sloth's diet matched sequences for numerous plant taxa, including Capparales, Liliaceae, and Vitaceae (Poinar *et al* , 1998)

The approach in my study differs from these earlier studies of Hoss *et al* (1992) and Poinar *et al* (1998) in that I attempt to identify the exact prey species consumed by the bats. In this study, I test species-specific DNA markers for the moths, corn earworm (*H. zea*) and tobacco budworm (*Heliothis virescens*), against fecal DNA of known insect content. I also assess the quantity and quality of DNA extracted from bat guano using four different extraction methods: 1) modified sodium lysis (Swiger, 1999), 2) guanidium thiocyanate (GuSCN) and silica (Boom *et al.*, 1990, Hoss and Paabo, 1993), 3) N-phenacylthiazolium bromide (PTB) (Poinar *et al* , 1998), and 4) modified DNeasy tissue kit (Qiagen)

METHODS

Fecal collections

Fecal samples for testing extraction protocols and moth DNA markers were collected from ten big brown bats (*Eptesicus fuscus*) captured and held in captivity for two and a half weeks. Both moth pupae of *H. zea* and *H. virescens* were obtained from USDA insect breeding facilities while the mealworms were bought at a local pet store. Moth pupae and adults were fed to bats. Bats were fed exclusively *H. zea*, *H. virescens*, or mealworms twice a day for five consecutive days. After each feeding, bats were placed in separate bags, feces were collected, stored in labeled sterile tubes, and frozen at -80°C.

Food can pass through a bat's gut within 24 hours of consumption (Kunz and Whitaker, 1983). Fecal samples that were collected 24 hours after the bats were captured or within 25 hours of transition of feeding of one insect species to the next were not analyzed. Only fecal samples known to contain one insect species were used. Fecal samples from bats fed only *H. zea* were used to provide a positive control for testing the consistency of the species-specific *H. zea* DNA

marker and to provide a negative control for the two *H. virescens* markers. Feces containing remains of only *H. virescens* were used as positive controls for the two *H. virescens* markers and as negative controls for the *H. zea* marker. Feces containing mealworms were employed as negative controls for the three moth species DNA markers.

DNA extraction

DNA extraction from moths

The extraction of genomic DNA from the moths is described in Chapter 1.

DNA extraction from feces

Four fecal extractions are described below. Great care was taken to avoid contamination with foreign DNA during all extractions of DNA from feces. Forceps used to weigh the feces were thoroughly cleaned with ethanol between each sample. Also, aerosol-barrier filter pipette tips were used when measuring liquids.

DNA extracted by each of the methods described below was visualized on a 2% agarose gel with ethidium bromide staining. Depending on the size and integrity of the bands visualized, the DNA was assessed as having high molecular weight (MW) DNA, high MW DNA + low MW DNA, low MW DNA, or no DNA. DNA from the extraction methods was quantified using Hoechst (33258, Sigma) fluorescence dye and a Hoeffer DynaQuant© 200 fluorometer. Six additional fecal extraction methods tested are described in Appendix A, with four of the six being modifications to the protocols described below. For each extraction protocol, at least twelve fecal samples were isolated with six containing insect remains of *H. zea* and six of *H. virescens*.

Method 1 Modified sodium lysis

Fecal pellets weighing 20 to 25 mg were broken apart with pestles in 1.5 ml sterile tubes, suspended in one ml PBS (0.15M NaCl, 0.05M NaHPO₄), and centrifuged at 8,000×G for 10 minutes. The supernatant was discarded and the pellet was washed with 1 ml PBS, followed by a subsequent wash using equal volumes of PBS and petroleum ether. The supernatant consisting of

ether and a middle layer suspected to contain polysaccharides and fatty acids was discarded. A glass wool column consisting of glass wool packed in a 2 ml pipette tip was used to filter fecal debris and contaminants. The bottom layer containing PBS and fecal material was pipetted over the glass wool column and the filtrate was collected in a 1.5 ml tube. The solution was centrifuged at 9,000×G for 10 minutes. The supernatant was discarded and cells in the pellet were lysed in 30 µl of 0.2M sodium hydroxide. After incubating at room temperature for 20 minutes, 180 µl of 20% Chelex 100 and 0.04M Tris/HCl, pH 7.5 were added to the lysed cells. Samples were centrifuged at 11,000×G for 5 minutes and the supernatant containing the DNA was stored in a sterile tube at 4°C. Twelve fecal samples can be isolated and quantified in approximately three hours. Further attempts to purify the DNA isolated with this protocol are discussed in Appendix B and C.

Method 2 GuSCN/ silica

The guanidium thiocyanate (GuSCN) and silica extraction protocol was modified from Boom *et al* (1990). The preparations for the different buffers and solutions (L6, L2, and silica suspension) required for the protocol are described in Figure 2-1. Approximately 20 to 25 mg of feces were suspended in 900 µL of L6 buffer, incubated at 60°C in a water bath, and agitated every 10 to 15 minutes for 1 ½ hours. After incubation, the fecal samples were centrifuged at 8,000×G for 10 minutes. Fresh L6 buffer (500 µL) and 40 µL of silica suspension were added to 500 µL of the supernatant. The silica suspension tends to settle and so the suspension was vortexed before it was added to the fecal solution. The feces plus L6 buffer and silica were incubated for an additional 1 ½ hours at room temperature. The samples were then centrifuged at 9,000×G for 5 minutes and the supernatant was discarded. The silica pellet was then washed once with L2 buffer, twice with 100% ethanol, and once with acetone, and dried in a vacuum. After adding 60µL of sterile distilled water, the solution was placed at 55°C in a water bath for 30

minutes. The supernatant containing the DNA was transferred to a new tube and stored at 4°C. Twelve fecal samples can be isolated and quantified within three hours.

Method 3 PTB

The N-phenacylthiazolium bromide (PTB) extraction protocol was used by Poinar *et al* (1998) to isolate DNA from coprolites of the extinct ground sloth. This protocol uses similar buffers as described by Boom *et al* (1990) with the exception of the use of PTB. PTB was synthesized in the laboratory of Dr. J. Turner (University of Tennessee, Knoxville) by refluxing 1M phenacyl bromide with 1M thiazole. The end precipitate (PTB) was cleaned with several washes using 90% ethanol.

A standard extraction buffer (0.1M Tris/HCl pH 7.2, 10mM EDTA pH 8.0, 0.5M LiCl, 1% SDS, 50mM DTT) and 20 µL of 20 mg/ml Proteinase K (Promega) was added to 20 to 25 mg of feces and the fecal pellets were broken apart with pestles. Subsequently, each sample received 100 µL of 0.1M PTB/0.01M NaPO₄ and was incubated at 37°C in a water bath shaker for 2 hours. After incubation, the samples were centrifuged at 16,000×G for 10 minutes. L6 buffer (1.4 ml) (Figure 2-1) was added to 700 µL of the supernatant and placed at 60°C in a water bath shaker for 2 hours, after which 50 µL of silica suspension (Figure 2-1) was added, and the samples were agitated for an additional hour at room temperature and centrifuged at 9,000×G for 30 seconds. The supernatant was discarded and the silica pellet was washed twice with L2 buffer (Figure 2-1), once with 100% ethanol, and once with acetone, and dried in a vacuum. The dried pellet was resuspended in 60 µL of 1/10 TE. Samples were incubated at 55°C for 15 minutes to elute the DNA. The supernatant containing the DNA was transferred to a sterile tube and stored at 4°C. Twelve fecal samples can be isolated and quantified within seven hours.

L6 (Lysis buffer): Dissolve 30.0 g GuSCN in 50 ml of 0.1M Tris/HCl (pH 6.4). Add 11 ml of 0.2M EDTA (pH 8.0). Add 1.3 g Triton X-100 to the solution. Vortex the solution and store at room temperature in the dark.

L2 (Washing buffer): Dissolve 30.0 g GuSCN in 50 ml of 0.01M Tris/HCl (pH 6.4)

For both the L6 and L2 buffers, the Tris/HCl plus GuSCN may need to be heated to 60°C before the GuSCN will go into solution. The L6 and L2 buffers are stable at room temperature in the dark for 3 weeks.

Silica suspension: Combine 2.4 g of silica particles and 20 ml of distilled water in 50 ml Falcon tubes and vortex. Place solution in the dark for exactly 24 hours. After 24 hours, pipette and discard 17.5 ml of supernatant. Bring volume to 20 ml with distilled water. Place solution in the dark for 5 hours and discard 17.5 ml of supernatant. Bring volume to 20 ml with distilled water and let settle for another 5 hours. Discard 18 ml of the supernatant. Transfer remaining silica solution in the falcon tube to 1.5 ml tubes. Wrap the tubes in aluminum foil and autoclave. The silica solution is stable at room temperature in the dark for 6 months.

Figure 2-1 Preparation of buffers for GuSCN/silica extraction protocol

Method 4 Modified DNeasy tissue protocol (Qiagen)

Fecal DNA was isolated using a modification of the tissue extraction protocol of the DNeasy kit (Qiagen). The kit provides the lysis (ATL and AL) and washing (AW2 and AW2) buffers used in the extraction protocol. For DNA isolation, 20 to 25 mg of feces were lysed with 40 μ L of 20 mg/ml of Proteinase K (Promega) and 350 μ L of ATL buffer. Pestles were used to break apart the fecal pellets. After incubation at 55°C in a water bath for 2 ½ to 3 hours, 380 μ L of AL buffer was added and incubated for another 10 minutes at 70°C. The samples were centrifuged at 16,000xG for 10 minutes, after which the supernatant was transferred to a new tube and the fecal debris pellet was discarded. The supernatant was diluted with 380 μ L of 100% ethanol and incubated at -20°C for 30 minutes. The samples were transferred to the filter columns provided by the kit and centrifuged at 5,000xG for 1 minute. The filter with the bound DNA was washed once with 100% ethanol, once with AW1 buffer, and once with AW2 buffer. After the final wash, 100 μ L of elution buffer, AE, was added to the filter. The filter column was centrifuged and the elution buffer containing the DNA was stored at 4°C. Twelve samples can be isolated and quantified in approximately three hours.

PCR (Polymerase Chain Reaction) amplification

Amplification of genomic DNA

The primers developed for *H. zea* and *H. virescens* are described in Chapter 1, Table 1-1 as are the PCR conditions for the species-specific amplifications of genomic DNA from the moths. Other moth primers and PCR-based techniques not discussed in Chapter 1 and 2 are described in Appendices D and E.

Amplification of fecal DNA

Because fecal DNA contains many PCR inhibitors, PCR reaction conditions were altered from those used to amplify genomic moth DNA by increasing the amount of *Taq* polymerase and adding BSA (Bovine Serum Albumin). PCR was carried out in 12 μ L volumes, each containing

2-20 ng of fecal DNA, 1X Promega PCR buffer, 1.04mM MgCl₂, 0.10mM dNTP's, 100 ng of each primer, 10 ng BSA, 2 – 3 units of *Taq* DNA polymerase, and sterile distilled H₂O to 12 µL. The PCR amplification profile involved initial denaturation for 2 min at 94°C, followed by 30 cycles of denaturing at 94°C for 1 min, annealing which varied for the three markers for 45 sec (Table 1-1), and elongating at 72°C for 45 sec. Different DNA *Taq* polymerases and PCR amplifications were tested against fecal DNA and the results are discussed in Appendix F.

A PCR reaction of genomic DNA of the target moth species was included as a positive control with every PCR run. All PCR runs were also performed with at least one negative control using either mealworm DNA or distilled water. PCR products were visualized on 2% agarose gels by ethidium bromide staining.

Although PCR is capable of generating thousands of copies of a desired DNA sequence from only a few copies of template DNA, amplification may not always occur. Therefore, the absence of a PCR amplicon using the moth primers in bat fecal DNA does not necessarily indicate that the moth species was not consumed.

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

Restriction sites within the target DNA sequence from each moth species were identified using Webcutter program. To differentiate between specific and non-specific fecal PCR products, PCR-RFLP was applied to fecal DNA samples isolated using the modified DNeasy tissue protocol. Restriction enzyme sites for the *H. zea* PBAN included *Nsi*I which cleaved the *H. zea* PCR product once, yielding 82 and 46 base pair fragments (Appendix G-1). The *Nsi*I restriction enzyme was tested against PCR products from *H. zea* genomic DNA and from *H. zea* and *H. virescens* fecal DNA. *Nsi*I was not tested against PCR products from *H. virescens* genomic DNA since products never amplified with the *H. zea* markers.

Restriction enzyme sites for the *H. virescens* mitochondrial p63 sequences included *Vsp*I enzyme which cleaved the *H. virescens* PCR product once, yielding 92 and 56 base pair

fragments (Appendix G-2) The *VspI* restriction enzyme was tested against amplicons from *H. virescens* genomic DNA and from *H. virescens* and *H. zea* fecal DNA. The *H. virescens* markers never amplified PCR products from genomic DNA of *H. zea* and therefore, *VspI* could not be tested against *H. zea*

For each PCR-RFLP reaction, twelve microliters of PCR product were incubated with 2.0 μ L 10X buffer, 0.5 μ L BSA, and 0.33 μ L restriction enzyme and brought to 20.0 μ L with sterile distilled water at 37°C for 2 hours. The PCR-RFLP reactions were visualized on a 2% agarose gel to determine if the products were cut with the restriction enzyme.

RESULTS

Quantity and quality of total fecal DNA

The quantity of the fecal DNA obtained differed among the extraction methods (Table 2-1). The modified sodium lysis protocol yielded the highest amount of fecal DNA with total DNA ranging from 0.20 to 80.0 μ g. The PTB and modified DNeasy extraction methods resulted in comparable DNA amounts with yields ranging from 0.08 to 5.0 μ g. Fecal DNA obtained from the GuSCN/silica protocol was the lowest with total DNA yields ranging from 0.02 to 1.0 μ g.

The quality of the fecal DNA also differed for the four extraction protocols (Table 2-1). Fecal DNA isolated from ten samples using the PTB protocol resulted in seven samples with high MW + low MW DNA and three samples with only low MW DNA. Of twelve samples extracted using the modified DNeasy tissue protocol, four samples had low MW DNA, four had high MW, and four had high MW + low MW DNA. Of six samples isolated using the GuSCN/silica extraction method, three samples had high molecular weight (MW) DNA and three samples had high MW + low MW DNA. Fecal DNA isolated using the modified sodium lysis protocol was not visible on agarose gels, even when microgram quantities of fecal DNA were electrophoresed (Figure 2-2). In brief, the PTB and modified DNeasy methods provided the best quality DNA from bat guano.

Table 2-1 Comparison of DNA quantity determined using a fluorometer and DNA quality assessed subjectively through visualization on 2% agarose gel for the four different extraction methods For each protocol, the DNA quantity is the total amount of DNA isolated from 20-25 mg of fecal material MW = molecular weight

	DNA extraction method			
	Modified sodium lysis ^a	GuSCN/silica ^b	PTB _c (N-phenacylthizolium bromide)	DNeasy tissue kit
DNA quantity (µg/20-25 mg)	0.2 - 80.0	0.02 - 1.0	0.1 - 3.0	0.08 - 5.0
DNA quality (number of samples)	No DNA visible	High MW + low MW (3), low MW (2), no DNA visible (1)	High MW + low MW (7), low MW (3)	High MW (4), High MW + low MW (4), Low MW (4)

^a - Swiger, 1999

^b - Boom *et al.*,

^c - Poinar *et al.*, 1998

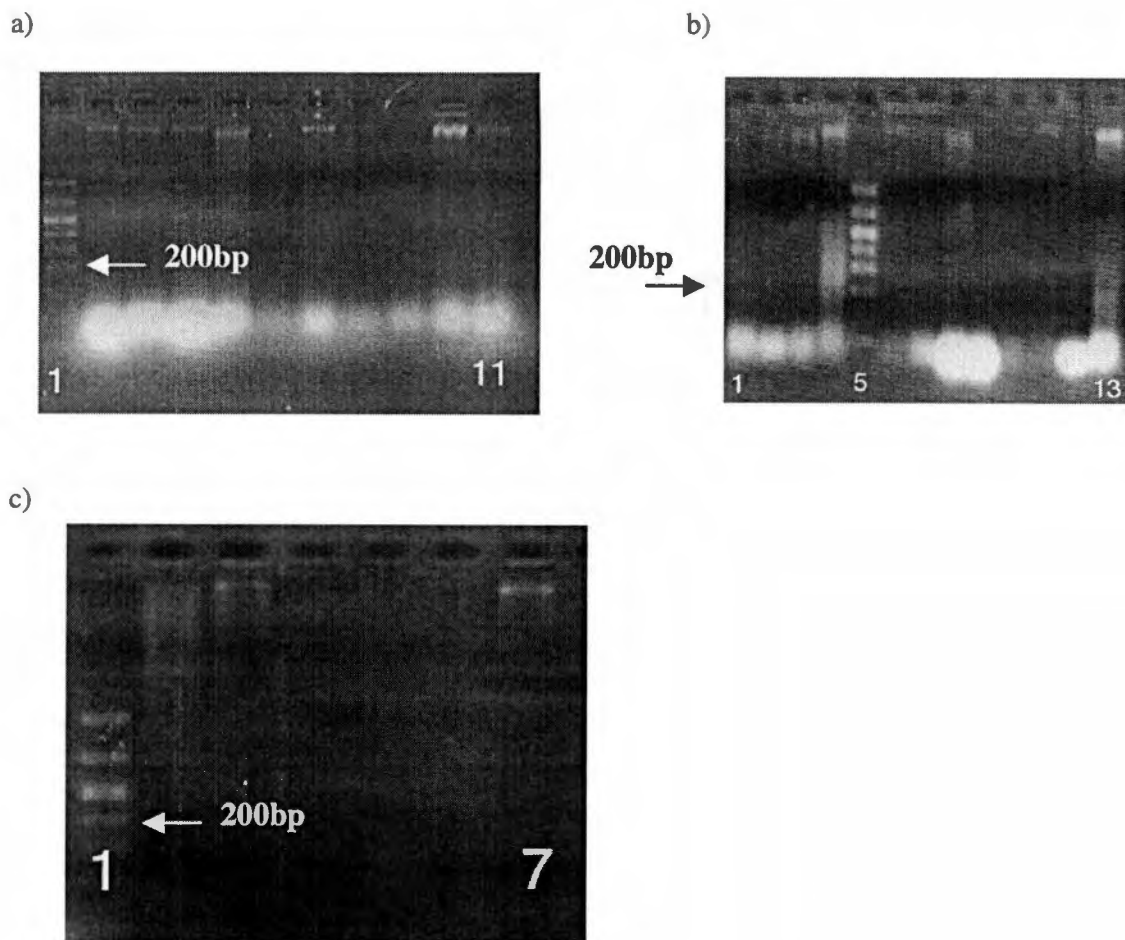


Figure 2-2: Quality of fecal DNA as assessed by electrophoresis. a) Fecal DNA isolated with PTB extraction method. Lane 1, 1 Kb, 50bp size marker; seven samples have high and low molecular weight (MW) DNA and three samples have high MW DNA b) Fecal DNA isolated with modified DNeasy tissue kit. Lane 5, 1 kb size marker; four samples have high and low MW DNA, four have high MW DNA, and four have low MW DNA c) Fecal DNA isolated with GuSCN/silica protocol. Lane 1, 1 Kb size marker; three samples have high and low molecular weight (MW) DNA and three samples have high MW DNA

PCR amplification

PCR amplification results are summarized in Table 2-2. PCR amplicon sizes were approximated using a size marker (BioVentures) of known base pairs.

Using the modified DNeasy tissue protocol, 13 of 14 (93%) of the fecal samples containing *H. zea* DNA amplified the expected *H. zea* PCR product. However, PCR products of the expected fragment size also resulted for five of 14 (36%) fecal samples containing only *H. virescens* DNA. The *H. zea* marker did not amplify a PCR product against mealworm fecal DNA (Figure 2-3). PCR products did not amplify for any of the *H. zea* fecal samples isolated using the modified sodium lysis method, GuSCN/silica, and PTB methods (Figures 2-4 to 2-6).

When tested against fecal DNA extracted with the modified DNeasy tissue kit, the *H. virescens* nuclear primers amplified PCR products of expected size for six of eleven *H. virescens* fecal DNA samples. PCR products also amplified for four of the remaining five *H. virescens* fecal DNA samples, however, the products were approximately 180 base pairs in length instead of the expected size of 200 base pairs. Eight of eleven fecal DNA samples containing *H. zea* amplified the shorter 180 base pair PCR product (Figure 2-7). For both *H. zea* and *H. virescens* fecal DNA, three of four samples resulted in a PCR product (Figure 2-8) using fecal DNA isolated with the GuSCN/silica method. However, the amplified product for *H. zea* and *H. virescens* fecal DNA was approximately 180 base pairs in length, not the expected 200bp PCR product. PCR products of the expected size were not visible for fecal samples containing *H. zea*, *H. virescens*, or mealworm DNA when the nuclear *H. virescens* primers were tested using DNA isolated with the modified sodium lysis and PTB methods (Figures 2-9 and 2-10).

Amplification products of the expected size were obtained with fecal DNA isolated using the modified DNeasy tissue protocol and *H. virescens* mitochondrial markers. Nine of ten PCR products amplified for fecal DNA containing *H. virescens* and eight of ten amplified from fecal DNA containing *H. zea* (Figure 2-11). PCR products were not observed for fecal DNA

Table 2-2 PCR amplification results for the four extraction methods using the *H. zea*, *H. virescens* nuclear, and *H. virescens* mitochondrial marker sets. The moth markers are all species-specific when applied to moth genomic DNA (Chapter 1)

Primer set	Modified sodium lysis ^a		GuSCN/silica ^b		PTB ^c		DNeasy tissue kit	
	<i>H. zea</i> feces	<i>H. virescens</i> feces	<i>H. zea</i> feces	<i>H. virescens</i> feces	<i>H. zea</i> feces	<i>H. virescens</i> feces	<i>H. zea</i> feces	<i>H. virescens</i> feces
<i>H. zea</i>	0/8	0/8	0/8	0/8	0/6	0/6	13/14	5/14
<i>H. virescens</i> , nuclear	0/11	0/11	3*/6	3*/6	0/6	0/6	8*/11	10**/11
<i>H. virescens</i> , mitochondrial	0/6	0/6	0/7	0/7	0/8	1/8	9/10	8/10

^a - Swiger, 1999

^b - Boom *et al.*,

^c - Poinat *et al.*, 1998

* - indicates PCR product was not the expected fragment size

** - 4 of 10 were not the expected fragment size

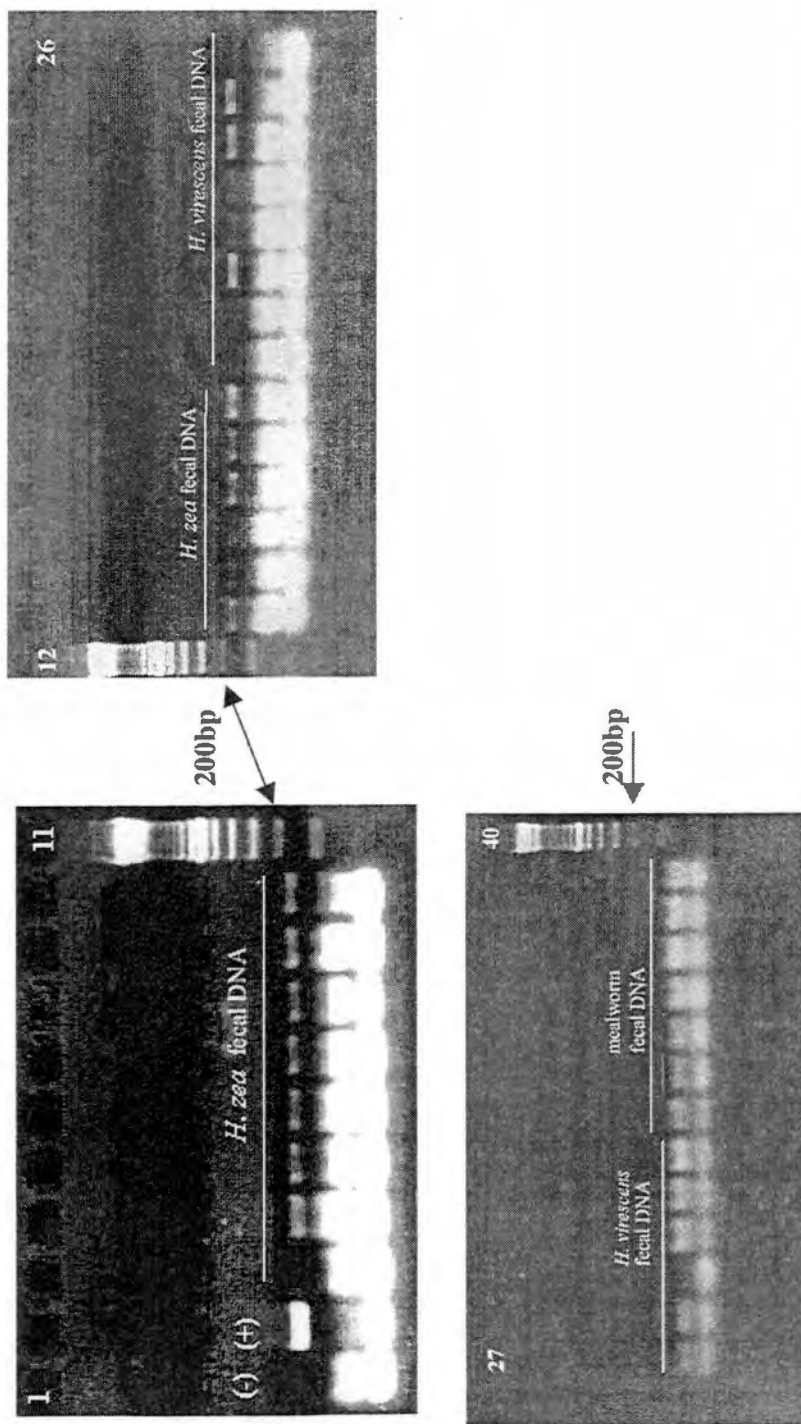


Figure 2-3: PCR amplification with the *H. zea* PBAN primers against fecal DNA isolated using modified DNeasy tissue kit. Lanes 11, 12, and 40, 2Kb, 100bp size markers; lane 2, positive control with *H. zea* genomic DNA (PCR product=145bp); lane 1, negative control of water; lanes 3-10 and 13-18, *H. zea* fecal DNA, expected PCR products amplified for 13 of 14 samples; lanes 19-32, *H. virescens* fecal DNA, PCR products amplified for 5 of 14 samples; lanes 33-39, mealworm fecal DNA, 0 of 7 amplified.

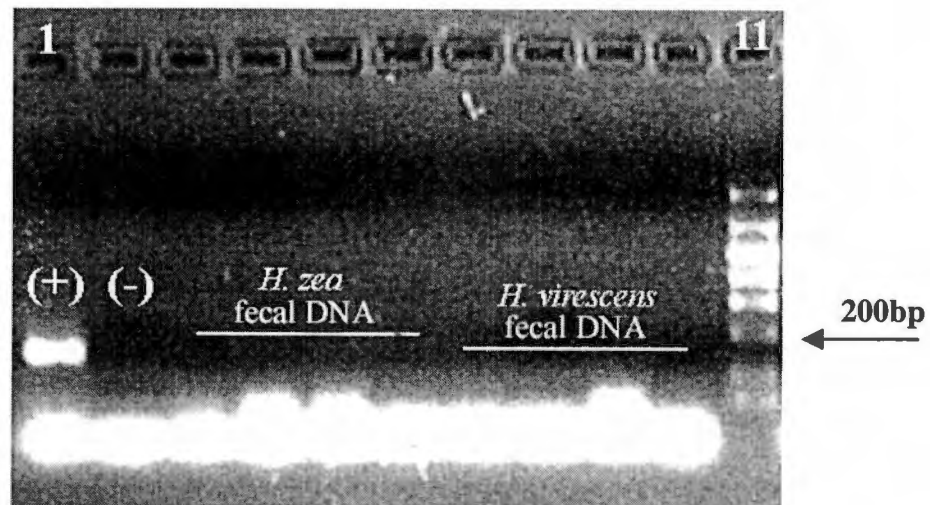


Figure 2-4: PCR amplification with the *H. zea* PBAN primers against fecal DNA isolated using the modified sodium lysis protocol. Lane 11, 1Kb, 50bp size marker; lane 1, positive control with *H. zea* genomic DNA (PCR product=145bp); lane 2, negative control of mealworm fecal DNA; lanes 3-6, *H. zea* fecal DNA; lanes 7-10, *H. virescens* fecal DNA. PCR products did not amplify from *H. zea* and *H. virescens* fecal DNA.

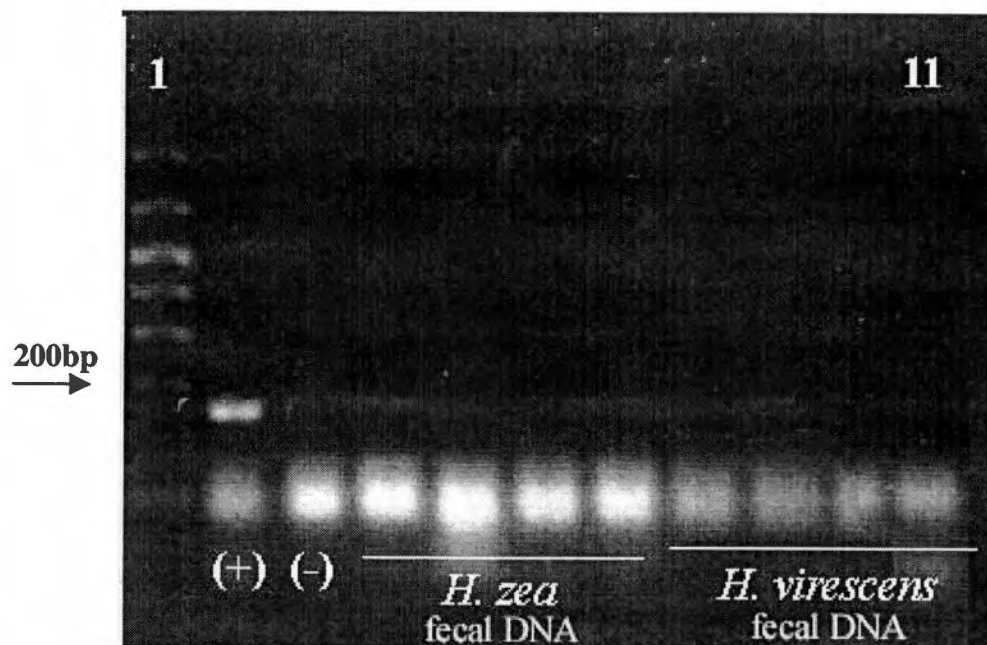


Figure 2-5: PCR amplification with the *H. zea* PBAN primer set against fecal DNA extracted using GuSCN/silica protocol. Lane 1, 1Kb, 50bp size marker; lane 2, positive control with *H. zea* genomic DNA (145bp); lane 3, negative control of mealworm fecal DNA; lanes 4-7, *H. zea* fecal DNA; lanes 8-11, *H. virescens* fecal DNA. No PCR products amplified from *H. zea* or *H. virescens* DNA.

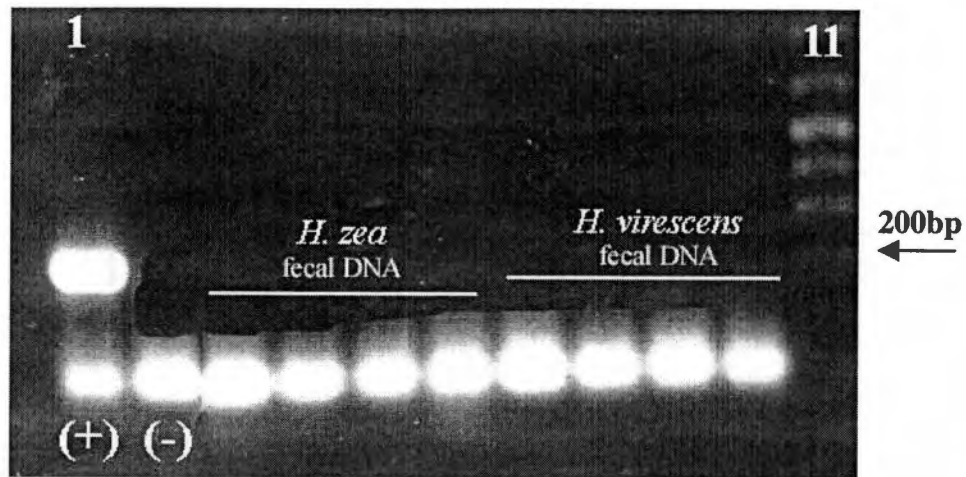


Figure 2-6: PCR amplification with the *H. zea* PBAN primer set against fecal DNA isolated using PTB extraction protocol. Lane 11, 1Kb, 50bp size marker; lane 1, positive control with *H. zea* genomic DNA (145bp); lane 2, negative control of mealworm fecal DNA; lanes 3-6, *H. zea* fecal DNA; lanes 7-10, *H. virescens* fecal DNA. No PCR products amplified from *H. zea* or *H. virescens* fecal DNA.

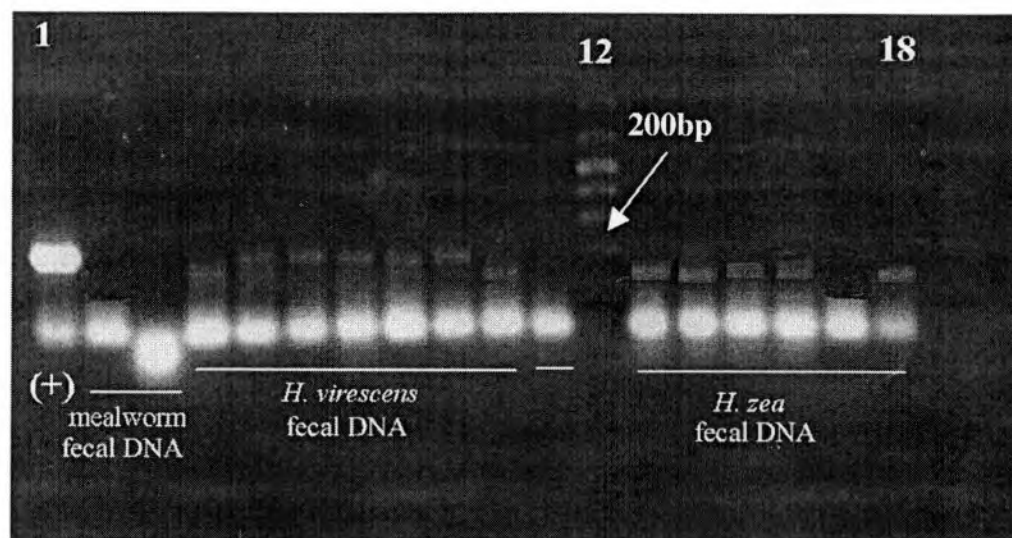


Figure 2-7: PCR amplification with the *H. virescens* nuclear primer set of fecal DNA isolated with modified DNeasy tissue kit. Lane 12, 1Kb, 50bp size marker; lane 1, positive control of *H. virescens* genomic DNA (200bp); lanes 2-3, mealworm fecal DNA, no PCR products amplified; lanes 4-10, *H. virescens* fecal DNA, PCR products that amplified were 200bp (expected size) and 180bp in length; lanes 13-18, *H. zea* fecal DNA, PCR products that amplified were 180bp in length.

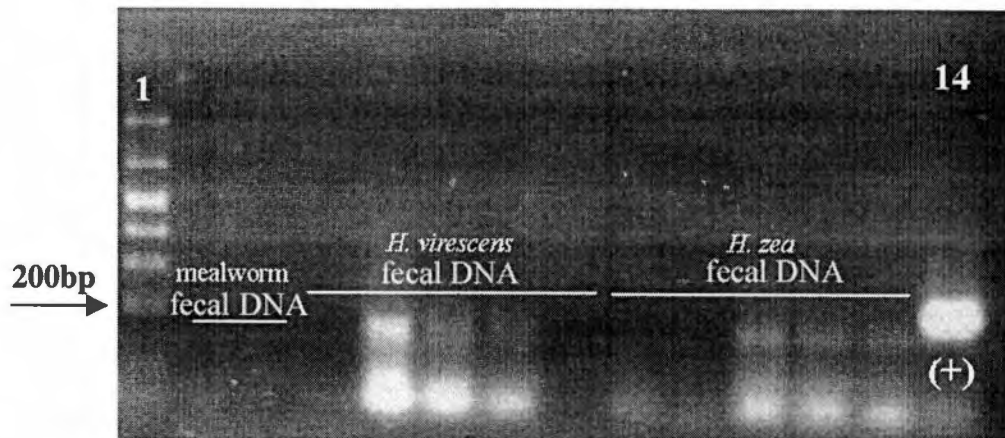


Figure 2-8: PCR amplification with the *H. virescens* nuclear primer set of fecal DNA isolated with GuSCN/silica protocol. Lane 1, 1Kb, 50bp size marker; lane 14, positive control of *H. virescens* genomic DNA (200bp); lanes 2-3, negative controls of mealworm fecal DNA; lanes 4-7, *H. virescens* fecal DNA, PCR product amplified for three of five samples, amplicons are 180bp, not expected size of 200bp; lanes 8-11, *H. zea* fecal DNA, PCR products amplified for three of five samples, again, the amplicons are 180bp, not 200.



Figure 2-9: PCR amplification with the *H. virescens* nuclear primer set of fecal DNA isolated with modified sodium lysis protocol. Lane 12, 1Kb, 50bp size marker; lane 1, positive control of *H. virescens* genomic DNA (200bp); lanes 2-3, negative controls of mealworm fecal DNA; lanes 4-7, *H. virescens* fecal DNA; lanes 8-11, *H. zea* fecal DNA. No PCR products amplified from *H. virescens* or *H. zea* fecal DNA.

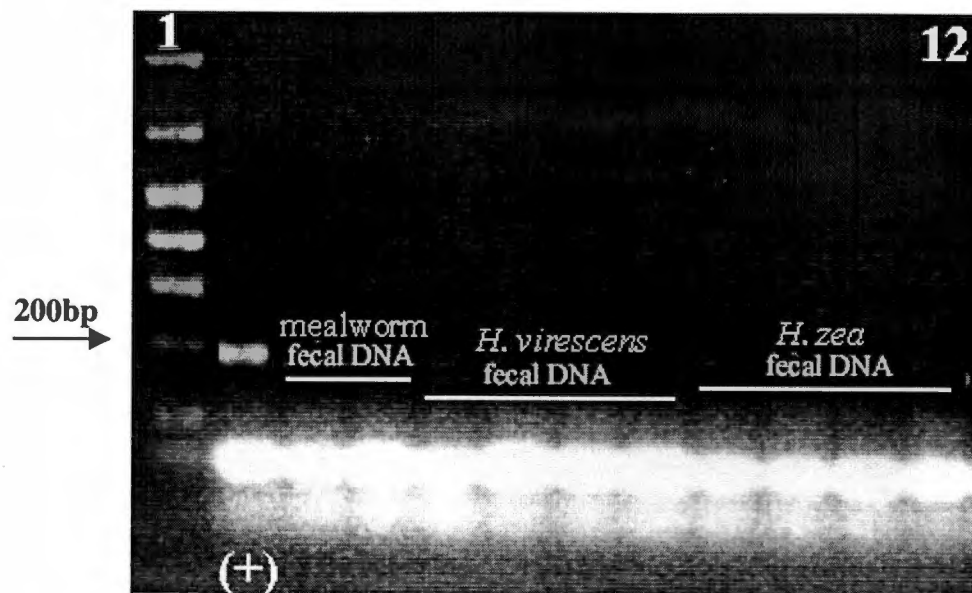


Figure 2-10: PCR amplification with the *H. virescens* nuclear primer set of fecal DNA isolated with PTB extraction method. Lane 1, 1Kb, 50bp size marker; lane 2, positive control of *H. virescens* genomic DNA (200bp); lanes 3-4, negative controls of mealworm fecal DNA; lanes 5-8, *H. virescens* fecal DNA; lanes 9-12, *H. zea* fecal DNA. No PCR products amplified from *H. virescens* or *H. zea* fecal DNA.

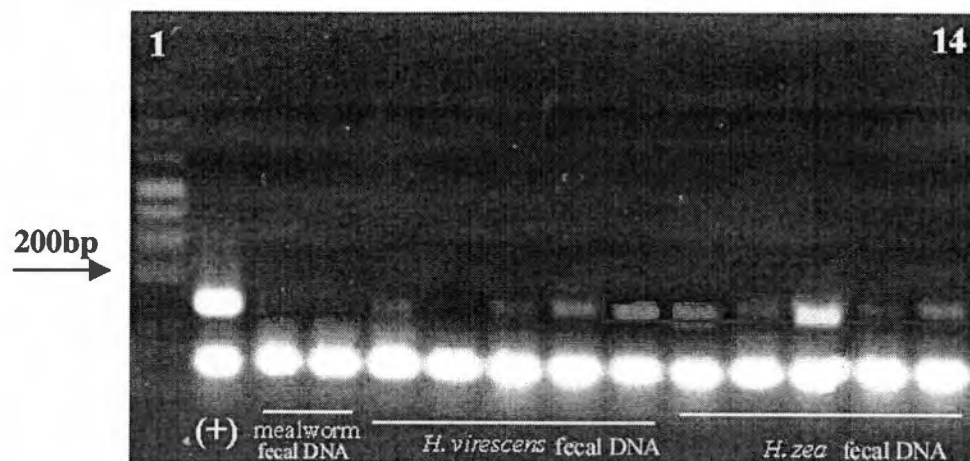


Figure 2-11: PCR amplification results with the *H. virescens* mitochondrial primer set of fecal DNA isolated using modified DNeasy tissue kit. Lane 1, 1Kb, 50bp size marker; lane 2, positive control of *H. virescens* genomic DNA (150bp); lanes 3-4, mealworm fecal DNA, no PCR products amplified; lanes 5-9, *H. virescens* fecal DNA; lanes 10-14, *H. zea* fecal DNA, PCR products amplified for *H. virescens* and *H. zea* fecal DNA.

containing mealworm. Attempts to amplify the *H. virescens* mitochondrial primers against *H. virescens* fecal DNA purified using the PTB protocol resulted in a product for one of eight samples (Figure 2-12). Fecal DNA isolated using the modified sodium lysis and GuSCN/silica methods did not result in any visible PCR products (Figures 2-13 and 2-14). In summary, the most consistent PCR amplifications were obtained with fecal DNA extracted with the modified DNeasy tissue method.

PCR-RFLP

The restriction enzyme *Nsi*I cut the PCR products amplified for *H. zea* genomic DNA to yield two fragments of approximately the expected sizes (80 base pairs and 50 base pairs). This restriction enzyme also cleaved PCR products from fecal DNA containing *H. zea* and from products from *H. virescens* fecal DNA (Figure 2-15), however, no distinct fragments were visible. The restriction enzyme *Vsp*I cut PCR products amplified for *H. virescens* genomic DNA. The digest yielded the expected 90 base pair fragment while the 60 base pair fragment was not visible. The *Vsp*I restriction enzyme also cleaved PCR products amplified against *H. virescens* and *H. zea* fecal DNA (Figure 2-16).

DISCUSSION

DNA extraction

The GuSCN/silica, PTB, and modified DNeasy tissue protocols provided not only sufficient amounts of DNA for molecular assays but also yielded DNA with samples containing both high molecular and low molecular weight DNA as assessed on a 2% agarose gel. However, the quality of the DNA as assessed on a 2% agarose gel proved not to be a good predictor of PCR success using the species-specific moth primers. I suspect that PCR inhibitors bound to the fecal DNA or present in minute quantities could not be visually detected. The modified sodium lysis protocol yielded the highest amount of fecal DNA, but the DNA obtained using the modified

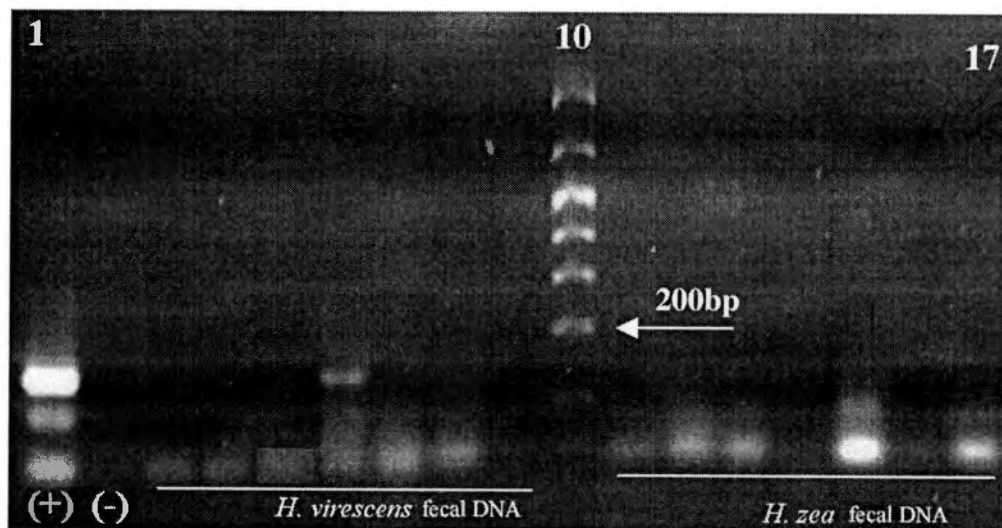


Figure 2-12: PCR amplification results with the *H. virescens* mitochondrial primers from fecal DNA isolated with PTB protocol. Lane 10, 1Kb, 50bp size marker; lane 1, positive control of *H. virescens* genomic DNA (PCR product=150bp); lanes 2, negative control of mealworm fecal DNA; lanes 3-9, *H. virescens* fecal DNA, one of seven samples amplified; lanes 11-17, *H. zea* fecal DNA, zero of seven samples amplified.

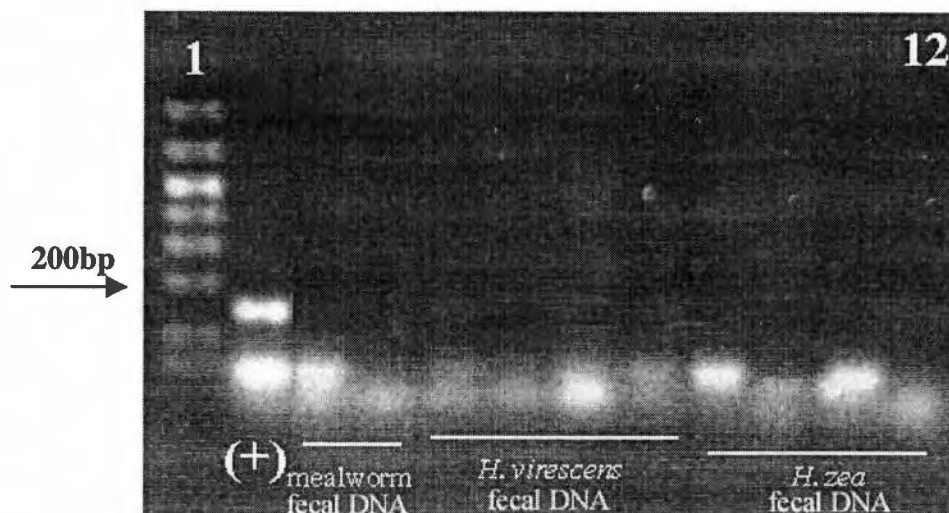


Figure 2-13: PCR amplification results with the *H. virescens* mitochondrial primer set of fecal DNA isolated using modified sodium lysis protocol. Lane 1, 1Kb, 50bp size marker; lane 2, positive control of *H. virescens* genomic DNA (150bp); lanes 3-4, negative controls with mealworm fecal DNA; lanes 5-8, *H. virescens* fecal DNA; lanes 9-12, *H. zea* fecal DNA. PCR products never amplified for *H. virescens* or *H. zea* fecal DNA.

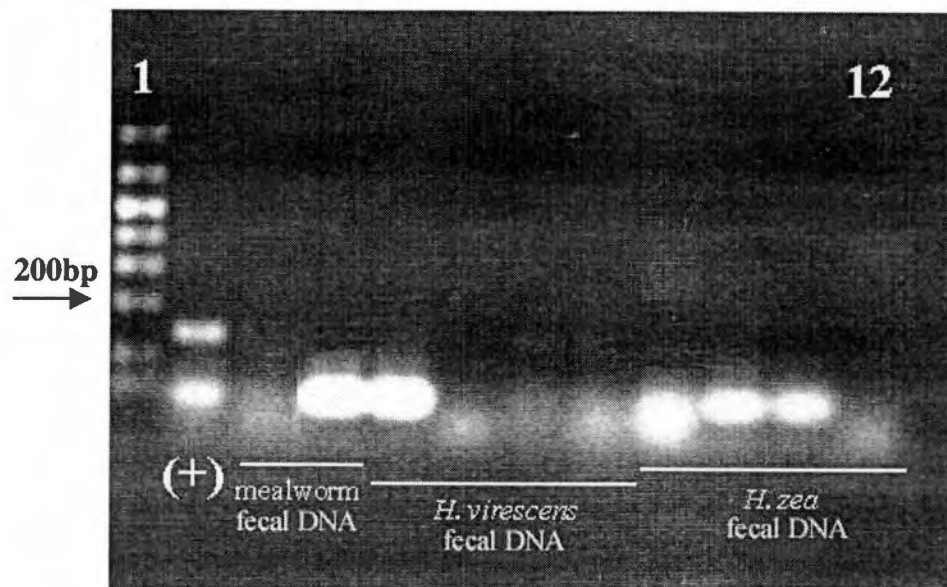


Figure 2-14: PCR amplification results with the *H. virescens* mitochondrial primers from fecal DNA isolated using GuSCN/silica protocol. Lane 1, 1Kb, 50bp size marker; lane 2, positive control of *H. virescens* genomic DNA (PCR product 150bp); lanes 3-4, mealworm fecal DNA; lanes 5-8, *H. virescens* fecal DNA; lanes 9-12, *H. zea* fecal DNA. PCR products never amplified against fecal DNA.

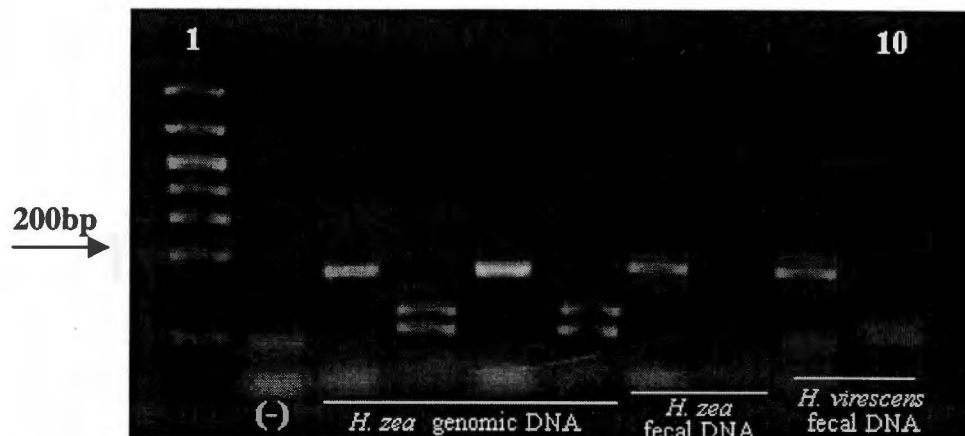


Figure 2-15: PCR-RFLP with the *H. zea* PBAN primer set of genomic *H. zea* DNA, fecal *H. zea* DNA, and fecal *H. virescens* DNA digested with *Nsi*I restriction enzyme. Lane 1, 1Kb, 50bp size marker; lane 2, negative control of water; lanes 3 and lane 5, undigested PCR products from *H. zea* genomic DNA; lanes 4 and 6, PCR fragments from *H. zea* genomic DNA digested with *Nsi*I; lane 7, undigested PCR product from *H. zea* fecal DNA; lane 8, *H. zea* fecal PCR product digested with *Nsi*I, product cut; lane 9, undigested PCR product of *H. virescens* fecal DNA; lane 10, *H. virescens* fecal PCR product digested with *Nsi*I. No visible fragments for digested lanes 8 and 10.



Figure 2-16: PCR-RFLP with the *H. virescens* mitochondrial primer set of genomic *H. virescens* DNA, fecal *H. virescens* DNA, and fecal *H. zea* DNA digested with *VspI* restriction enzyme. Lane 1, 1Kb, 50bp size marker; lane 10, negative control of water; lane 2 and lane 4, undigested PCR products from *H. zea* genomic DNA; lanes 4 and 6, PCR fragments from *H. zea* genomic DNA digested with *NsiI*; lane 7, undigested PCR product from *H. zea* fecal DNA; lane 8, PCR product from *H. zea* fecal DNA digested with *NsiI*; lane 9, undigested PCR product of *H. virescens* fecal DNA; lane 10, PCR products from *H. virescens* fecal DNA digested with *NsiI*.

sodium lysis protocol was never visualized on a gel nor would it amplify. The sodium lysis protocol was originally designed for extraction of bacterial DNA from soil samples. I suspect that the lysis buffer may have been too concentrated for use with feces which could have further degraded the fecal DNA beyond detection on a 2% agarose gel. Alternatively, contaminants or other unknown components bound to the fecal DNA may have caused faulty readings on the fluorometer, with total fecal DNA perhaps overestimated.

PCR amplification

The PCR amplification results differed for the four extraction methods. The modified DNeasy protocol gave the most consistent amplification results, with PCR products visible for all of the three primer sets. The modified DNeasy protocol was the only one of the four protocols that amplified PCR products from fecal DNA for both the *H. virescens* mitochondrial and *H. zea* nuclear markers. Although, the markers were not species-specific in the fecal samples, this is not a result of poor quality DNA. The probable reasons for the non-specific binding are discussed below.

While the GuSCN/silica protocol (Boom *et al*, 1990) has been used successfully in previous fecal studies (Hoss and Paabo, 1993, Taberlet *et al*, 1996, Reed *et al*, 1997), I had poor amplification results using this same protocol, with fecal DNA not amplifying for either the *H. virescens* mitochondrial or the *H. zea* nuclear markers, and only six of twelve amplifying for the *H. virescens* nuclear marker. Studies have shown that silica particles will bind to DNA rather than inhibitors in the presence of high concentrations of GuSCN. However, GuSCN and silica are both potential PCR inhibitors, (Boom *et al*, 1990), and trace amounts of either substance can interfere with PCR. This possible inhibition may be the underlying reason for the observed failure of the GuSCN/silica protocol in this study.

The PTB extraction method yielded poor PCR-quality DNA. The PTB extraction method was used to examine the diet of the extinct ground sloth (*N. shastensis*, Poinar *et al*, 1998). In

that study, the authors found that DNA from coprolites could not be amplified until the addition of PTB to the extraction buffer. PTB is a reagent that cleaves glucose-derived protein crosslinkers (Vasan *et al* , 1996; Poinar *et al*, 1998) that form from a browning reaction (Evershed *et al*, 1997) or Maillard process that is a “biochemical aging process initiated by the reaction of proteins with reducing sugars” (Bucala *et al*, 1984, Vasan *et al* , 1996). In fecal DNA, the brown pigments can be attributed to Maillard products. In the ground sloth study, PTB added to the fecal extraction released fecal DNA bound to the Maillard products, allowing for PCR amplification (Poinar *et al*, 1998). However, Maillard products which are associated with decaying and aging materials are not always present in fecal DNA. Fecal samples collected in this study were frozen at -80°C within 12 hours of the bats defecating, allowing for little decay time. For this reason, PTB may not have been useful for isolating PCR-quality DNA from bat feces. The failure of PCR amplifications using this protocol may also be attributed to the presence of GuSCN or silica in the fecal DNA.

In summary, the modified DNeasy tissue protocol, in comparison to the other three protocols, yielded the most consistent PCR amplifications with the three moth primers. Although the DNeasy kit is more costly than the other methods, the advantages offered by the kit outweigh its expense.

PCR controls

Because of the sensitivity of PCR to a few copies of template DNA, contamination is a potentially important factor in fecal analysis. The spread of minute volumes of foreign DNA can result in false positives. Therefore, in this study, extra precautions, such as the use of new sterile chemicals and filter pipette tips, were taken to avoid contamination. Negative controls were also important, allowing for contamination of DNA to be quickly detected and removed. Negative controls using water or mealworm fecal DNA and positive controls using genomic moth DNA were useful for identifying false positives and negatives.

Non-specific primer binding

Fecal extractions contain DNA of the diet, the organism, and gut bacteria, with the DNA of the diet and organism present in both lower quantity and quality than that of bacteria (Morin and Woodruff, 1996, Kohn and Wayne, 1997). While PCR is sensitive to only a few copies of template DNA (Arnheim *et al* , 1990), amplification of targeted DNA in feces may not always occur. Therefore, PCR amplification using species-specific moth primers of fecal DNA containing the target moth species may not always be 100%.

The species-specific moth primers tested against fecal DNA isolated using the DNeasy extraction amplified products were not specific to the target moth species. For example, while the *H. zea* marker did not amplify with *H. virescens* genomic DNA, feces containing *H. virescens* fecal DNA amplified the PCR product that was expected for *H. zea*. The two species, *H. zea* and *H. virescens*, are closely related (Fang *et al* , 1997, Roehrdanz, 1997) and are expected to be similar in DNA sequences. The *H. zea* primers were designed from an *H. zea* intron region (Davis *et al* , 1992). Because introns are not under selective control and are often variable between species, the primers were specific to *H. zea* genomic DNA. However, since, fecal DNA is sheared and highly fragmented, the *H. zea* primers that would not bind to intact *H. virescens* genomic DNA, might bind to the cleaved primer template sequence and amplify a non-specific PCR product of similar size in fecal DNA containing *H. virescens*. The same logic applies to the non-specific binding in fecal DNA for the other two markers.

In a study of ancient DNA, Paabo *et al* (1990) sequenced PCR products to describe a possible mechanism that can account for non-specific primer binding. When template DNA is sheared, as is the case with ancient and fecal DNA, few molecules contain intact primer sites. This damage may promote the 'jumping polymerase chain reaction' (Paabo *et al* , 1990) which allows for the amplification of longer DNA segments than are present in the sample. In the process, primers extend onto different DNA segments until the other priming site is reached and

amplify a fragment of the expected size. The end result is the inaccurate amplification of template sequences. I hypothesize that this model may apply to the non-specific products that amplified in fecal DNA using the three moth primers.

PCR-RFLP digests

Sequencing of all specific and non-specific fecal PCR products should differentiate the two moth species, however, the ultimate goal of this study was to apply the DNA moth marker to hundreds of fecal samples collected from wild bats. Sequencing hundreds of PCR products is impractical for this application. Therefore, PCR-RFLP was chosen as a more practical method to differentiate between the moth species in bat guano. The PCR-RFLP studies were not informative because fecal PCR products from both the target and non-target moths, were cut with the restriction enzymes.

While the test with genomic *H. zea* DNA demonstrated that the primer binding sites are specific to *H. zea*, it is not known what sequence differences may occur between primer sites. The *H. zea* sequence could not be compared to the homologous *H. virescens* sequence since PCR product never amplified with *H. virescens* genomic DNA. Therefore, the restriction sites for *NsiI* may be present in the homologous sequence of both moth species. The presence of the same restriction site would explain why the non-specific PCR products that amplified from *H. virescens* fecal DNA were cut with *NsiI*. The same explanation may account for the digestion of fecal PCR products with the restriction enzyme *VspI*.

Future possibilities

Because primers that were specific against genomic DNA resulted in non-specific binding and amplification of apparently random PCR products in fecal DNA, I believe that future studies should target more conservative DNA sequences by using genus-specific or even family-specific markers. The genus-specific or family-specific markers would be expected to amplify PCR products for several taxa. These PCR products could then be differentiated by taxon using

PCR-RFLP with each species possibly having unique restriction sites and therefore, unique banding patterns. Sequences used in phylogenetic studies (Pashley *et al* , 1993, Taylor *et al* , 1993, Brower and DeSalle, 1994, Weller *et al* , 1994, Friedlander *et al* , 1996, Fang *et al.*, 1997) for moth species are potential sources of genus-specific and family-specific markers. Such sequences would be ideal since complete sequences have already been determined, allowing for the identification of species-specific restriction sites (Appendix H)

While it may be difficult to differentiate moth species using PCR-RFLP of a 200 base pair sequence, the chances of detecting base pair differences increase with larger PCR fragments. By amplifying larger PCR products up to 300 and 400 base pairs in length and targeting conserved sequences, unique restriction sites could be determined for different moth species. While moth markers amplifying fragments less than 200 base pairs were discussed in this chapter, fragments of 280 and 400 base pairs for the moths have been amplified in fecal DNA. These markers were only tested against fecal DNA isolated using the modified DNeasy protocol and the results are presented in Appendix I. I believe PCR-RFLP presents a promising solution to problems associated with identifying prey items from fecal DNA.

Significance

The application of molecular scatology to dietary studies of bats has the potential to confirm the economic value of bat populations to agriculture. In the Lower Rio Grande Valley of Texas, agricultural fields of corn, cotton, and other commercially significant crops cover much of the landscape. These fields experience damage from infestations of migratory and local moth populations of *H. zea* and *H. virescens*. The combined losses to U.S. agricultural producers due to crop damage by such insect pests total over \$1 billion annually. In Texas alone, cotton producers in 1995 spent \$12.6 million treating agricultural fields and lost 182,000 bales due to *H. zea* infestations (McCracken and Westbrook, 1999).

Efforts to control insect populations involve host plant resistance, conservation of natural predators, introduction of alien predators, and use of pesticides, with pesticides the most common practice (Allen and Hoelscher, 1997). With increasing societal concern of extensive insecticide use, and the growing resistance to insecticides by many pests (McCaffery, 1998), management programs should focus more attention on conservation of natural enemies. Biological control involving the use of natural predators of the pest species is one of the oldest and most effective means of insect pest control and offers a safer and more efficient approach than increased pesticide use. Natural predators such as bats can not only significantly reduce insect populations and potential crop damage caused by pests but can also better track insect outbreaks and regulate insect populations (Pedigo, 1989).

Future molecular fecal studies that are aimed towards verifying the consumption of pest species by bats can build upon and extend the results of this project. Further research into the diets of bats has the potential of confirming the economic value of bat populations to the agricultural industry as existing biological control agents. Since in recent years populations of many insectivorous bats have been declining (Barbour and Davis, 1969, Cockrum, 1969, McCracken, 1986), application of molecular scatology to their diet also provides powerful ammunition for the conservation and protection of populations of these bats (McCracken, 1989).

CHAPTER 3

Use of microsatellites for identifying bats through guano

INTRODUCTION

Fecal analysis, while traditionally applied to questions of diseases and parasitic loads in medicine and pathology (Balatbat *et al* , 1996, Schubbert *et al* , 1997, Makristathis *et al* , 1998, Matsuki *et al.*, 1998, Kageyama and Benno, 2000), is becoming increasingly prevalent in the fields of ecology and population genetics (Hoss *et al.*, 1992, Gerloff *et al.*, 1995, Kohn *et al.*, 1995, Taberlet *et al* , 1996; Van der Kuyl *et al* , 1996, Reed *et al* , 1997, Poinar *et al* , 1998, Gerloff *et al* , 1999, Whittier *et al* , 1999, Ernest *et al* , 2000, Farrell *et al* , 2000) Recent advances in molecular genetics allow for the amplification of DNA from minute quantities of tissue (Paabo, 1989, Boom *et al* , 1990, Hoss and Paabo, 1993, Deuter *et al* , 1995, O'Rourke *et al* , 1996, Yang *et al* , 1997), including tissue that slough off an animal's intestinal tract as it defecates For this reason, molecular scatology which is the application of molecular techniques to fecal analyses, can be used to answer an array of questions regarding difficult to capture, endangered, and even extinct organisms (Hoss *et al* , 1992, Kohn *et al* , 1995, Tikel *et al* , 1996, Reed *et al* , 1997, Poinar *et al* , 1998)

Microsatellites of the host organism have been successfully amplified from fecal DNA of wild Bonobos (*Pan paniscus*, Gerloff *et al* , 1999), dugongs (*Dugong dugong*, Tikel *et al* , 1996), baboons (*Bison donasus*, Whittier *et al* , 1999), and grey (*Halichoerus grypus*) and harbour (*Phoca vitulina*) seals (Reed *et al* , 1997) to assess paternity and kinship, sex, and for species identification Levels of genetic variation were estimated from fecal DNA for the highly endangered European brown bears (*Ursus arctos*, Hoss *et al* , 1992) by sequencing a portion of the mitochondrial control region In that study, the control region sequence also allowed for comparison of genetic relationship of brown bears from the Brenta region of Europe to those from Romania In another study, DNA extracted and amplified from 11,000-31,000 year old

coprolites confirmed that the coprolites were from the extinct ground sloth (*Nothrotheriops shastensis*, Poinar *et al* , 1998)

Although molecular scatology has been applied to several mammal populations (Hoss *et al* , 1992, Gerloff *et al*, 1995, Kohn *et al.*, 1995, Reed *et al* , 1997, Gerloff *et al* , 1999, Ernest *et al* , 2000, Farrell *et al* , 2000), it has not yet been used to investigate the ecology of bats. Fecal analysis offers a valuable tool for studying bats due to many aspects of their ecology. Bats are distributed throughout the world and occupy a wide range of habitats including caves, rock crevices, tree canopies, and man-made structures (Altringham, 1996). Due to the inaccessibility of many roost sites, it is often difficult to capture bats to collect tissue samples for molecular analyses. As an example, many species of flying foxes (*Pteropus spp*) roost in large colonies in trees many meters above the ground. While giant harp traps have been used to catch random bats (Tiddeman and Loughland, 1993), capturing specific individuals to collect tissue for genotyping is difficult and has hindered much-needed research on their social organization and population structure.

A variety of molecular markers are available for studies of population genetics. Microsatellite markers are among the most recently developed and useful of these as they are highly variable, nuclear DNA markers that are inherited in a co-dominant fashion (Bruford and Wayne, 1993). Microsatellites offer many advantages for fecal studies since they require just picogram amounts of DNA and the target PCR products are typically less than 300bp in length. Here, I report the first fecal extraction protocol that allows for the isolation of DNA of bat guano. In this project, I 1) test the accuracy of the protocol by comparing microsatellite genotypes obtained from bat feces to the genotypes obtained from tissue from the same bats, and 2) test the reliability and reproducibility of the protocol by determining if genotypes obtained from multiple fecal samples are identical for the same individual.

MATERIALS AND METHODS

Fecal collections

Fecal samples for the genotyping of bats using microsatellites were obtained from ten big brown bats (*Eptesicus fuscus*) captured at Maryville College in Tennessee. During captivity, the bats were fed mealworms twice a day for two days. After each feeding, the bats were placed in separately labeled bags until they defecated. The guano was collected and placed in sterile tubes labeled with the number of the bat and stored at -80°C until analysis. Two 3mm wing biopsy punches were obtained from the same bats and refrigerated in saturated NaCl-DMSO solution (Worthington Wilmer and Barratt, 1996). Genotypes from the bats' genomic DNA isolated from these wing tissue samples were compared to genotypes obtained from their feces.

In a separate experiment, genotypes from multiple fecal samples from same individual were compared using ten different big brown bats. The bats were fed mealworms twice a day for three days and placed in labeled cloth bags after each feeding to collect feces. Multiple fecal samples were collected from the ten bats and stored in labeled tubes at -80°C. Wing biopsies were not taken from these bats.

Hair samples shed by the bat while in the bag could potentially contaminate the fecal material. However, hair shed during the bats grooming rarely contain follicles and are unlikely sources of contamination.

DNA extraction

The wing biopsies were digested in a standard DNA extraction buffer (100mM NaCl, 10mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 0.5% SDS) with 20 µL of 20 mg/ml Proteinase K (Promega) overnight at 55°C. After the tissue was digested, the DNA was isolated with phenol/chloroform. The DNA was precipitated with 100% ethanol and eluted in 1/10 TE (Sambrook *et al* , 1989).

Fecal DNA was isolated using a modification of the DNeasy tissue extraction protocol (Qiagen) which included two lysis buffers (ATL and AL), two washing buffers (AW1 and AW2), and an elution buffer (AE). For DNA isolation, 20 to 25 mg of feces was placed in 1.5 ml tubes and lysed with 20 μ L of 20 mg/ml of Proteinase K (Promega) and 350 μ L of ATL buffer. Pestles were used to break apart the fecal pellets. Forceps used to weigh fecal material were thoroughly cleaned with ethanol between different samples and buffers were handled only with filter pipette tips. After incubation at 55°C in a water bath for 2 ½ to 3 hours, 380 μ L of AL buffer was combined with each sample and incubated for another 10 minutes at 70°C. The samples were centrifuged at 16,000xG for 10 minutes and the supernatant was transferred to a new tube. The fecal debris pellet was discarded. The supernatant was diluted with 380 μ L of 100% ethanol and stored at -20°C for 30 minutes. The samples were transferred to filter columns provided in the kit and centrifuged at 5,000xG for 1 minute. The filter with the bound DNA was washed once with 100% ethanol, once with AW1 buffer, and once with AW2 buffer. After the final wash, 100 μ L of AE buffer was added to the filter. The filter column was centrifuged and the elution buffer containing the DNA was stored at 4°C. The tissue and fecal DNA were quantified using a Hoeffer DynaQuant© 200 fluorometer.

DNA analysis

Five microsatellite primers were tested against the fecal and tissue DNA of *E. fuscus* (Table 3-1). Two of the primers, EF1 and EF6, were designed for *E. fuscus* by Marteen Vonhof (pers. comm.). The other primers were originally designed for other Vespertilionid bats, primers MM5 (Petri *et al.*, 1997) for *Myotis myotis*, Paur02 (Burland *et al.*, 1998) for *Plecotus auritus* and NN8 (Petri *et al.*, 1997) for *Nyctalus noctula*. Microsatellite PCR reactions for tissue DNA were carried out in 12 μ L volumes, each containing 5-10 ng of template DNA, 1X Promega PCR buffer, 1.04mM MgCl₂, 0.10mM dNTP's, 0.10 pmol of the unlabeled primer, and 1 pmol of the ³²P end-labeled primer, 1 unit of *Taq* DNA polymerase, and sterile distilled H₂O to 12 μ L. The

Table 3-1 A list of the microsatellite primers tested for *E. fuscus*. Included are the sequences for the forward and reverse primers and the approximate product size in base pairs. The subscript denotes the source of the primer sequence.

Species	Locus/ Primer set	Primer sequence (5'-3')	Approx PCR product size (bp)
<i>E. fuscus</i>	EF1 _a	F ATCTGGGCAATGATACCTTT R GCAGGCTGGGCTGAG	200
<i>E. fuscus</i>	EF6 _a	F ATCTGTTTTTCTCTCCTTAT R ATCACATTTTTGAAGCAT	200
<i>E. fuscus</i>	MM5 _b	F TTCATCCAGTTCTGG R GTTGATTTAACATGC	150
<i>E. fuscus</i>	Paur02 _c	F GGATTCAATTCTGGTCAAGGAC R AACCACCCATTCTCTGTAGTGAG	200
<i>E. fuscus</i>	NN8 _b	F TTGTGTTTTTAAAGAAAATCC R ATAGGTGATTTCATTCCCA	150

_a – designed for *E. fuscus* by Marteen Vonhof at York University, Toronto Canada

_b – Petri *et al.*, 1997

_c – Burland *et al.*, 1998

PCR amplification profile involved initial denaturation for 2 min at 94°C, followed by 30 cycles of 94°C for 1 min , 50 or 55°C for 30 sec , depending on the primer (Table 3-2), and 72°C for 30 sec MgCl₂ concentrations varied for the primers (Table 3-2)

PCR reactions for the fecal DNA samples were similar to those used for the tissue DNA. The only variations were that the fecal DNA PCR reactions contained 2-20 ng template DNA, 10 ng Bovine Serum Albumin, and 2 units of *Taq* Polymerase. All PCR runs were accompanied by a negative control reaction using distilled water in place of template DNA. For fecal DNA samples that would not amplify on the first PCR, a second PCR was done. However, if fecal DNA samples still did not amplify after the second PCR, a second fecal extraction was done. PCR products were separated on 6% polyacrylamide sequencing gels and visualized using X-ray sensitive film. A 1 Kb ladder (BioVentures) and a G-A-T-C sequence (Promega) were included on each gel to facilitate scoring of alleles.

RESULTS

DNA was successfully extracted from all the tissue and fecal samples. The amount of DNA from tissue samples ranged from 0.02 to 1.0 µg. The amount of fecal DNA ranged from 0.08 to 3.0 µg.

Three of the microsatellite loci (MM5, EF1, and EF6) were highly variable with nine to eleven alleles identified at each locus (Table 3-2). Paur02 and NN8 were monotypic. Therefore, only MM5, EF1, and EF6 were included in the subsequent genotyping of bats. For the experiment comparing the genotypes from bat's tissue to the same bat's feces, 100% (30/30) of the tissue DNA amplifications and 93% (28/30) of the fecal DNA amplifications yielded microsatellite PCR products upon the first PCR attempt. The two reactions that did not produce PCR products amplified after the second PCR. For the experiment comparing the genotypes

Table 3-2 PCR annealing temperatures and magnesium concentrations used for the three microsatellite primers used in this study The total number of alleles per locus are also included

Locus	Annealing temp. (° C)	Mg concen.	# alleles
EF1	55	0.71mM	11
EF6	50	0.75mM	9
MM5	50	1.04mM	9

obtained from multiple fecal extractions from the same bat, 92 % (55/60) of the fecal DNA amplifications yielded PCR products on the first attempt. Of the five reactions that did not produce PCR products, three did after the second PCR. For the remaining four samples, two amplified after the second fecal extraction (Table 3-3). Two fecal samples never amplified using the MM5 primers even after a second PCR and a second extraction. These two fecal samples were collected from the same bat. Wing tissue was not collected for this bat.

In all cases where a fecal sample amplified, the genotype obtained from feces consistently matched the genotype obtained from the wing biopsy of the bat (Figures 3-1 to 3-3). Though the genotypes from the feces were usually fainter than the genotypes from tissue DNA, they could all be reliably scored. Multiple extractions from fecal samples that were obtained from the same bat always gave the same genotype (Figures 3-4 to 3-6).

DISCUSSION

Fecal analysis, when coupled with molecular techniques such as PCR, provides a powerful tool for studying animal populations (Morin and Woodruff, 1996, Schlotterer, 1998). With PCR, a region of DNA can be replicated to yield millions of copies from a single template molecule (Arnheim *et al* , 1990). However, the use of fecal material presents unique problems for DNA isolation and PCR amplification. Feces consist not only of sloughed intestinal cells from the organism but also of fragments of consumed prey species and intestinal bacterial cells, which can complicate the isolation of the DNA of the defecator. PCR amplification also can be inhibited by contaminants in feces such as bile salts (Deuter *et al* , 1995) and polysaccharides (Monteiro *et al* , 1997). The modified DNeasy tissue protocol (Qiagen) used in this study overcomes these problems and yields good quality DNA from bat guano.

Since feces contain DNA of the organism in very low concentrations and in a fragmented condition, it is conceivable that some microsatellite alleles, especially those with larger product

Table 3-3· Results of the genotyping of bats using three microsatellite primer sets

	# tissue DNA samples amplified	# fecal DNA samples amplified			% of genotypes matched	
Locus	1 st PCR	1 st PCR	2 nd PCR	2 nd fecal extraction	Tissue to feces	Feces to feces
Ef1	10/10	29/30	1/1	-----	100	100
EF6	10/10	27/30	1/3	2/2	100	100
MM5	10/10	27/30	1/3	0/2	100	100

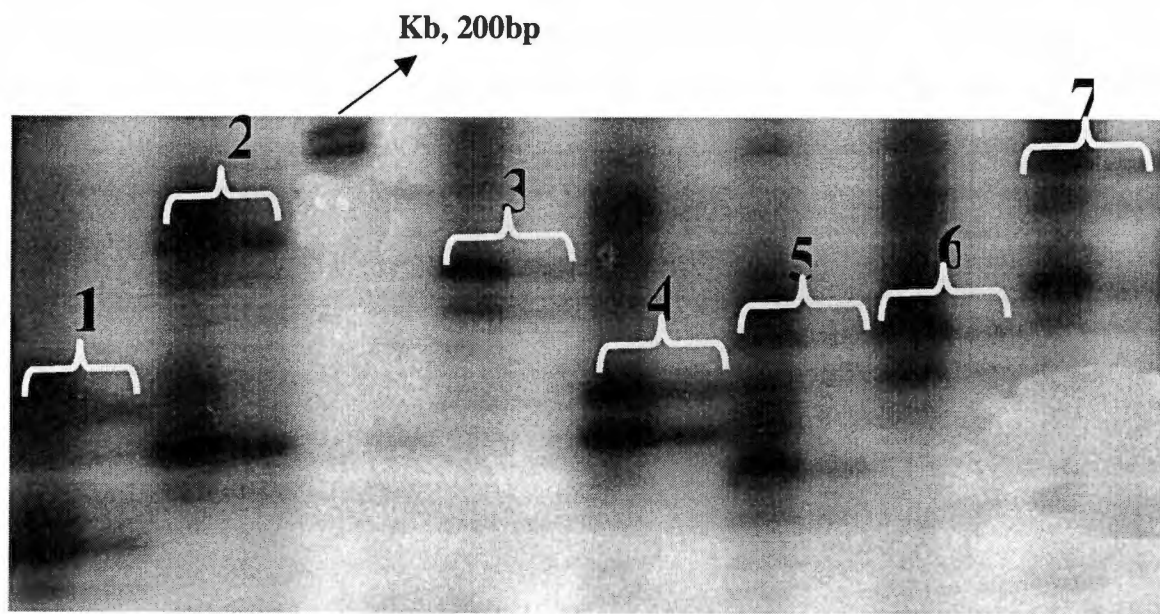


Figure 3-1: Autoradiograph of amplification results with microsatellite locus EF1, comparing the bats' tissue genotype to its fecal genotype. The brackets and numbers indicate 2 samples from the same bat. The first sample represents the bats' genotype from tissue DNA and the second sample represents the bats' genotype from fecal DNA. In all cases, the genotype from the bat and its feces matched. A 1Kb, 50bp size marker was used to estimate PCR product size.

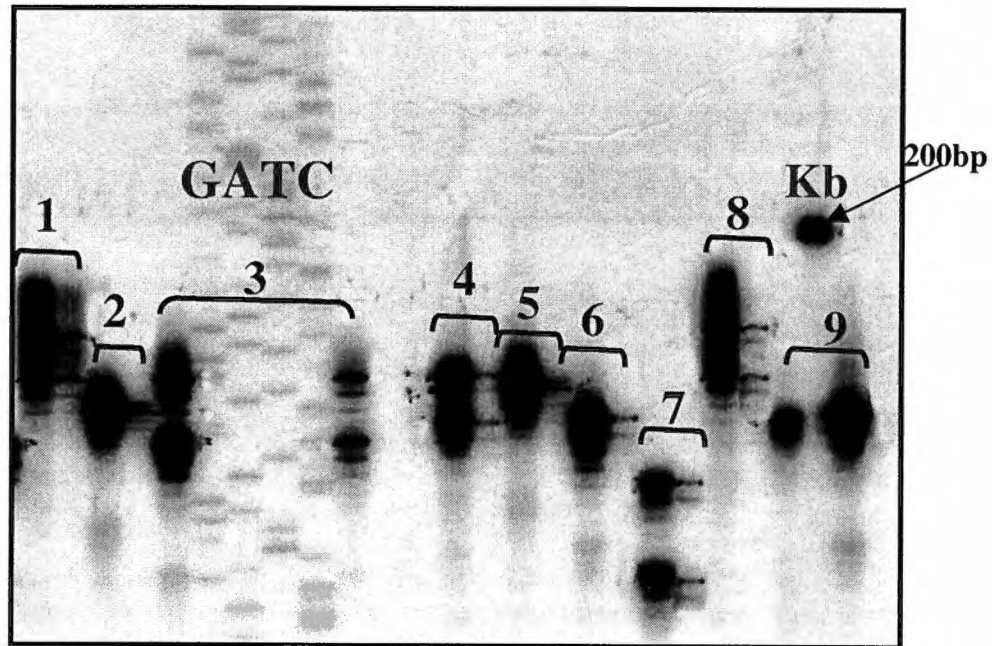


Figure 3-2: Autoradiograph of amplification results with EF6, comparing the bats' genotype to its feces genotype. The brackets and numbers indicate 2 samples from the same bat. The first sample is the bats' genotype from tissue DNA. The second sample is the bats' genotype from fecal DNA isolated. For #3, the tissue genotype is to the left of the G-A-T-C sequence and the fecal genotype to the right. For #9, the tissue genotype is to the left of the 1 Kb marker and the fecal genotype to the right. In all cases, the genotype from the bat and its feces matched. A 1 Kb, 50bp size marker was used to estimate PCR product size. A sequence (G-A-T-C) was used to facilitate scoring of genotypes.

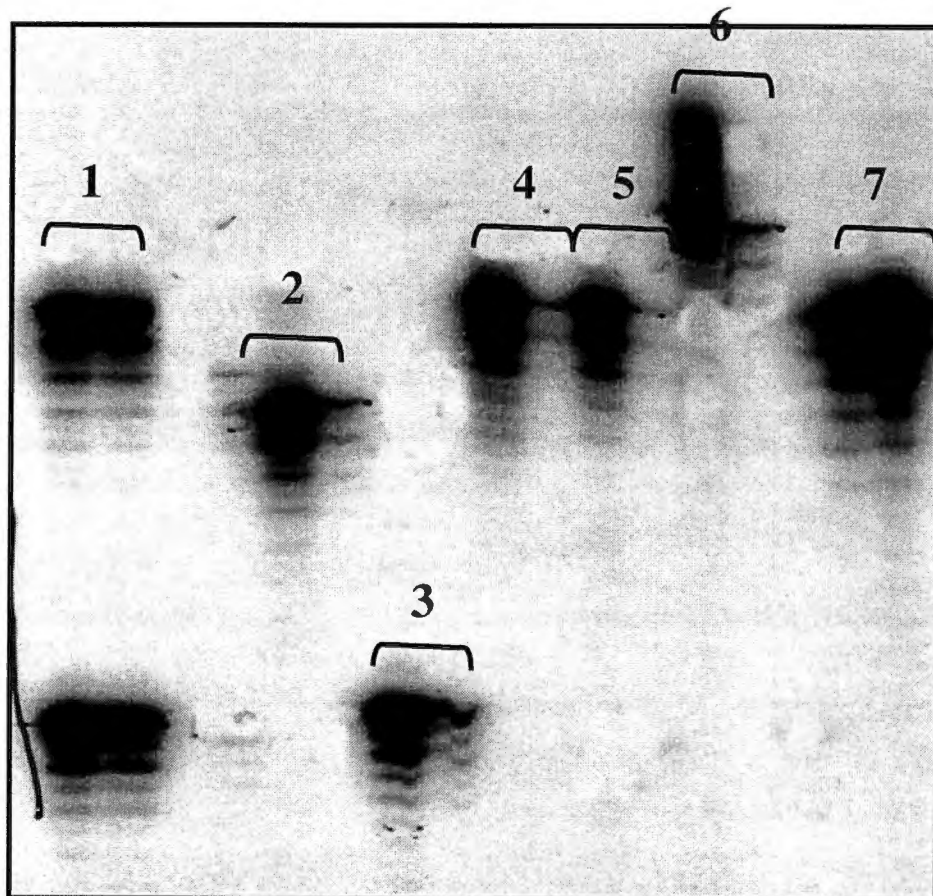


Figure 3-3: Autoradiograph of amplification results with microsatellite locus MM5, comparing the bats' tissue genotype to its fecal genotype. The brackets and numbers indicate 2 samples from the same bat. The first sample is the bats' genotype from tissue DNA. The second sample is the bats' genotype obtained from fecal DNA. In all cases, the genotype from the bat and its feces matched.

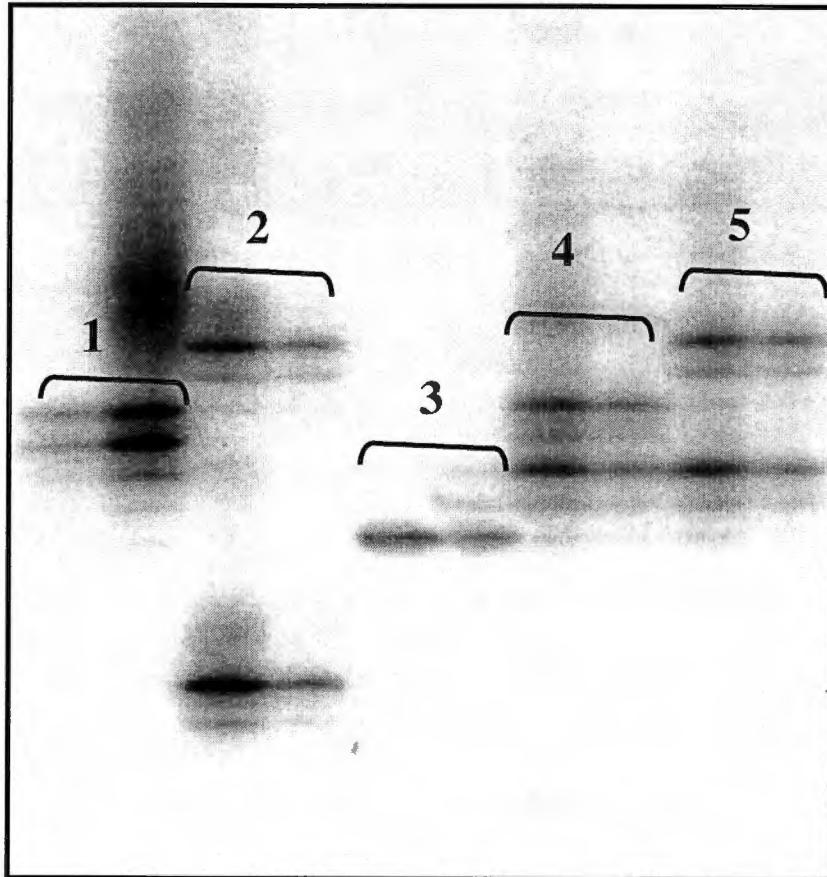


Figure 3-4: Autoradiograph of amplification results with microsatellite EF1, comparing genotypes from two fecal DNA samples obtained from the same bat. The brackets and numbers indicate two fecal genotypes from two fecal DNA samples for the same bat.

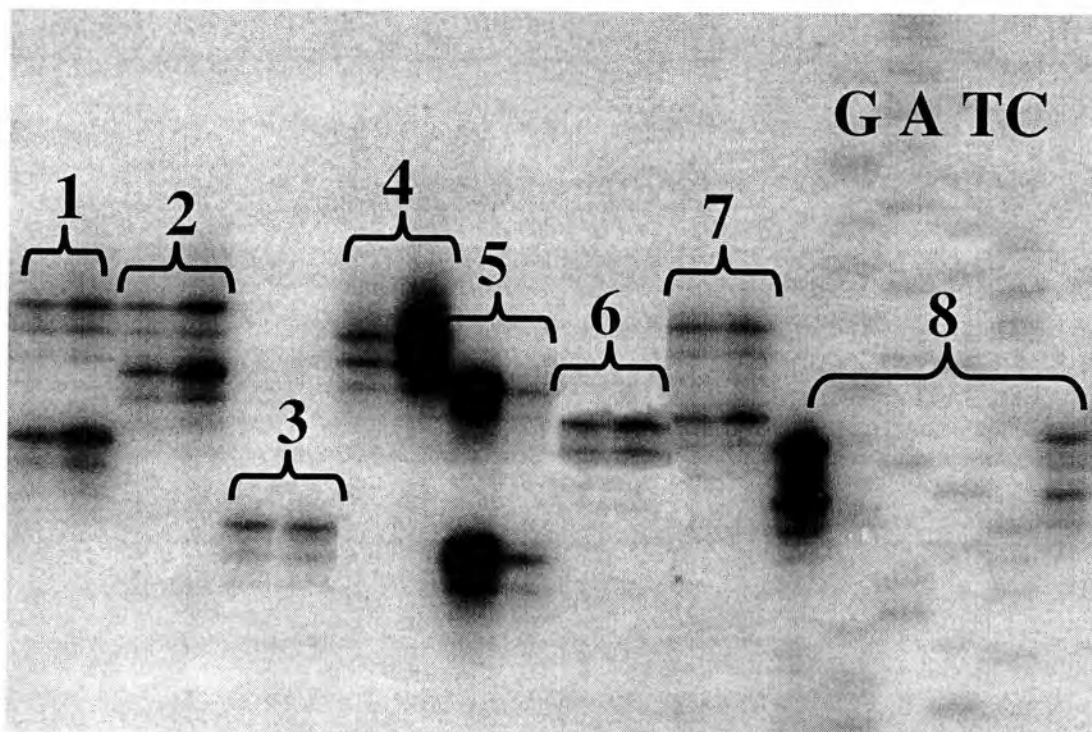


Figure 3-5: Autoradiograph of amplification results with microsatellite EF6, comparing genotypes from two fecal DNA samples obtained from the same bat. The brackets and numbers indicate two fecal genotypes from two fecal DNA samples for the same bat. For #8, one fecal sample is to the left of the G-A-T-C sequence and the other to the right. G-A-T-C sequence was used to facilitate scoring of genotypes.

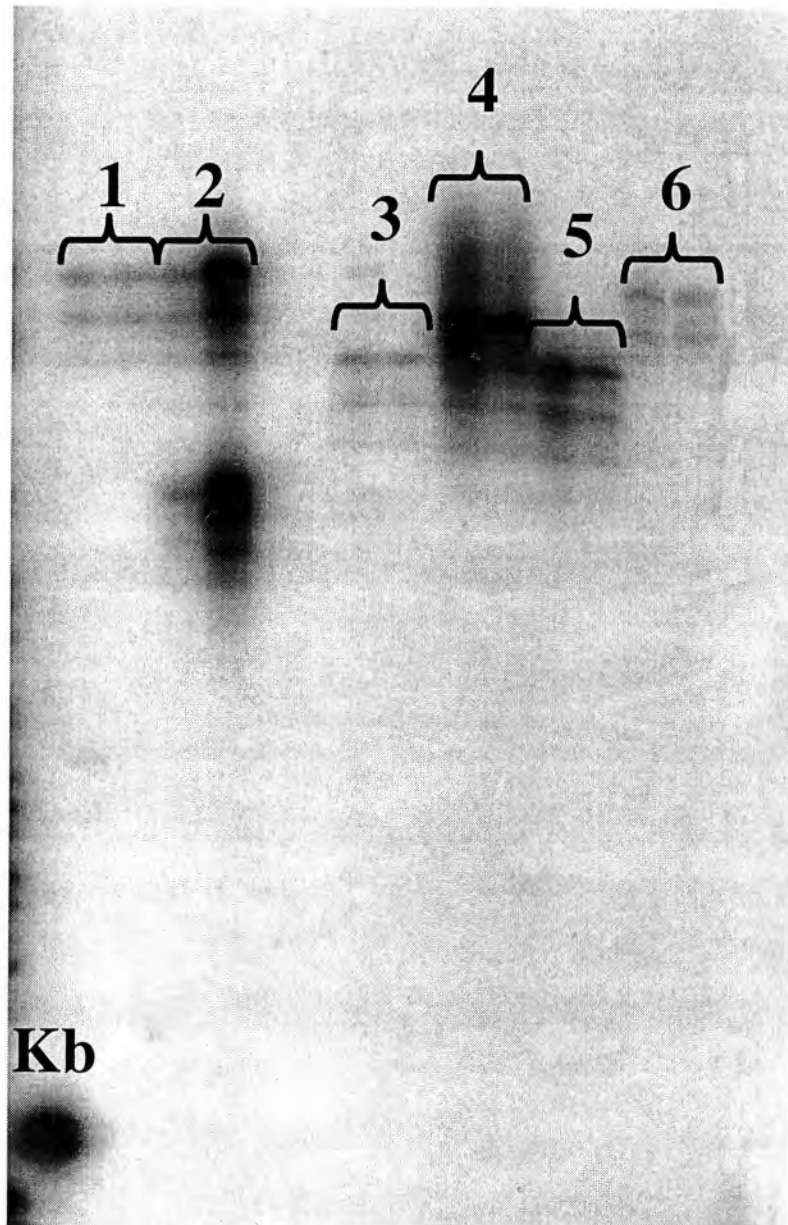


Figure 3-6: Autoradiograph of amplification results with microsatellite MM5, comparing genotypes from two fecal DNA samples obtained from the same bat. The brackets and numbers indicate two fecal genotypes from two fecal DNA samples for the same bat. A 1 Kb, 50bp size marker was used for estimating PCR fragment size.

sizes, may not amplify. Studies on Bonobos (*Pan pygmeus*, Gerloff *et al* , 1995, Gerloff *et al* , 1999) and brown bears (*Ursus arctos*, Taberlet *et al* , 1996) have shown that some microsatellite alleles can fail to amplify causing individuals that are heterozygous to be incorrectly genotyped as homozygous. This problem of misidentification of genotypes was not encountered in this study. The comparisons of tissue and fecal amplifications as well as fecal amplifications from multiple DNA isolations from the same individual demonstrated that non-amplifiable alleles were not a problem. While the genotypes obtained from guano were usually fainter than that from tissue, they were confidently scored.

These consistent results may have occurred because the microsatellite PCR products of the loci used were less 200 base pairs in length with the largest allele for the three loci only 192 base pairs. PCR product sizes are not mentioned in the studies on Bonobos (Gerloff *et al* , 1995, Gerloff *et al* , 1999) and brown bears (Taberlet *et al* , 1996), but it is possible that larger alleles did not amplify due to extensive fragmentation of the target DNA. A second possibility for the prevalence of null alleles in the previous studies may have resulted from the failure to quantify the fecal DNA. In these studies, PCR amplifications were performed by diluting samples in increasing increments and determining the dilution that worked best. DNA yields for fecal samples vary with some feces containing a higher concentration of tissue from the animal's rectal wall than others. By quantifying fecal DNA in this study, the total amount of DNA for the microsatellite PCR reactions was controlled.

While the modification to the DNeasy extraction method isolated high quality DNA, multiple fecal samples from one bat could not be amplified for one microsatellite locus (MM5). Even after attempting to amplify DNA from a second extraction, this individual could never be genotyped at this locus. However, the two other loci reliably amplified for this bat. It is possible that this particular bat had a point mutation in the primer-binding sequence that did not allow at

least one of the microsatellite primers to bind. Tissue samples from the bat were not available to test this theory.

The genotyping of bats through their feces has applications in ecology and conservation biology. For many endangered and especially difficult-to-capture organisms, tissue samples may not be easily or safely collected for molecular analyses. Feces, on the other hand, can be obtained without the need for CITES permits and without undue stress to the organisms (Morin and Woodruff, 1996; Schlotterer, 1998). Many endangered organisms go undetected because of their low numbers and the difficulties in tracking them. However, organisms leave their mark in an area through their feces, allowing individuals and populations to be identified and studied.

CONCLUSIONS

The research opportunities available through fecal analyses have increased the range of questions that can be addressed for remote and endangered species. In this research, bats were reliably genotyped through their feces, which has broad applications for population genetics, for kinship and paternity studies, and provides possibilities of collecting fecal samples from inaccessible roosts to answer questions on various aspects of the bats' ecology and behavior.

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APPENDICES

APPENDIX A

Fecal extraction protocols

The extraction protocols (lysis, modified lysis, modified GuSCN/silica, and modified PTB) are all modifications to the protocols described in Chapter 2. However, the modifications did not yield good quality DNA. Also, the Promega and Dynal protocols are described below. Table A-1 includes the total DNA yields and moth primers tested for each extraction protocol

Lysis protocol (Alcivar *et al* , 1989, Whittier *et al* , 1999)

- 1) Weigh 20 to 25 mg of feces into 1.5 ml tubes
- 2) Add 1 ml of lysis buffer (0.02M Tris/HCl, 0.01M EDTA, 1% SDS, 0.15M NaCl, 0.01M DTT) and 25 μ L of 25 mg/ml Proteinase K (Promega) to feces --- mix
- 3) Incubate samples @ 37°C overnight.
- 4) Bring the samples back to 1M NaCl
- 5) Add 1/10 volume of 10% hexadecyl trimethylammonium bromide (CTAB)/0.7M NaCl --- mix
- 6) Add equal volumes of chloroform --- mix
- 7) Centrifuge @ 16,000xG for 15 minutes.
- 8) Transfer supernatant to new 1.5 ml tube
- 9) Repeat steps 6 and 7
- 10) Add equal volumes of phenol/chloroform/isoamyl (25:24:1 by volume) --- mix
- 11) Centrifuge @ 16,000xG for 20 minutes.
- 12) Transfer supernatant to new 1.5 ml tube
- 13) Add 2.5 times volume of 100% EtOH and place @ 4°C overnight
- 14) Centrifuge @ 16,000xG for 20 minutes --- discard supernatant
- 15) Wash pellet with 75% EtOH
- 16) Dry pellet in vacuum
- 17) Elute DNA with 1/10 TE

Modifications

- 1) Add 1 ml of extraction buffer (0.1M Tris/HCl pH 7.2, 10mM EDTA pH 8.0, 0.5M LiCl, 1% SDS, 50mM DTT) to 20 to 25 mg feces --- mix
- 2) Add 100 μ L of 0.1M PTB/0.01 NaPO₄ and 20 μ L of 20mg/ml Proteinase K (Promega) --- mix
- 3) Incubate overnight @ 37°C
- 4) Add 1/10 volume of 10% hexadecyl trimethylammonium bromide (CTAB)/0.7M NaCl --- mix
- 5) Incubate for 10 minutes @ 65°C
- 6) Go to step 4 in the Lysis protocol

Promega wizard kit

Buffers provided with the kit. NucleiLysis, Rnase, Protein precipitate

- 1) Weigh 20 to 30 mg feces into 1.5 ml tubes
- 2) Add 800 μ L of chilled NucleiLysis buffer to feces --- mix
- 3) Incubate for 45 minutes @ 65°C
- 4) Add 3 μ L of Rnase and incubate for 30 minutes @ 37°C
- 5) Cool samples to room temperature
- 6) Add 200 μ L of Protein precipitate --- vortex
- 7) Centrifuge @ 13,800xG for 5 minutes
- 8) Transfer supernatant to a 1 ml tube containing 600 μ L of room temperature isopropanol
- 9) Centrifuge @ 13,800xG for 1 minute
- 10) Discard supernatant
- 11) Wash pellet with 70% EtOH
- 12) Dry pellet in vacuum
- 13) Elute DNA with 50 μ L of water

Dynal DNA Direct Kit

TEN buffer 500mM Tris, 16mM EDTA, 10mM NaCl, pH – 9.0

- 1) Add 75 μ L of TEN buffer to 20 to 25 mg feces --- mix
- 2) Centrifuge @ 8,000xG for 2 minutes
- 3) Transfer supernatant to 1.5 mL tube
- 4) Proceed with DNA Direct kit.
- 5) Add 200 μ L Dynabeads to the supernatant ---do not vortex
- 6) Let sit at room temperature for five minutes
- 7) Place tube in Dynal MPC (Magnetic Particle Concentrator) for 1 to 2 minutes --- DNA Dynabeads move towards magnet
- 8) Discard supernatant.
- 9) Remove tube from MPC
- 10) Add 200 μ L washing buffer in a single pipetting motion --- do not break pellet
- 11) Replace in MPC for 30 seconds
- 12) Discard supernatant
- 13) Remove tube from MPC
- 14) Repeat steps 10 – 14 twice
- 15) Re-suspend DNA Dynabeads in 40 μ L elution buffer ----mix
- 16) Replace in MPC for 30 seconds
- 17) Keep supernatant

Modified Guanidium/silica protocol

- 1) Add 1 ml of extraction buffer (0.1M Tris/HCl pH 7.2, 10mM EDTA pH 8.0, 0.5M LiCl, 1% SDS, 50mM DTT) to 20 to 25mg of feces.
- 2) Add 100 μ L of 0.1M PTB/0.01 NaPO_4 and 20 μ L of 20mg/ml Proteinase K (Promega) --- mix
- 3) Add equal volumes of phenol/chloroform/isoamyl (25:24:1 by volume) --- mix.
- 4) Centrifuge @ 16,000xG for 20 minutes
- 5) Transfer 700 μ L of supernatant to new 1.5 ml tube
- 6) Add 1.4 ml of L6 buffer (Figure 2-1, Chapter 2) --- mix.
- 7) Incubate in 60°C water bath shaker for 2 hours.
- 8) Add 50 μ L silica solution (Figure 2-1, Chapter 2) --- mix
- 9) Incubate at room temperature for 1 hour
- 10) Centrifuge @ 9,000xG for 30 seconds
- 11) Discard supernatant
- 12) Wash pellet twice with 1 ml L2 buffer (see Figure 2-1, Chapter 2), once with 100% EtOH, and once with acetone
- 13) Between each wash step, centrifuge @ 9,000xG for 30 seconds and discard supernatant.
- 14) Elute DNA with 100 μ L of 1/10 TE.

Modified PTB protocol

- 1) Add 1 ml extraction buffer (0.1M Tris/HCl pH 7.2, 10mM EDTA pH 8.0, 0.5M LiCl, 1% SDS, 50mM DTT) to 20 to 25 mg of feces
- 2) Add 100 μ L of 0.1M PTB/0.01 NaPO_4 and 20 μ L of 20 mg/ml Proteinase K (Promega) --- mix
- 3) Incubate overnight @ 37°C
- 4) Add 20 μ L of 20 mg/ml Proteinase K (Promega)
- 5) Incubate @ 37°C for 2 hours
- 6) Bring to 1M NaCl
- 7) Pipette solution over column of glass wool
- 8) Add 100 μ L of fresh extraction buffer.
- 9) Incubate @ 55°C for 2 hours
- 10) Add equal volumes of phenol/chloroform/isoamyl (25:24:1 by volume) --- mix
- 11) Centrifuge @ 16,000xG for 20 minutes
- 12) Transfer supernatant to new 1.5 ml tube
- 13) Add 2.5 times volume of 100% EtOH and place @ 4°C overnight
- 14) Centrifuge @ 16,000xG for 20 minutes --- discard supernatant
- 15) Wash pellet with 75% EtOH
- 16) Dry pellet in vacuum
- 17) Elute DNA with 1/10 TE

Table A-1 List of different fecal extraction protocols tested, including total fecal DNA yielded and moth primers tested*

Fecal extraction protocol	Total DNA yields (µg)	Moth DNA primers tested
Lysis	0.20 – 24.00	Hze1/2, Hzea2/3, Hvir1/2
Modified Lysis	0.03 – 25.00	Hzea2/3, Hvir1/2
Promega Wizard	0.01 – 2.00	Hzea2/3, Hvir1/2
Dynal DNA Direct	0.06 – 1.00	Hzea2/3
Modified GuSCN/silica**	0.01 – 0.70	Hzea2/3, Hvir1/2
Modified PTB**	0.40 – 4.50	Hzea2/3, Hvir1/2

*- Moth DNA primers are described in Chapter 1. The primers did not amplify PCR products against fecal DNA isolated using the different extraction protocols listed.

** - GuSCN/silica and PTB protocols are described in Chapter 2.

APPENDIX B

Spectrophotometer

The modified sodium lysis protocol yielded poor quality fecal DNA. To test if the fecal DNA contained high concentrations of protein which could have inhibited PCR, spectrophotometer readings were taken for fecal DNA samples at two different wavelengths, 260nm and 280nm. Readings were also taken for *H. zea* and *H. virescens* genomic DNA to serve as controls (Table B-1). The 260nm wavelength measures the concentration of DNA while the 280 wavelength measures protein concentrations in the fecal DNA samples. If the ratio of the DNA to protein ranges from 1.4 to 1.8, the DNA is relatively clean. If the ratio is less than 1.1, the sample contains high protein levels (Sambrook *et al*, 1989).

The spectrophotometer readings showed that moth genomic DNA was of good quality, however, the fecal DNA contained high protein concentrations. Only one *H. virescens* fecal DNA sample had a reading that indicated good quality DNA.

Table B-1: Spectrophotometer readings taken for *H. zea* and *H. virescens* genomic DNA as well as *H. zea* and *H. virescens* fecal DNA isolated using the modified sodium lysis protocols. The readings taken at the wavelengths 260 and 280, along with the ratios, were obtained from spectrophotometer printouts. Ratios 1.4-1.8 = good quality DNA/low protein concentrations, ratios <1.1 = low quality DNA/high protein concentrations.

Sample	260 λ	280 λ	Ratio (260:280)
<i>H. zea</i> genomic moth	0.030	0.020	1.6000
<i>H. zea</i> genomic moth	0.010	0.006	1.6200
<i>H. virescens</i> genomic moth	0.024	0.016	1.4776
<i>H. virescens</i> genomic moth	0.017	0.011	1.6250
<i>H. zea</i> feces	0.067	0.060	1.1245
<i>H. zea</i> feces	0.021	0.051	0.4109
<i>H. zea</i> feces	0.026	0.062	0.4257
<i>H. zea</i> feces	0.020	0.036	0.5570
<i>H. zea</i> feces	0.024	0.036	0.6429
<i>H. zea</i> feces	0.024	0.021	1.0921
<i>H. virescens</i> feces	0.010	0.006	1.6200
<i>H. virescens</i> feces	0.007	0.013	0.5673
<i>H. virescens</i> feces	0.016	0.017	0.9638
<i>H. virescens</i> feces	0.006	0.009	0.6883
<i>H. virescens</i> feces	0.007	0.009	0.7763
<i>H. virescens</i> feces	0.013	0.013	1.0097

APPENDIX C

Filtration methods

The high protein concentrations in the fecal DNA could inhibit PCR amplification. Therefore, fecal DNA samples isolated with the modified sodium lysis protocol were further purified using 2 methods 1) polypropylene filtration (Cavallini *et al.*, 2000) and 2) standard phenol/chloroform extraction (Sambrook *et al.*, 1989) In addition, *H. virescens* mitochondrial and *H. zea* nuclear primers were tested against these purified fecal DNA samples

Filtration methods have been widely used for isolating DNA, with the various methods using different filter matrices Cavallini *et al.* (2000) tested the ability of five filter matrices in isolating fecal DNA The authors found polypropylene filters purified fecal DNA by removing bacterial polysaccharides The filters bound the DNA while allowing inhibitors to pass through.

In this study, fecal DNA was centrifuged through microcon filter columns that have a polypropylene matrix However, microcon filters did not allow inhibitors to pass through the filters, rather the inhibitors and DNA stayed on the filters and the DNA could not be eluted Therefore, I could not quantify the DNA or test PCR amplification

For the phenol/chloroform purification method, fecal DNA ranging from 4.0 to 5.0 µg was incubated for 2 hours at 55°C with 30 µL of 20 mg/ml Proteinase K (Promega) After incubation, equal volumes of phenol were mixed with the fecal samples and centrifuged at 5,000xG for 15 minutes The supernatant was transferred to a clean tube and equal volume of chloroform was added and centrifuged at 5,000xG for 10 minutes The DNA contained in the supernatant was precipitated with 100% ethanol and resuspended with distilled water

The phenol/chloroform method yielded low amounts of total fecal DNA with quantities ranging from 0.0 to 0.30 µg When the *H. virescens* mitochondrial marker (F GCATCTCAAGTGGTTCCAGC, R GCTTTGACTGCTACTACCGGC) was tested against three samples of *H. virescens* fecal DNA and three of *H. zea* fecal DNA, the expected product did not amplify for any of the samples (Figure C-1) The *H. zea* marker set (F

GGTACCGCCAAGACCCTGAAC, R GTAGTTCTTCGGGACGCGGG) when tested against the same fecal samples also did not amplify any PCR products (Figure C-2)

There results indicate that neither microcon filters not phenol/chloroform method is suitable for purifying fecal DNA isolated using the sodium lysis protocol

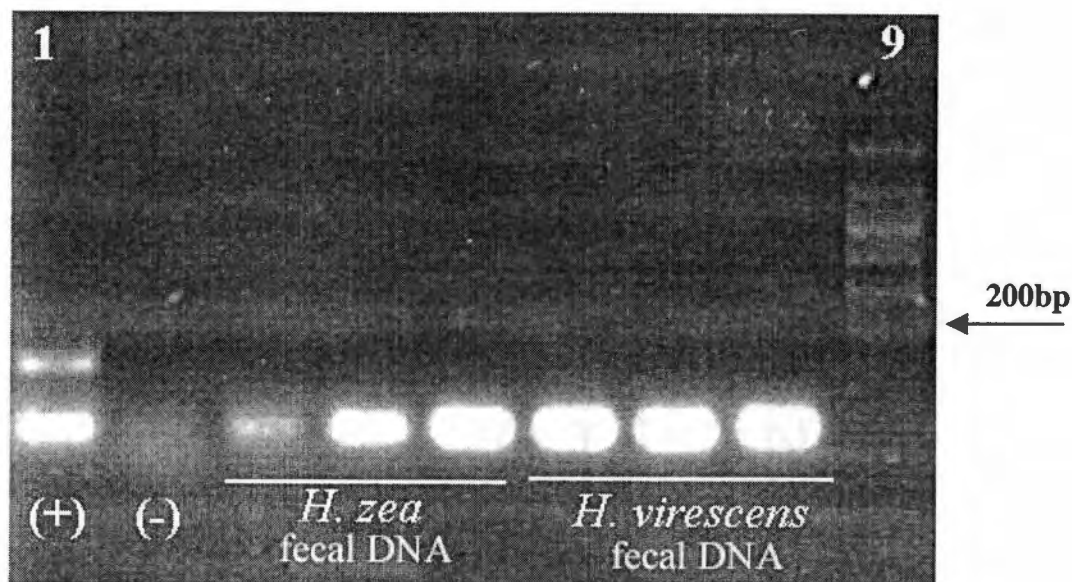


Figure C-1: PCR amplification with *H. virescens* mitochondrial primer set of fecal DNA isolated first with modified sodium lysis protocol and then further purified with phenol/chloroform extraction method. Lane 9, 1Kb, 50bp size marker; lane 1, positive control with PCR template of *H. virescens* genomic DNA (125bp); lane 2, negative control with PCR template as water; lanes 3-5, fecal DNA containing remains of *H. zea*; lanes 6-8, fecal DNA containing remains of *H. virescens*. No PCR products amplified from fecal DNA of *H. zea* or *H. virescens*.

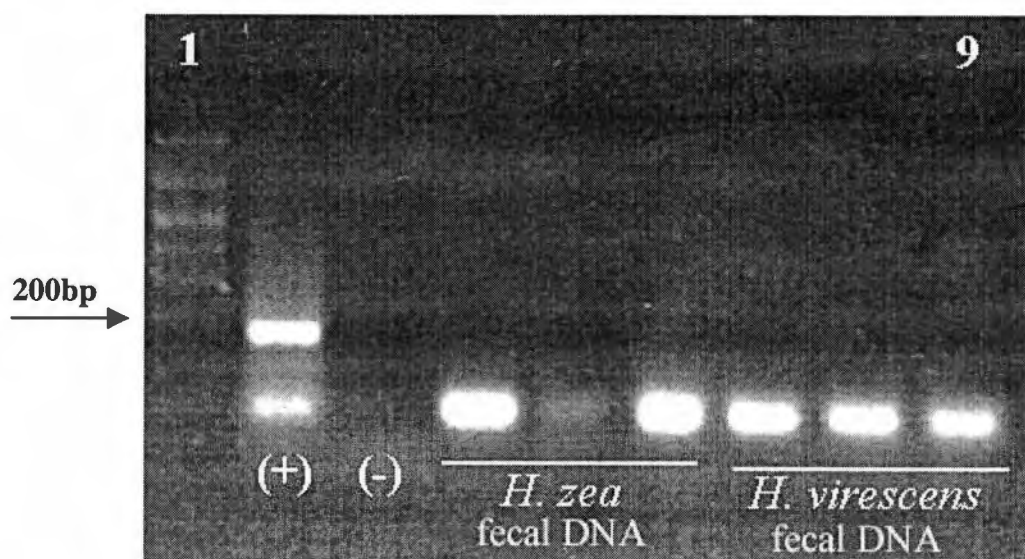


Figure C-2: PCR amplification with *H. zea* PBAN primer set of fecal DNA isolated first with modified sodium lysis protocol and then further purified with phenol/chloroform extraction method. Lane 1, 1 Kb, 50bp size marker; lane 2, positive control of *H. zea* genomic DNA (160bp); lane 3, negative control of water; lanes 4-6, fecal DNA containing remains of *H. zea*; lanes 7-9, fecal DNA containing remains of *H. virescens*. No PCR products amplified from fecal DNA of *H. zea* or *H. virescens*.

APPENDIX D

***Spodopteran* primers**

Chapter 1 and 2 described primers designed for *H zea* and *H virescens*. Here, I describe three primer sets designed for moths of the genus *Spodopteran*.

With the exception of annealing temperatures (Table D-1), DNA extraction, PCR reaction cocktails, and amplification profiles are described Chapter 1.

The SC1/2 primer set was originally designed by Adamczyk *et al* (1996) for studying variation within and between *Spodopteran* species. The primers were tested against genomic DNA isolated from the four moth species. PCR products amplified for five of eight *S exigua*, three of six *S frugiperda*, and twelve of twelve *H virescens*. PCR products never amplified for twelve *H zea* individuals. The PCR products for *H. virescens* were differentiated from the two *Spodopterans* by fragment size with the *H virescens* amplicons approximately 170 base pairs in length and *Spodopteran* amplicons 200 base pairs (Figure D-1).

A primer set for *S frugiperda* was designed from an amylase gene sequence (Da Lage *et al* , 1996, Table D-1). The primers amplified PCR products from genomic DNA for six of seven *S frugiperda* individuals, seven of eight *S exigua*, five of ten *H zea*, and four of ten *H virescens*. The amplified PCR product was approximately 180 base pairs for the two *Spodopteran* species and 150 base pairs for *H zea* and *H virescens* (Figure D-2). The Sfrug1/2 primer set differentiates to the genus level.

A second primer set for *S frugiperda* was designed from a ND-1 region (Pashley and Ke, 1992, Table D-1). PCR products amplified from genomic DNA for all seven *S frugiperda* individuals tested. While products did not amplify for genomic DNA for any of the ten *S exigua* individuals, one of seven *H zea* and two of eight *H. virescens* amplified the same size fragment as for *S frugiperda* (Figure D-3).

While the primers were not tested against DNA isolated from bat feces, the genus specific markers may be useful for future dietary studies for identifying *Spodopterans* consumed.

Table D-1 *Spodoptera* primer sets Included in the table are the primer sequences, gene location, PCR annealing temperature, and approximate product size

Primer set	Primers (5'-3')	Locus	Annealing temp. (°C)	Product size (≈bp)
SC1/2 ^a	F TTCAAAGGTTGGATCCAGATTATG R TACATGTAGATGTTGGTCTC	Sodium channel	48	200, 185
Sfrug1/2 ^b	F GTTCTTGCTCGTCTTCTCC R GCACCTAAGTCTAAGTTTCAAAGTC	Amylase	56	150, 120
Sfmito1/2 ^c	F GACTGTACAAAGGTAGCATAATC R CTCTATAGGGTCTTCTCGTC	ND-1	55	150

^a- Adamczyk *et al*, 1996

^b- Da Lage *et al*, 1996

^c- Pashley and Ke, 1992

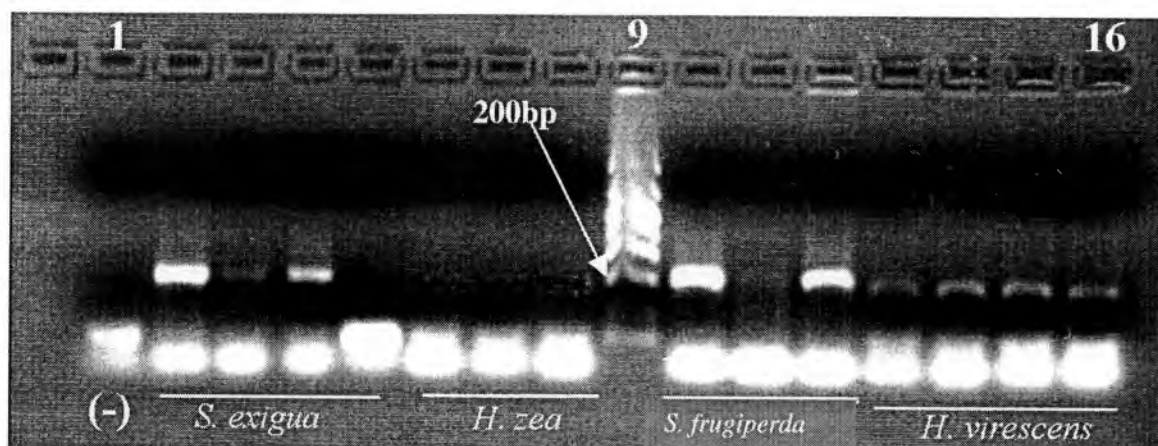


Figure D-1: PCR amplification from genomic moth DNA with *Spodopteran* primer set SC1/2. Lane 9, 1 Kb, 50bp size marker; lane 1, negative control of water; lanes 2-5, *S. exigua* genomic DNA; lanes 6-8, *H. zea* genomic DNA; lanes 10-12, *S. frugiperda* genomic DNA; lanes 13-16, *H. virescens* genomic DNA. The PCR fragments amplified for the two *Spodopteran* species are approximately 200bp in length while the *H. virescens* amplicons are approximately 185bp. PCR products did not amplify for genomic DNA of *H. zea*.

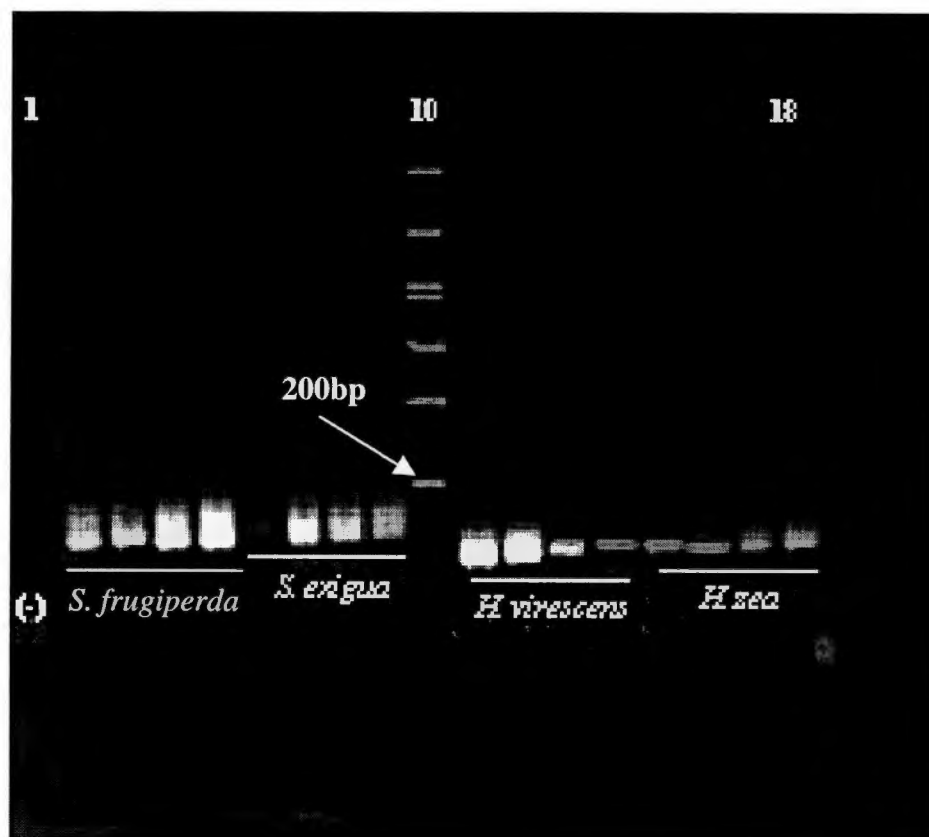


Figure D-2: PCR amplification from genomic moth DNA with *Spodopteran* primer set Sfrug1/2. Lane 10, 1 Kb, 50bp size marker; lane 1, negative control of water; lanes 2-5, *S. frugiperda* genomic DNA; lanes 6-9, *S. exigua* genomic DNA; lanes 11-14, *H. virescens* genomic DNA; lanes 15-18, *H. zea* genomic DNA. The PCR fragments amplified for the moth species vary in size. The PCR product is approximately 150bp for the two *Spodopteran* species and 120bp for *H. virescens* and *H. zea*.

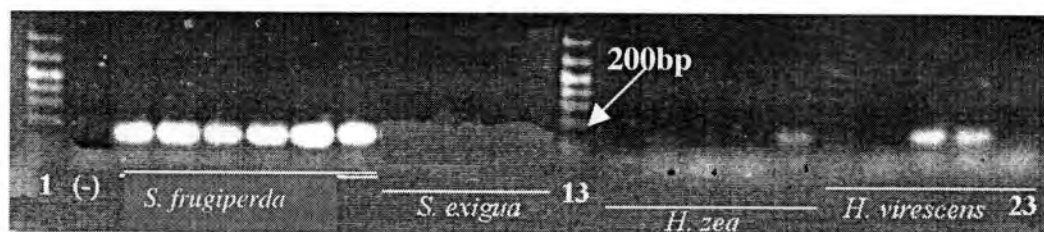


Figure D-3: PCR amplification from genomic moth DNA with *Spodopteran* primer set Sfmito/2. Lanes 1 and 13, 1 Kb, 50bp size marker; lane 2, negative control of water; lanes 3-8, *S. frugiperda* genomic DNA, 150bp PCR product amplified; lanes 9-12, *S. exigua* genomic DNA, no PCR products amplified; lanes 14-18, *H. zea* genomic DNA; lanes 19-23, *H. virescens* genomic DNA; PCR product of same size as from DNA of *S. frugiperda* amplified in one *H. zea* and two *H. virescens* samples.

APPENDIX E

RAPD (Randomly Amplified Polymorphic DNA)

Published DNA sequence information on the moth species is limited, making it difficult or impossible to design species-specific primers. An alternative source for sequence information is Randomly Amplified Polymorphic DNA (RAPD). RAPD is a PCR based (Polymerase Chain Reaction) technique that uses arbitrarily binding primers that typically amplify numerous fragments per template DNA (Welsh and McClelland, 1990, Williams *et al* , 1990). Several RAPD primers (Table E-1) were tested against genomic DNA of the four moths identify fragments that were unique to a particular species. Although, fragment sizes for RAPDs can be large (greater than 500 base pairs) and may not be useful for amplification against damaged DNA found in feces, it is possible to sequence the larger fragments and design new primers to amplify a PCR product less than 200 base pairs in length.

In total, seven RAPD primers were tested. Each 10 μ L PCR reaction consisted of 100 μ M/ μ L nucleotide triphosphate (Idaho Technologies), 0.4 pmoles/ μ L primer, 0.06 units of *Taq* DNA polymerase (Promega), 1X MgCl₂, and 20ng template DNA. Reactions were performed using an Air Thermo Cycler 1605 (Idaho Technologies). The PCR amplification profile involved two cycles of denaturation at 92°C for 60 seconds, annealing at 42°C for 7 seconds, and elongation at 72°C for 70 seconds, followed by 38 cycles of 92°C for 1 second, 42°C for 7 seconds, and 72°C for 70 seconds. PCR products were visualized on a 2% agarose gel with ethidium bromide staining.

Only one primer (OPA-1) yielded a fragment (600 base pairs) that was unique to *S. frugiperda* (Figure E-1). It was not possible to sequence this band either directly by excising it from an agarose gel or by using blunt-end cloning. RAPDs were not useful for identifying moth DNA markers for this study.

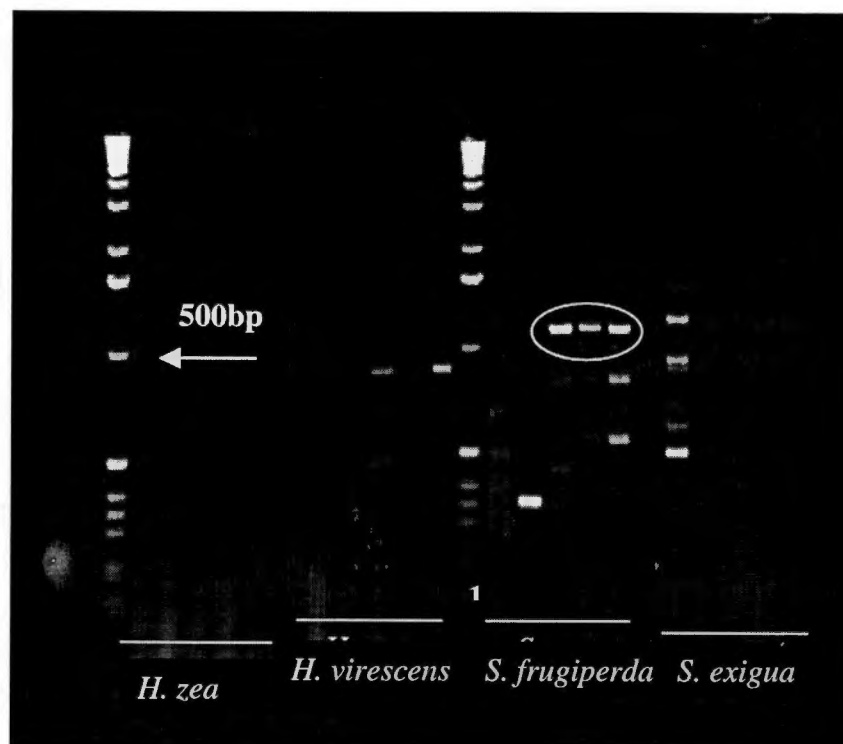


Figure E-1: RAPD results of primer OPA-1 tested against DNA of four moth species. Lanes 1 and 13, 1 Kb, 50bp size markers; lanes 2-7, *H. zea* genomic DNA; lanes 8-12, *H. virescens* genomic DNA; lanes 14-18, *S. frugiperda* genomic DNA, circled is a 600bp fragments that amplified for three of five *S. frugiperda* genomic DNA samples; lanes 19-23, *S. exigua* genomic DNA. PCR products amplified for one of five *S. exigua* and for no *H. zea* or *H. virescens* genomic DNA samples.

Table E-1 RAPD primer codes and sequences tested in this study

Primer Code	Primer Sequence (5' – 3')
OPA-01	CAGGCCCTTC
OPA-03	AGTCAGCCAC
OPA-09	GGGTAACGCC
OPA-12	TCGGCGATAG
OPA-15	TTCCGAACCC
OPA-16	AGCCAGCGAA
OPB-17	AGGGAACGAG

APPENDIX F

PCR Troubleshooting

Bile salts and polysaccharides in fecal DNA have been found to inhibit PCR (Polymerase Chain Reaction) through the inactivation of *Taq* DNA polymerases (Kohn and Wayne, 1997). With the fecal extraction protocols tested in this study, PCR amplification either failed or non-specific PCR products resulted from fecal DNA. The use of different *Taq* DNA polymerases (Al-soud and Radstrom, 1998) and changes to PCR amplification profiles (Roux, 1995) have been shown to overcome problems associated with sensitive PCR reactions. Here, I report the results of 1) HotStar *Taq* (Qiagen) and Master *Taq* (Promega) and 2) different PCR amplification profiles in their abilities in overcoming PCR inhibitors (Table F-1).

Only the *H. zea* PBAN primers (Hzea2/3; Chapter 1) were tested against fecal DNA. Fecal DNA extractions for the two objectives are described in Chapter 2. PCR reactions, and amplification profiles for the different *Taq* polymerases were similar to those in Chapter 2. The amplification profile only varied in that the *Taq* polymerases had an initial denaturation of 15 minutes, versus 2.

HotStar *Taq* was tested against fecal DNA isolated using the modified DNeasy, modified sodium lysis, and GuSCN/silica protocols. PCR products amplified for two of eight *H. zea* and zero of eight *H. virescens* fecal DNA samples extracted with the modified DNeasy protocol. HotStar *Taq* was not successful in amplifying the PCR products against DNA isolated using the modified sodium lysis or GuSCN/silica protocols.

PCR cocktails and amplifications with Master *Taq* resulted in amplicons for two of nine *H. zea* and zero of six *H. virescens* fecal DNA samples isolated using the modified sodium lysis protocol. Master *Taq* was not successful in amplifying PCR products against fecal DNA isolated using the modified DNeasy or GuSCN/silica methods.

The touchdown and touchup amplification profiles were only tested against fecal DNA isolated using the modified sodium lysis and GuSCN/silica protocols. The touchdown and touchup profiles are described in Box F-1.

By applying touchdown amplification, the expected PCR products amplified for ten of twelve *H. zea* and nine of twelve *H. virescens* fecal DNA samples isolated with the modified lysis protocol. Touchdown resulted in the amplification of one of six *H. zea* and zero of six *H. virescens* fecal DNA samples extracted using the GuSCN/silica method.

For the modified sodium lysis protocol, touchup amplification resulted in one of ten *H. zea* and zero of ten *H. virescens* fecal DNA samples. None of the fecal DNA samples isolated with the GuSCN/silica extraction amplified PCR products.

In summary, the different *Taq* polymerases and touchup amplification were not useful in this study for amplifying fecal DNA. The touchdown amplification while having more stringent PCR conditions resulted in the non-specific amplicons. PCR products amplified for the target (*H. zea*) and non-target moth species (*H. virescens*).

Table F-1 PCR amplification results of the different *Taq* DNA polymerases and PCR amplification conditions with *H. zea* PBAN primers against fecal DNA samples. The fecal DNA was extracted with using the modified DNeasy, modified sodium lysis, and GuSCN/silica protocols

	Modified DNeasy		Modified sodium lysis		GuSCN/silica	
	<i>H. zea</i> fecal DNA	<i>H. virescens</i> fecal DNA	<i>H. zea</i> fecal DNA	<i>H. virescens</i> fecal DNA	<i>H. zea</i> fecal DNA	<i>H. virescens</i> fecal DNA
HotStar <i>Taq</i>	2/8	0/8	0/12	0/4	0/4	0/4
Master <i>Taq</i>	0/4	0/4	2/9	0/6	0/4	0/4
Touchdown	---	---	10/12	9/12	1/6	0/6
Touchup	---	---	1/10	0/10	0/4	0/4

Touchdown:

- 1) 95°C for 15 min
- 2) 95°C for 1 min
- 3) 65°C for 45 sec
- 4) 72°C for 45 sec
- 5) Repeat 2-4 for 19 cycles
- 6) After each cycle, decrease annealing temperature (65°C) by 0.5°C
- 7) 95°C for 1 min
- 8) 55°C for 45 sec
- 9) 72°C for 45 sec
- 10) Repeat 7-9 for 9 cycles
- 11) Hold @ 4°C

Touchup:

- 1) 95°C for 15 min
- 2) 95°C for 1 min
- 3) 45°C for 45 sec
- 4) 72°C for 45 sec
- 5) Repeat 2-4 for 19 cycles
- 6) After each cycle, increase annealing temperature (45°C) by 0.5°C
- 7) 95°C for 1 min
- 8) 55°C for 45 sec
- 9) 72°C for 45 sec
- 10) Repeat 7-9 for 9 cycles
- 11) Hold @ 4°C

Box F-1: Touchdown and touchup PCR amplification profiles

APPENDIX G

Graphic map | Table by enzyme name

gcgatcaggtaaccgacctttcatcatctgaatagtttttcccaactatgttgaggtcggcattcagtataacc base pairs
cgctagtccattggctggaaagtagtagacttatcaaaaaagggttgatacaactccagccgtaagtcattgg 1 to 75

Mph1103I

NsiI

Zsp2I

ggatgcactctgagtatctttgtgctacttgagcgactgcctacctggctttc base pairs
cctacgtagactcatagaaacacgatgaacctcgctgacggatggaccgaaag 76 to 128

Enzyme name	No. cuts	Positions of sites	Recognition sequence
Mph1103I	1	82	atgca/t
NsiI	1	82	atgca/t
Zsp2I	1	82	atgca/t

Figure G-1: Restriction map of *H. zea* neuropeptide gene sequence. The sequence was analyzed with the Webcutter software. The restriction enzyme *NsiI* cuts at base 82.

Graphic map | Table by enzyme name

PshBI
AsnI
ccgcgaaaggcttattgatcgtttgggtaggctacagctcgggtagctgtattattaataggtggctgtagcga base pairs
ggcgctttccgaataactagcaaaccatccgatgtcagaccacatcgacataataattatccaccgacatcgct 1 to 75
VspI
AseI

Ggttgaagtgaacgagaaaaaagatcgtgtcaatgacgcactaaatgctactagagcagcagttgaagaaggc base
ccaacttcacttgctctttttctagcacagttactgcgtgattacgatgatctcgtcgtcaacttcttccg 76 to 148

Enzyme name	No. cuts	Positions of sites	Recognition sequence
AseI	1	56	at/taat
AsnI	1	56	at/taat
PshBI	1	56	at/taat
VspI	1	56	at/taat

Figure G-2: Restriction map of a portion of *H. virescens* p63 isoform gene. The sequence was analyzed with the Webcutter software. The restriction enzyme *VspI* cuts at base 56.

APPENDIX H

PCR-RFLP

The results from the primers tested in Appendix I demonstrated that larger fragments of the diet can be amplified from fecal DNA isolated with the modified DNeasy tissue protocol. With the amplification of larger DNA fragments, PCR-RFLP of a conserved region could be used to differentiate between moth species (discussed in Chapter 1). One individual for each moth species (*H virescens*, *H zea*, *S exigua*, and *S frugiperda*) was amplified using the 12S primer set. The PCR products were cleaned using Qiagen's PCR purification kit and sequenced (Figures H-1 to H-4). Clustal X (1.8) program aligned the sequences (Figure H-5). Nucleotide bases specific to a moth species represent potential species-specific restriction sites.

In addition to the 12S alignment, published dopa decarboxylase gene sequences (Fang *et al* , 1997) for the four moth species were aligned (*H virescens*, *H zea*, *S exigua*, and *S frugiperda*) using Clustal X (Figure H-6). Again, possible species-specific nucleotides indicate potential restriction sites. While the sequence size is too large to amplify in feces, new primers could be designed off the conserved sequence to amplify smaller fragments.

Figure H-5 Alignment of *H. virescens*, *H. zea*, *S. exigua*, and *S. frugiperda* with the Clustal X (1.8) program. The moth PCR products that were sequenced were amplified with the 12Sf primer. The boxed nucleotides represent possible species-specific bases and potential restriction sites.

```

H. virescens
---CACCTTCAATTAAATTATTACAAATTAATATTCATTTAAATAAAATTTATTGTAATCCA
H. zea
-----TATTANTATCAGTAAATAAAATTTATTGTAATCCA
S. exigua
ATCCACCTTTAAATTAAATTTTACAAATTAATATCCATTTAAATAAAATTTATTGTAATCCA
S. frugiperda
-----ATTTAAATAAAATTTATTGTAATCCA
* *****

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H. virescens
TTATATTCTTAATTATAATCTGCATCTTGATCTGATTTAAATTAAAAATTTAATTTTTAA
H. zea
TTATATTCTTAATTATAATCTGCATCTTGATCTGATTTAAATTAA-TTCAATTTTTAA
S. exigua
TTATATTCTTAATTATAATCTGCATCTTGATCTGATTTAA-TTTTAAATTTAATTTTTAA
S. frugiperda
TTATATTCTTAATTATAATCTGCATCTTGATCTGATTTAAATTNTAATTAATTTAATTTTTAA
***** ** *

```

```

H. virescens
ATATTAATTTTTTTTAAAAATATTTTTTTTAAACAACGATATACAAAATTATAAA--TTAAG
H. zea
ATATTAATTTTTTTTAAAAATATTTTTTTTAAACAACGATATACAAAATA-TAAA--TTAAG
S. exigua
ATATTA-TATTA-TTAAAAAATATTTTTTTTAAACAACGATATACAAAATAA-AAAAAATTAAG
S. frugiperda
ATATTAATATN-TTAAAAAATATTTTTTTTAAANAACGATATANAAAATTATAAA--TTAAG
***** * * ** *****

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```

H. virescens
TAAATTTATTCGTGGATTATCAATTATTAAACAGATTCCTCTAAATGAACTAAAATACCG
H. zea
TAAATTTATTCGTGGATTATCAATTATTAAACAGATTCCTCTAAATGAACTAAAATACCG
S. exigua
TAAATTTATTCGTGGATTATCAATTA-TAAACAGATTCCTCTAAATGAACTA-GAATACCG
S. frugiperda
TAAATTTATNCGTGGATTATCAATTATTAAACAGATTCNTCTAAATGAACTAAAATACCG
*****

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```

H. virescens
CCNAATTATTTAAGTTT-CAAGAGGTAATTATCTACTATTTTCAGTATTATAAATTTAAAT
H. zea
CCNAATTATTTAANTTTTCAGGAGGTAATTA-TACTATTTTAGTATTATAAATTTAAAT
S. exigua
CCAAATTATTTAAGTTT-CAAGANATAATTA-TACTATTTTAGTATTTTCAATTTAAAT
S. frugiperda
CCAAATTATTTAAGTTT-CAAGAAATAATTATCTACTATTTTCAGTATTNTAAATTTAAAT

```

** ***** ** ** ***** ***** ***** * *****

H virescens	TTTAATAATAGG-TATCNA-----
H. zea	TTTAATAATAGGGTATCTA-----
S. exigua	TTTAATAATAGG-TATCA-----
S frugiperda	TTTAATAATAGGGTATCTAACATTNA
	***** ****

Figure H-6 Alignment of *Heliothis virescens*, *Helicoverpa zea*, *Spodoptera exigua*, and *S. frugiperda* dopa decarboxylase sequences with the Clustal X (1.8) program. The gene sequences were downloaded from GenBank. The boxed nucleotides represent possible species-specific bases and potential restriction sites.

```

H. virescens
TTAGAGGTTGTGATGCTAGACTGGTTGGGCCAGATGTTGGGTGTGCCCGAGTCGTTCCCTG
H. zea
TTGGAGGTTGTGATGCTGGACTGGTTGGGTTCAGATGTTGGGTCTGCCGTGAGTCGTTCCCTG
S. frugiperda
TTGGAGGTTGTGATGCTTGACTGGCTGGGCCAGATGCTGGGTCTGCCCCGACGTCGTTCCCTG
S. exigua
TTGGAGGTTGTGATGCTTGACTGGTTGGGCCAGATGTTGGGTCTGCCCGAGTCGTTCTGTG
** ***** ***** ***** ***** ***** ***** ***** ** ***** **

H. virescens
GCTCGGTCGGGAGGCGAGGCCGGTGGCGTCATCCAGGGCACTGCCAGCGAGGCCACGCTT
H. zea
GCTCGGTCGGGTGGCGAGGCCGGTGGCGTCATCCAGGGCACTGCCAGCGAGGCCACGCTG
S. frugiperda
GCTAAGTCTGGTGGTGAGGCAGGTGGTGTTCATTCAAGGCACCGCCAGTGAGGCCACGCTG
S. exigua
GCTCGGTCGGTGGTGAGGCAGGTGGGTGTTCATTCAAGGCACCGCCAGCGAAGCCACGCTG
*** ** * * * ***** ***** ** * * * ***** ***** ** *****

H. virescens
GTCGCTCTGCTAGGAGCCAAAGAACCGCACCATCATACGCGTGAAGGAACAACATCCTGAA
H. zea
GTCGCTTTACTGGCGCCAAGAACCGCACCATTATACGCGTGAAGGAACAACACCCTGAA
S. frugiperda
GTGGCGTTACTTGAGCCAAGAACCGCACCATCTTACGTGTGAAGGAAAAGCACCCGAA
S. exigua
GTCGCTTTACTTGAGCCAAGAACCGCACCATCTTACGTGTGAAGGAAAAGCACCCCTGAA
** * * * * * ***** ***** ** * * * ***** ***** ** * * *

H. virescens
TGGACCGATACAGAAATCATTTCAAACCTCGTCGGTTATTGCAACAAGCAAGCTCACTCT
H. zea
TGGACTGATATGAGATCATTTCGAAACCTCGTCGGTTATTGCAACAAGCAAGCTCATTCT
S. frugiperda
TGGAGCGAGACGGAGATTCTTTCGAAACCTCGTCGGTTATTGCAACAAGCAAGCCCATTCT
S. exigua
TGGAGCGACACTGAGATTCTGTGAAACCTCGTCGGTTATTGCAACAAGCAAGCCCATTCA
**** ** * * * * * ***** ***** ***** ***** ** *

H. virescens
TCGGTGGAAGGGCTGGTCTCCTGGGCGGTGTGAAACTGCGTTCGCTGCAGCCAGACGAA
H. zea
TCGGTGGAAGGGCTGGTCTCCTGGGTGGTGTAAGCTGCGTTCGCTACAACAGACGGA
S. frugiperda
TCCGTGGAACGTGCTGGACTTTTGGGAGGAGTAAAGCTTCGTTCACTCAGCCTGATGGA

```

S. exigua
 TCCGTGGAACGTGCTGG[C]CTTTTGGGAGGAGTAAAGCTTCGTT[T]ACTACAGCCTGACGGA
 ** ***** * ***** ** ***** ** * * * * * * * * * * * * * *

H virescens
 AAGAGGCGACTTCGTGGTGACACTCTCCAGGAAGCCAT[A]GAGGAAGATATTCGCAATGGA
 H zea
 AAGAGGCGCCTTCGTGGTGACACTCTGCAGGAAGCCATCGAGGAAGATATCCGCAATGGA
 S frugiperda
 AAGAGGAGACTTCG[C]GG[C]GATACTCTCCGAGA[G]GC[TAT]T[GAGGAAGATACTCGCAACGGA
 S. exigua
 AAGAGGAGCCTTCGTGG[A]GATAC[C]CTCCGAGA[C]GCCATCGAGGAAGATACTCGCAACGGA
 ***** * ***** ** * * * * * * * * * * * * * * * * * *

H. virescens
 TTGATTCCATTCTATGTGGTGGCCACTCTGGGTAC[G]AC[G]TCTTCATGCGCATTCGACGCC
 H zea
 TTGATTCCATTCTATGTGGTGGCCAC[A]TT[A]GGTACAACATCTTC[T]TGCGCATTCGACGCC
 S. frugiperda
 CTTATTCCATTCTATGTCGT[T]GCTAC[C]CTGGGTACAACATCATCCTGCGCATTTGATGCA
 S. exigua
 CTTATTCCATTCTATGTCGTGGCTACTTTGGG[A]ACAACATCATCCTGCGC[C]TTTATGCA
 * ***** ***** ** * * * * * * * * * * * * * * * * * *

H. virescens
 TTGGACGAAATTGGTGATGTGTGCTCCAAGCACGAAATCTGGTTACATGTGACGCAGCC
 H zea
 TTGGACGAAATTGGTGACGTGTGCTCCAAGCATGACATCTGGTTACATGTGACGCAGC[T]
 S. frugiperda
 TTGGACGAAATTGG[A]GACGTGTG[TAA]TGAACATGG[T]GTTTGG[C]TGCA[C]GTAGACGCTGCC
 S. exigua
 TT[A]GACGAAATTGG[C]GATGT[A]TGCAACGAACATGGCGTTTGGTTGCATGTAGACGCTGCC
 ** ***** ***** ** * * * * * * * * * * * * * * * * *

H virescens
 TATGCCGGATCCGC[T]TTCATCTGTCCTGAATACCGTTACCTCATGAAAGGCGTCGAAAAA
 H. zea
 TATGCCGGATCAGC[C]TTCATCTGTCCTGAATATCGTTACCTCATGAAAGGCGTTGAAAAA
 S. frugiperda
 TACGCCGG[C]TC[T]GCATTCATCTG[C]CCTGAGTACCGTTACCTCATGAAGGG[TGT]AGAGAAG
 S exigua
 TACGC[TGG]TTCAGCATTCATCTGTCCTGAATATCG[C]TACCTCATGAAGGGCGT[T]GAGAAG
 ** *

H virescens
 GCGGATTCTTTCAATTTCAATCCGCACAAATGGTTGCTTGTAACTTCGACTGTTCCGCC
 H zea
 GCGGATTCTTTCAATTTCAATCCACACAAG[TGG]TGTCTCGTTAACTTCGACTGTTCCGCC
 S. frugiperda
 GCAGA[C]TCCTTTCAATTTT[AACCCACACAAATGGTTACTGGTTAACTTTGATTGTTCCGCC
 S exigua
 GCAGATTCTTTCAATTTCAACCCACACAAATGGTTACTGGTTAACTTTGATTGTTCCGCC

** ** ** ***** ** ** ***** *** * ** ***** ** ***** **

H. virescens

ATGTGGCTCAAAGAACCTCGTTGGATCGTCGATGCCTTCAATGTCGACCCCTTGTACTTG

H. zea

ATGTGGCTCAA[GAACC]CGTTGGATCGTCGATGCCTTCAATGTCGA[CCCTT]TACTTG

S. frugiperda

TTGTGGCTGAAAGAACC[CGTTGGATCATCGATGCCTTCAATGTGGACCCACTATACCTG

S. exigua

TTGTGGCTGAAAGAACC[A]CGTTGGATCATCGATGCCTTCAATGTGGACCCACTATA[CTG

***** ** ***** ***** ***** ** ** * ** **

H. virescens

AAACACGACCAACAGGGATCAGCACCAGAC

H. zea

AAACACGACCAGCAGGGATCAGCTCCAGAC

S. frugiperda

AAACACGATATGCAAGGATCTGCTCCAGAC

S. exigua

AAACACGATATGCAAGGATCTGCC[CC]TGAC

***** ** ***** ** ** *

APPENDIX I

Amplification of larger DNA fragments from feces

DNA fragments up to 400 base pairs in length have been amplified from DNA of the defecator extracted from feces (Poinar *et al* , 1998, Whittier *et al* , 1999), however, the DNA from the defecator's diet is suspected to be more fragmented due to either shearing that occurs in the digestive tract or poor quality fecal DNA extraction protocols. I tested two primers that amplified larger DNA fragments of the diet. an *H virescens* mitochondrial marker set that amplifies a 300 base pair product and an *H zea* and *H virescens* 12S ribosomal marker set that amplifies a 400 base pair product (Roehrdanz, 1997). The fecal DNA was extracted using two protocols, modified DNeasy (Qiagen) and PTB (Poinar *et al* , 1998), as described in Chapter 2. PCR conditions were similar to those described in Chapter 2.

The *H virescens* mitochondrial marker set (Hvf-CCGCGAAAGGCTTATTGA, Hvr-CGTCGAAACCAGCATTAGAAGC) was designed from a p63 isoform sequence (Miller *et al* , 1990) and was specific to *H virescens*. The primers were tested against DNA extracted using the modified DNeasy tissue kit. PCR products amplified against nine of ten *H virescens* and eight of ten *H zea* fecal DNA samples (Figure I-1). While this primer set was not species-specific in fecal DNA, it is significant that a 300 base pair product amplified from the DNA of the diet. PCR products never amplified against fecal DNA samples isolated with the PTB protocol (Figure I-2).

The 12S ribosomal RNA primers (12Sa-AAACTAGGATTAGATACCCTATTAT, 12Sf-CGGGCGATGTGTACATAATT) were designed by Roehrdanz (1997) to amplify a 400 base pair product from DNA of *H zea* and *H virescens*. The primers were tested against genomic DNA of the four moth species and the expected fragment amplified in three of seven *H zea*, ten of ten *H virescens*, eight of eight *S exigua*, and three of six *S frugiperda*. The primers when tested against fecal DNA isolated using the modified DNeasy protocol, the 400 base pair product amplified in two of two *H zea*, zero of two *H virescens*, and one of two mealworm fecal

DNA samples (Figure I-3) PCR products never amplified against fecal DNA isolated with the PTB method (Figure I-4)

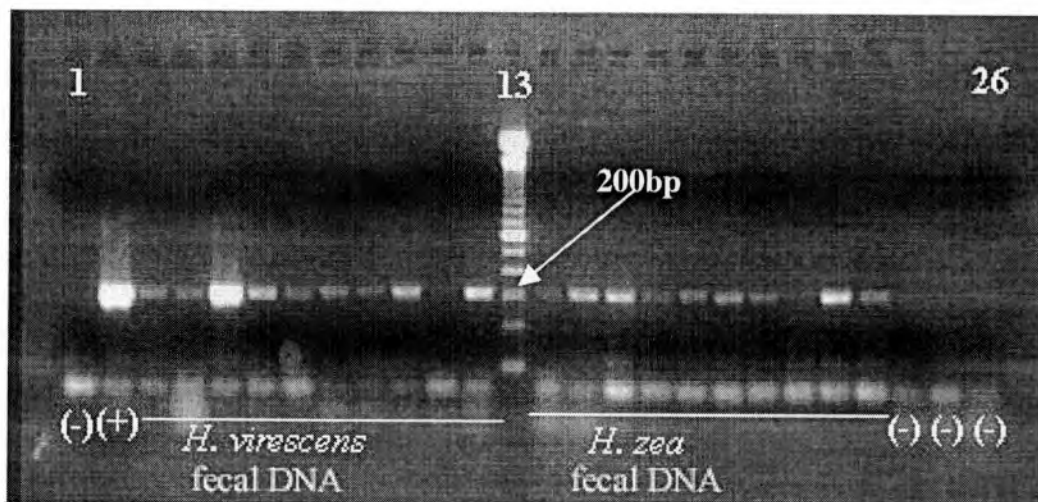


Figure I-1: PCR amplification results of *H. virescens* primer set tested against fecal DNA isolated with modified DNeasy tissue kit. Lane 13, 2 Kb, 100bp size marker; lane 1, negative control with water; lanes 24-26, negative controls with mealworm fecal DNA; lane 2, positive control with *H. virescens* genomic DNA (280bp), lanes 3-12, *H. virescens* fecal DNA, expected PCR product amplified for nine of ten samples; lanes 14-23, *H. zea* fecal DNA, expected PCR product amplified for eight of ten samples.

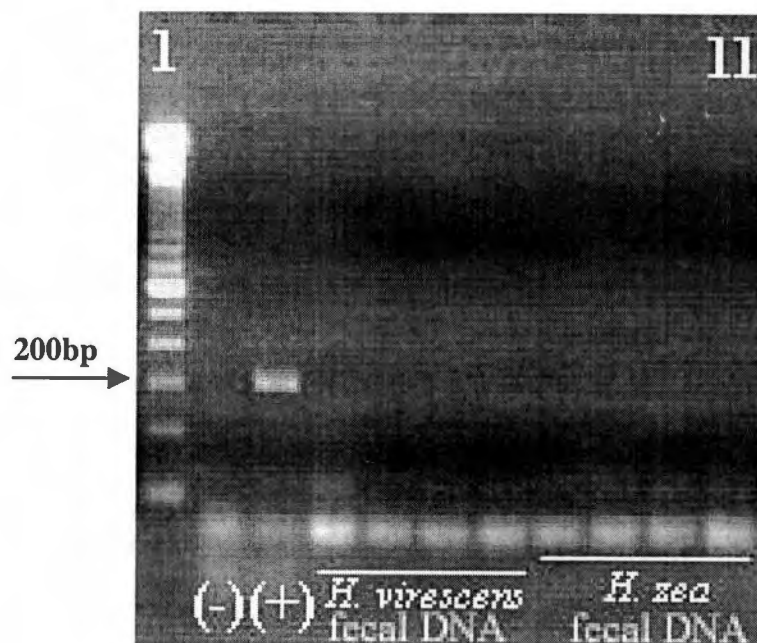


Figure I-2: PCR amplification results of *H. virescens* primer set tested against fecal DNA isolated with PTB extraction protocol. Lane 1, 2Kb, 100bp size marker; lane 2, negative control with water; lane 3, positive control with *H. virescens* genomic DNA (280bp), lanes 4-7, *H. virescens* fecal DNA; lanes 8-11, *H. zea* fecal DNA. No PCR product amplified.

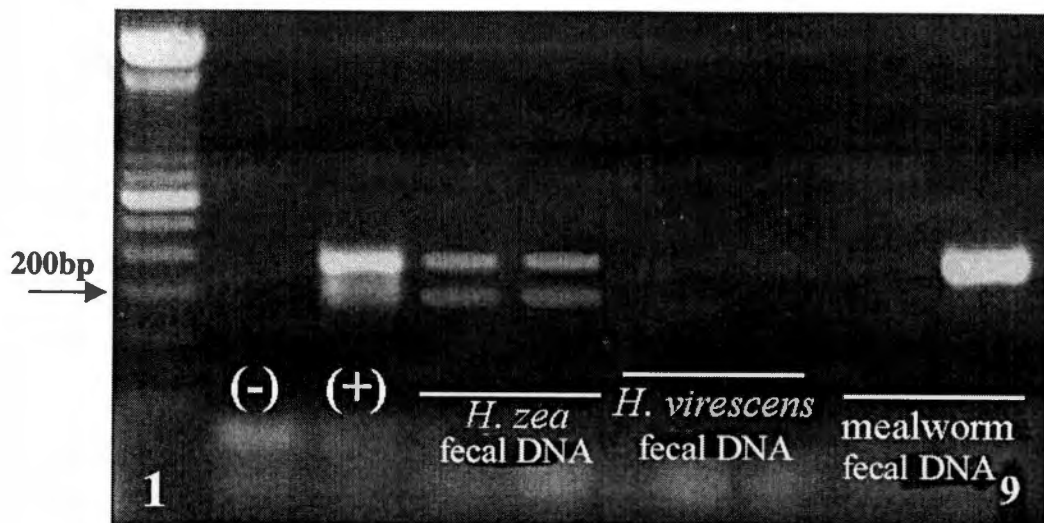


Figure I-3: PCR amplification with 12S ribosomal RNA primer set and with fecal DNA isolated with modified DNeasy tissue kit. Lane 1, 2 Kb, 100bp size marker; lane 2, negative control with water; lane 3, positive control with *H. virescens* genomic DNA (400bp), lanes 4-5, *H. zea* fecal DNA, expected PCR product amplified for two of two samples; lanes 6-7, *H. virescens* fecal DNA, no PCR products amplified; lanes 8-9, mealworm fecal DNA, PCR product of expected size amplified for one of two samples.

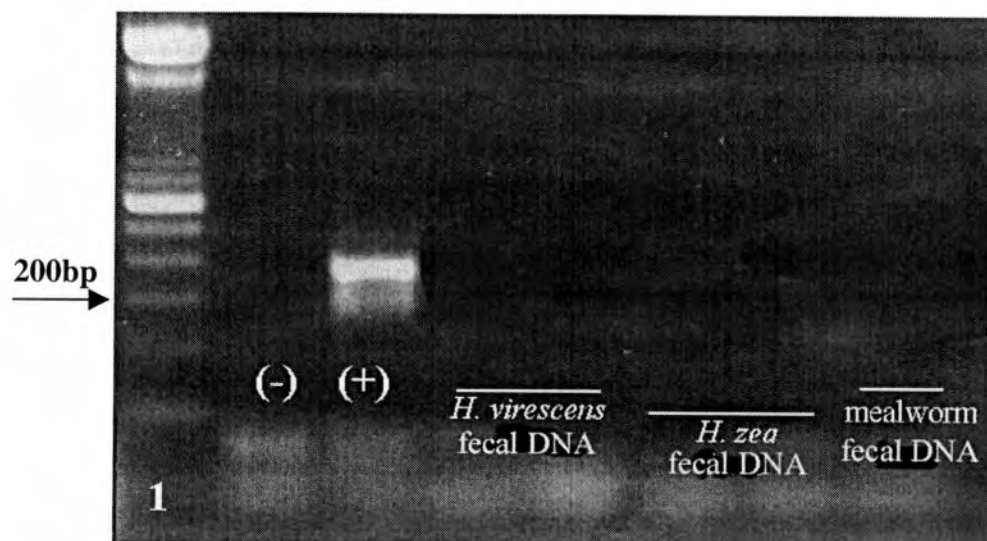


Figure I-4: PCR amplification with 12S ribosomal RNA primer set and with fecal DNA isolated using PTB extraction protocol. Lane 1, 2 Kb, 100bp size marker; lane 2, negative control with water; lane 3, positive control with *H. virescens* genomic DNA (400bp), lanes 4-5, *H. virescens* fecal DNA; lanes 6-7, *H. zea* fecal DNA; lanes 8-9, mealworm fecal DNA. No PCR products amplified.

VITA

Sunitha Vege was born in Tenali, India on February 5, 1974. She moved to the United States with her parents when she was four. She graduated from Marion High School in Marion, South Carolina in June 1992. She attended Furman University in Greenville, South Carolina from 1992 to 1994. She transferred to University of Georgia in Athens, Georgia in 1994 and there, she received her Bachelor of Science degree with a major in biology in 1996. She took an absence from school to travel and work. She entered graduate school in the department of Ecology and Evolutionary Biology at the University of Tennessee, Knoxville in fall 1998. She received her Master of Science with a major in ecology and evolutionary biology in December 2000.