Extraction and Characterization of DNA from *Demodex canis*

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ABSTRACT

Canine demodicosis occurs when *Demodex* mites colonize hair follicles in large numbers. Their characterization is based on morphologic differences. A molecular genetic analysis within *Demodex* mite species and between other mite species would better characterize their phylogenetic diversity and could assist in treatment strategies as well as improve our understanding of disease progression. *Demodex canis* mites were obtained during routine deep scrapings of client-owned dogs and collected in Tris EDTA buffer (TE). The mites were separated from debris with a microhematocrit suction apparatus. Their outer chitin layer was ruptured using chitinase, glass beads, and vortexing. Membranes and proteins were disrupted with Sodium Dodecyl Sulfate (SDS) and proteinase K. Phenol/chloroform extraction, ethanol precipitation, and resuspension in TE further purified the nucleic acids. One hundred ng of mite DNA was amplified by a Random Amplified Polymorphic DNA (RAPD) method as DNA yields were low. The amplified DNA was randomly cloned into plasmids and 10 clones were sequenced. The BLAST program in Macvector version 9.0™ was used to identify sequences that matched insect-type DNAs. Two oligonucleotide primer sets were designed to amplify the mite DNA by polymerase chain reaction (PCR). One sequence was homologous to ubiquitin and the other matched a bacterial species related to those commonly found as commensals in insects. The latter primer set also amplified a similar sequence from flea and mosquito DNA. Neither amplified dog DNA. This method was successful in isolating DNA from *Demodex canis* mites. Oligonucleotide primers were developed that amplify *Demodex* sequences and will be useful in analyzing phylogenetic relationships and may assist in understanding demodicosis progression.

INTRODUCTION

Canine demodicosis is a dermatologic disease that occurs when mites colonize hair follicles and sebaceous glands and greatly increase in number.[1] Dermatological changes include, but are not limited to, erythema, alopecia, follicular hyperkeratosis, comedones, follicular casts, pustules, crusts, and seborrhea. Often, a secondary pyoderma further complicates the disease.[1] Two species and one as yet unnamed type of *Demodex* mite have been identified in canines based on morphological differences. Genetic variation within and between the different species and types may be determined more specifically utilizing molecular markers.[2]

The three recognized canine *Demodex* mites are: *Demodex canis*, *Demodex injai*, and the unnamed short-bodied mite. *Demodex canis* was the first to be identified and named.[3,4] The two additional *Demodex* mites may be mutations of *Demodex canis*, or separate species.[1] Hillier and Desch described *Demodex injai*, a long-bodied demodecid, in 1997.[5] Males are more than twice the length of the males of *D. canis*; females are 1.5 times longer than females of *D. canis*. Differences were also observed in opisthosomal length, length of the egg, and the number of ventral scutes present on the nymph.[6] The unnamed mite was described in 1988 by Scarff.[7] In a report by Chesney, this "stubby" form of the *Demodex* was described as being about one half of the length of the female of *D. canis*.[8]

The use of molecular markers may determine more accurately the genetic diversity between different species and types of demodecid mites.[2] A variety of markers are available, each with its own benefit. Examples include the non-coding regions of genes such as the internal transcribed spacer (ITS) region of nuclear ribosomal genes. Non-coding areas are not under selection pressure, and as a result, evolve quickly. ITS sequences are better used to compare the relationships within a species or to compare species that are closely related. Mitochondrial genes are also used as molecular markers. Mitochondrial genes have high copy numbers, and thus higher sensitivity. Consequently, they are preferred targets when DNA recovery is difficult. However, mitochondrial genes can be transferred from the mitochondrial genome to the nuclear genome, resulting in phylogenetic inaccuracies. The gene encoding mitochondrial cytochrome oxidase also is often used to establish phylogenetic relationships. Methodologies using this gene have advantages similar to the internal transcribed spacer region of nuclear ribosomal genes.[9] These markers are examples of sequences that are useful for studying phylogenetic relationships among organisms, and could be useful in evaluating genetic differences among mites. The use of Random Amplified Polymorphic DNA (RAPD) techniques to amplify *Demodex canis* DNA may also be useful in differentiating mites. Cloning and sequencing of RAPD products would aid in the creation of specific primers to random genomic DNA.[10]

At the time of this research, a single *Demodex canis* gene sequence (partial chitin synthase) has been reported in GenBank.[11] Since this deposit was not accompanied by publication, there exists no published method for isolating DNA from *Demodex canis* mites. The goals of this research include development of a method to isolate *Demodex canis* DNA, development of a Polymerase Chain Reaction (PCR) protocol to amplify one or more *Demodex canis* DNA sequences, and to determine the nucleotide sequence of the cloned amplicons.

MATERIALS AND METHODS

Mite Collection and Purification

Demodex canis mites were collected and identified during routine deep skin scrapings of client-owned dogs admitted to the dermatology service of the Auburn University Small Animal Teaching Hospital. Twenty-four dogs were sampled during the study period. Mites and scraped debris were collected using a #10 surgical blade. The scraped material was placed into either

mineral oil, 1mL of Tris EDTA (TE), or directly into microcentrifuge tubes and stored at room temperature. TE was added to the dry samples to make them more conducive to mite separation. Various methods were evaluated to separate the mites from hair and skin debris. The goal was to extract mites free of scraped debris. Debris could contain canine DNA that might overwhelm the yield of mite DNA. The material was shaken in the microcentrifuge tube to evenly suspend the material in TE. A 1mL syringe was used to place approximately 0.02-0.03 mL of the scraped debris in Tris EDTA on a slide. Materials attempted for mite separation included a wire inoculating needle, 1mL syringe with a 25 gauge needle, a hand-pulled glass pipette that held 400µL, and a microhematocrit suction apparatus (Wiretrol® capillary tubes, 5uL size). The latter proved most useful in separating the mites from the debris.

Sufficient numbers of mites were isolated from eight dogs and were used for DNA isolation and PCR protocols (discussed below). Not all scrapings produced yielded samples acceptable for DNA isolation. Separating the mite material from other debris was difficult when the mites were suspended in mineral oil. Suspension in TE allowed for easier separation of mites from other DNA. Also, not all samples had sufficient numbers of mites for DNA isolation. Initially, only 10-20 mites were manually separated from scraped debris. Using this number of mites, yields of DNA were less than 100 ng. Subsequent sampling yielded approximately 75-220 mites. The separation technique was performed using a light microscope at either 40 or 100X total magnification. Mites from different canine cases were collected separately in 1 ml TE in a microcentrifuge tube. All samples were morphologically similar to *D. canis*.

We attempted to use gradient centrifugation at various speeds to separate the mites from additional debris in one sample because the patient had severe seborrhea oleosa. The gradient was created by adding 2mL of Percoll® and 1.5mL of Histopaque-1077® to a 10mL glass centrifuge tube. The sample in 1 ml TE was applied to the top of this solution. Centrifugation at 11,000 rpm for 10 minutes yielded three distinct layers. The uppermost lipid layer was removed. The second layer contained many mites with little contamination. The mites were manually extracted from this layer as described above.

Mite DNA Isolation

Many methods were attempted to successfully isolate DNA from *Demodex canis* mites. In the early isolation attempts, 10-20 mites were used (dogs 1- 3) and suspended in approximately 1mL of TE. These tubes were labeled Tubes 1, 2, and 3a (Table 1). A fourth sample was the remainder of the material collected from dog 3 in approximately 1mL of TE (tube 3b). This sample was used to determine if manual separation of mites from the other material was necessary for successful isolation of DNA from mites. These samples were centrifuged for 5 minutes at approximately 3,000 rpm. The supernatant was removed and 500 µL of lysis buffer (20 mM tris, pH 8.8, 1 % SDS, 1 mM EDTA) was added to each of the pellets. The material was vortexed, and placed in the sonicator for 10 minutes. Ten uL of proteinase K (50 mg/ml) was added to each tube. These samples were placed in a 42°C water bath for 1 hour followed by incubation in a dry bath incubator at 65°C for 15 minutes. The sample was mixed with 275 µL of 3 M potassium acetate, pH 7.0 and placed in an ice bath for 20 minutes. Following centrifugation at 14,000 rpm, the supernatant from each sample was poured into a fresh tube and a volume of isopropanol equivalent to half the volume in the tube was added. The samples were mixed gently, incubated at room temperature for 5 minutes, and briefly centrifuged at 14,000 rpm. The supernatant was removed and saved, and the pellet rinsed with cold 70% ethanol. The tubes were inverted on paper towels to drain. After 5-10 minutes, the DNA pellet was re-suspended in10 µL TE with 10 µg/

***Table 1**: DNA Yields and Purity*

Sample	Concentration ng/µL	Purity (ratio of absorbance at 260 and 280 nm)
1	4	1.12
2	6.4	1.56
3a	5.7	1.72
3b	3.7	1.72
4a-1	1655.6	1.63
4a-2	2000.2	1.67
4b-1	2800.7	1.59
4b-2	2480.5	1.63
5a-1	1427.5	1.48
5a-2	1199.5	1.51
5b-1	13.0	1.91
5b-2	15.4	1.73
6a	200.5	1.63
6b-1	682	1.50
6b-2	461.7	1.74
6b-3	0.1	0.20
6c	4162.7	1.99
6d	3042.0	1.70
6e-1	540.2	1.70
6e-2	346.3	1.75
*6f-1	1754.6	1.58
6f-2	134.6	1.63
6f-3	2460.0	1.36
6f-4	365.5	1.62
7a-1	435.6	1.41
**7a-2	283.5	1.59
7a-3	1490.9	1.34
7a-4	403.6	1.74
7b-1	218.2	1.60
7b-2	312.9	1.53
8a-1	889.1	1.34
8a-2	3765.9	1.52
8a-3	1198.4	1.53
**8b-1	294.1	1.63
8b-2	84.1	1.35
8b-3	74.1	1.42
Flea a	938.3	1.70
Flea b	1160.0	1.73
Mosquito a	3079.0	1.77
Mosquito b	3065.3	1.81

**Indicates those samples that were used with the RAPD technique.*

***Indicates those samples that were used with the RAPD primers.*

ml RNAse A. The tubes were vortexed and centrifuged briefly. The DNA yield was quantified with a Nanodrop spectrophotometer. The ratio of absorbance at 260 and 280 nm established DNA purity. The concentration was found to be 0. However, the tubes containing the pellets remaining after the addition of 3M acetate pH 7.0 were thought to still contain the DNA. Trizol (0.5 mL) and TE (0.5mL) were added to the pellets. Approximately 50 µl of 0.5 mm diameter glass beads were added to each tube and the samples were vortexed.

The samples were then centrifuged for 1 minute at 14,000 rpm. The supernatants were removed and placed into new microcentrifuge tubes. Two to three drops of chloroform were added to each tube. After thorough mixing, the tubes were centrifuged at 14,000 rpm for 2-3 minutes. The top layer containing RNA was removed. A volume of 95% ethanol equivalent to half the existing volume was added to each tube. After 3 minutes at room temperature, the tubes were centrifuged at 3,000 rpm for 5 minutes. The supernatant was removed and 0.5 mL of 0.1 M sodium citrate in 10% ethanol was added to each tube. After 30 minutes at room temperature with periodic mixing, the tubes were centrifuged at 3,000 rpm for 5 minutes. The supernatant was removed with a pipette and 0.75 to 1 mL of 70% cold ethanol was added to each tube. The tubes were kept at room temperature for 10-20 minutes with periodic mixing. After centrifuging 5 minutes at 3,000 rpm, the supernatant was removed. The samples were allowed to air dry for 5-15 minutes, and the pellets were dissolved in 10 µL 10 mM NaOH, mixed thoroughly, and centrifuged briefly.

Another attempt was made to isolate *Demodex canis* DNA from scrapings from one additional dog, but without manual separation of mites. This procedure incorporated several variations from the procedure described above. The sample was divided into two tubes (4a and 4b). Following centrifugation at 3,000 rpm for 10 minutes and removal of the supernatant, 500 µL lysis buffer was added to each pellet. The samples were mixed with a small quantity of 0.5 mm diameter glass beads, and 2 µL of proteinase K. These samples were placed in a 42°C water bath for 1 hour followed by incubation at 65°C for 15 minutes in a dry bath incubator. The samples were centrifuged at low speed for 1 minute, and the supernatants were removed with a 200 µL pipetteman to avoid disturbing the glass beads. Fifty uL of 1M NaCl was added to yield 0.3 M NaCl, and followed by 500µL of phenol and 2-3 drops of chloroform. The samples were mixed, and then centrifuged for 10 minutes at 14,000 rpm. Three layers were observed. The uppermost layers were removed and placed into new tubes. A total of 500 µL was removed from each sample. This was subdivided to yield two 4a tubes (4a-1 and 4a-2) and two 4b tubes (4b-1 and 4b-2) (Table 1). Three volumes of 95% ethanol was added to each tube, mixed briefly, and placed in an ice bath for 15 minutes. After centrifugation at 14,000 rpm for 5 minutes, the supernatant was poured off and the pellets were rinsed with 2-3 drops of 70 % cold ethanol. The tubes were inverted onto paper towels to air dry for 5-15 minutes. Ten µL of TE containing 50 µg/ml RNAse was added to each. The tubes were mixed and centrifuged briefly.

The same protocol used for sample 4 was repeated with sample 5. However, 75 mites were manually separated from this sample. Sample 5 was divided into 2 samples, 5a-1, and 5b-1 (Table 1)

Many attempts, with several modifications, were made at isolating DNA from the sample acquired from dog 6. For the first 2 DNA extractions, chitinase (Sigma/Aldrich™) was added to the protocol, and glass beads were not used. Sample 6a contained 177 mites, and 6b contained 217 mites (Table 1). Following centrifugation at 3,000 rpm and removal of the supernatant, 450 µL of chitinase buffer (10 mM KPO4, pH 6.0, 2 mM CaCl2) and 50 µg chitinase (1 mg/ml) was added to each sample. The samples were incubated at 37° C for 30 min-

utes. The samples were centrifuged at 3,000 rpm for 10 minutes and the supernatant removed. One hundred µL of lysis buffer was added to each of the remaining pellets. The DNA isolation procedure was performed as previously described for samples 1,2,3, and 4a and 4b. However, the final pellet was resuspended in 10 µL STE (sodium chloride Tris EDTA). Also, following incubation on ice for 15 minutes and centrifugation at 14,000 rpm for 5 minutes, the supernatant from 6b-1 was removed, labeled 6b-2, and stored at -20°C. Sample 6b-2 was centrifuged at 14,000 rpm for 5 minutes. The supernatant was removed, the pellet was rinsed with 70% ethanol, and air dried for 5-15 minutes over paper towels. The pellet was re-suspended in 25 µL STE, mixed thoroughly, and repelleted by centrifugation.

For sample 6c (Table 1), approximately 100 mites were separated manually and added to 1 mL of TE in a microcentrifuge tube. This sample was centrifuged at 3,000 rpm and the supernatant was removed. The sample was pipetted with a 200 µL pipetteman into a 0.5 mL microtube. Approximately two to three 20 µL samples of TE were added to the microcentrifuge tube, mixed, centrifuged, and transferred to the microtube. A small amount of 0.5 mm diameter glass beads was added to the sample and manually ground using a pellet Pestle® and a vortex. Approximately 2 µL of sample was removed and examined using a light microscope at 10X magnification. A single intact egg was visualized. The sample was placed in the sonicator for 6-7 minutes. Again, another 2 µL sample was evaluated and a single intact egg was visualized. This was followed by manual grinding and additional mixing. Neither intact mites nor eggs were observed in the 2 µL sample. Fifty µL of 1 mg/mL chitinase was then added to the sample mixture. No additional buffer was added. Following incubation at 37°C in a water bath, 200 µL of lysis buffer was added. The remainder of the procedure utilized the methods described for samples 1, 2, and 3. The final pellet was re-suspended in 10 µL STE. Also, the supernatant, following incubation on ice and high speed centrifugation was saved and labeled as 6d. A portion of the pellet was lost in the supernatant. Sample 6d was centrifuged at high speed for 5 minutes. The supernatant was removed, and the pellet was rinsed with 70% ethanol. It was inverted over paper towels and allowed to air dry for 5-15 minutes, and re-suspended in 10 µL STE, mixed, and re-centrifuged.

Standardization of Mite DNA Isolation (samples 6 and 7)

The final procedure that was developed was applied to samples obtained from dogs 6, and 7. Two DNA isolation procedures were performed utilizing sample 6 (6e and 6f-1), because additional DNA was needed for further experiments. The number of mites obtained by manual separation from each sample was as follows: 200 from 6e, 191 from 6f-1, and 157 from 7a-1. These samples were placed in1 mL of TE in microcentrifuge tubes and centrifuged at 3,000 rpm for 10 minutes. The supernatants were removed, except for a small residual amount to allow for re-suspension of the pellet. The samples were then pipetted into a 0.5 mL microtube. Approximately two to three 20 µL samples of TE were added to each microcentrifuge tube, mixed, and centrifuged. The re-suspensions were pooled and approximately 50 µL of 0.5 mm diameter glass beads were added. The sample was ground using a pellet Pestle® and vortex. Fifty µL of 1 mg/mL chitinase was added to each sample and incubated for 30 minutes in a 37°C water bath. Two hundred µL of lysis buffer (20 mM Tris pH 8.8, 1% SDS, 10 mM EDTA) and 2 µL of 50 mg/ml proteinase K were added to the samples. The samples were mixed and placed in the 42°C water baths for 1 hour. They were transferred to a 65°C dry bath incubator for 15 minutes, and then centrifuged at approximately 3,000 rpm for 1 minute. The supernatants were removed. Ten µL of sample 6e-1 sample were evaluated at 10X with the light microscope and no intact mites were seen. One M NaCl was added to the supernatants to yield a final

concentration of 100 mM NaCl. A volume of phenol equal to the residual amount in the tubes and 2-3 drops of chloroform were added to the samples.

After mixing and centrifugation at 14,000 rpm for 10 minutes, the top layer was removed without disturbing the underlying layers, and three times the volume of 95% of ethanol was added to each. The samples were mixed, placed on ice for 15 minutes, and centrifuged at 14,000 rpm for 5 minutes. After removal of the supernatants, the pellets were rinsed with 2-3 drops of cold 70% ethanol, allowed to air dry, and 10 µL STE were added to re-suspend the pelleted DNA. The samples were mixed and briefly centrifuged. Following incubation on ice and high speed centrifugation, the supernatants from samples 6e, 6f-1, and 7a-1 were saved and labeled as 6e-2, 6f-2, and 7a-2. Some of the pellet was lost in this supernatant. The supernatant samples were centrifuged at high speed for 5 minutes. The resulting supernatants were removed and saved (6e-3, 6f-3, 7a-3), and the pellets were rinsed with 70% cold ethanol. The samples were inverted over paper towels to air dry for 5-15 minutes, re-suspended in 10 µL STE, mixed, and centrifuged. This procedure was later repeated for samples 6f-3 and 7a-3. DNA concentrations and purity of these samples can be found in Table 1.

Mite DNA Isolation for sample 8

DNA was isolated initially using the protocol performed on samples 6 and 7. This was carried out with 170 manually separated mites. These samples were denoted 8a-1 and 8a-2 (Table 1).

In an attempt to achieve a higher purity DNA sample, 100 mites were removed from sample 8 via centrifugation as described above. The DNA was isolated using the protocol described for samples 6 and 7. Exceptions to this isolation protocol were as follows: after removal of the top layer after phenol-chloroform extraction,1M NaCl was added to obtain an NaCl concentration of 200 mM, followed by the addition of 2/3 volume of 5 M NaCl and 1 volume of isopropanol. The final product was suspended in 80 µl TE and labeled as 8b-1. Following incubation on ice and centrifugation at 14,000 rpm, the supernatant was divided into two samples (8b-2 and 8b-3) and saved. These tubes were filled with isopropanol and placed on ice for 20-30 minutes. They were then centrifuged at 14,000 rpm for 5 minutes. The supernatants were removed, and the tubes were filled with 70% cold ethanol. The tubes were then centrifuged at 14,000 rpm and the supernatants removed. The tubes were inverted over paper towels to air dry and the pellets were re-suspended in 10 µL TE. DNA concentrations and purity of these samples are presented in Table 1.

The DNA isolation protocol used for sample 8 excepting centrifugation was used to isolate additional DNA from sample 7 to achieve a more pure sample. This sample was re-suspended in 10 µL TE and labeled as 7b-1. Following incubation on ice and centrifugation at 14,000 rpm the supernatant was also re-centrifuged at 14,000 rpm. The supernatant was removed and the pellet rinsed with 70% cold ethanol. The tube was inverted over paper towels to air dry, re-suspended in 10 µL TE, and labeled as 7b-2. DNA concentrations and purity of these samples can be found in Table 1.

Mosquito and Flea DNA Isolation

DNA from mosquitoes and fleas served as a positive control for the Random Amplified Polymorphic DNA technique. Fleas and mosquitoes were obtained from the Auburn University College of Veterinary Medicine's Parasitology Laboratory. Ten dead fleas and five dead mosquitoes were each placed in microtubes. Seven hundred fifty µL TE were added, and specimens were centrifuged at approximately 14,000 rpm for 10 minutes. The supernatants were removed, and 500 µL lysis buffer and approximately 50 µL of 0.5mm diameter glass beads were added. The samples were mixed and manually homogenized using a Pellet Pestle.™ Two µL of 50 mg/ml proteinase K was added to each tube. The tubes were then incubated in a water bath for 1 hour at 42°C and

then 65°C for 25 minutes. The tubes were centrifuged at 3,000 rpm for 1 minute. The supernatants were removed and placed into separate microcentrifuge tubes. Fifty µL of 1M NaCL, 500 µl phenol, and 2-3 drops of chloroform were then added to each tube. The tubes were mixed and then centrifuged at 14,000 rpm for 10 minutes. The top layer was removed and placed into separate tubes. Three volumes of 95% ethanol were added. The sample was mixed briefly and placed on ice for 30 minutes. The sample was centrifuged at 14,000 rpm for 5 minutes. The supernatant was stored at -20°C in the event that additional DNA might be needed. The pellets were rinsed with cold 70% ethanol and the tubes were inverted over paper towels to dry for 10 minutes. Ten µL TE was added to each tube. Each specimen was mixed thoroughly and centrifuged. This yielded two final tubes for each sample: Flea A and B, and Mosquito A and B (see Table 1).

DNA Yield and Purity Measurement

The DNA yields were quantified with a Nanodrop spectrophotometer. The 260/280 nm ratio measured nucleic acid purity

DNA cleanup

Additional DNA purification procedures were performed for samples whose 260/280nm ratio was less than 1.60, but appeared to have sufficient quantities of DNA. The following procedures were performed on sample [5].

Carbohydrate removal: Ninety µL TE, 25 µL isopropanol, and 25 µL 1.2 M sodium citrate was added to each sample. The samples were mixed and then centrifuged at 14,000 rpm for 5 minutes. The supernatant was removed, and the pellets were rinsed with cold 70% ethanol. After drying for 5-15 minutes, the DNA was re-suspended in 50 µL TE plus 10 µg/ml RNAse. The samples were mixed and centrifuged briefly. These samples were denoted as 5a-2 and 5b-2 (Table 1).

The following procedures were performed on sample 6b-1.

Phenol removal: Sample 6b-1 was diluted with 40 µL STE to a total of 50 µL, and the DNA re-extracted with 2-3 drops of chloroform. After mixing and centrifugation at 14,000 rpm for 10 minutes, the top layer was removed and placed in a separate tube. Three volumes of 95% ethanol was added, and after mixing, placed at -20 °C for 30 minutes. The sample was centrifuged at 14,000 rpm for 5 minutes, and the supernatant was removed and saved. After rinsing with 70% ethanol, the DNA was air dried for 5-15 minutes, and re-suspended in 50 µL STE. The tube was mixed to re-suspend the DNA and briefly centrifuged. This tube was labeled as 6b-3.

In an attempt to improve purity of samples 8a-2, 6f-3 and 7a-3, phenol-chloroform extraction was repeated. The pellets did not appear to adhere well to the microcentrifuge tube wall, and therefore, additional salt was added before ethanol precipitation. Five µL of 5 M NaCl was added to the DNA samples, previously re-suspended in 100 µL of STE, to yield a 0.3 M salt concentration. Three volumes of ethanol were then added. The various supernatants and pellets from samples 6, 7, and 8 were combined and subjected to additional phenol-chloroform and ethanol precipitation. The final samples were labeled 8a-3, 6f-4, and 7a-4 (Table 1).

Random Amplified Polymorphic DNA (RAPD)

The 6f-1 sample was diluted to 100 ng/µL in TE. Random Amplified Polymorphic DNA (RAPD) reactions were set up with 12 mer primers that had been useful for RAPD studies with parasites.12 Each reaction contained 2.5 µL 10X Thermopol II buffer without Mg (New England Biolabs), 0.9 µL 25 mM MgCl2, 0.4 µL 25 mM dNTPs, 0.5 µL FideliTaq™ DNA polymerase, 2 µL of 0.1 mg/ml primer (25 pM final concentration), 1 µL of 100 ng/uL DNA, and 17 µL of deionized water to yield a final volume of 25 µl. The negative control sample contained primer, but no DNA. Cycling conditions were as follows, initiation at 94 °C for 5 minutes, 40 cycles of 94 °C for

1 minute, 35 °C for 1 minute, 72 °C for 2 minutes, followed by an additional 72 °C for 15 minutes. Five µL of each sample were combined with 5 µL loading dye and subjected to electrophoresis in a 1.2% agarose gel with TBE buffer for 4 hours at 50 mA. This reaction was performed with both mite (6f-1) DNA and canine DNA (obtained from a prior research experiment) to verify that the DNA collected was from mites.

PCR

Canine DNA contamination testing: To verify that the mite DNA was not contaminated with canine DNA, a canine specific primer was used in a PCR procedure using mite sample 6f-1 DNA, canine DNA as a positive control, and negative controls of flea DNA, mosquito DNA, and zero DNA. Two µL of 100 ng/µL of mite 1, flea, and mosquito were used. One µL of 100 ng/µL canine DNA was used. The PCR assay mix contained 5µL 10X Thermopol II buffer, 0.75µL of 25 mM MgSO4., 0.4µL 25 mM dNTPs, 0.50 µl Fidelitaq DNA polymerase, 4uL of DLA-DRA primer (Dog leukocyte antigen primer), 100 ng DNA and 37.5µL sterile deionized water. The PCR reaction conditions were 2 minutes at 94°C, 40 cycles of 94°C 30 seconds, 60°C 1 minute, and 72°C 1 minute and 5 minutes at 72°C. The PCR samples were run on a 2% agarose gel with TBE buffer for 2 hours at 60mA.

PCR with Known Primers: Various PCR protocols with candidate genes and rRNA markers were attempted in efforts to amplify *Demodex canis* DNA. Table 2 displays the protocols utilized with the various primers. These protocols were based on published PCR procedures. In general, a 50 µL reaction was set up for each trial with 100 ng DNA from the mite samples (utilizing samples 1-6e).

PCR with Novel Primers: Sequences of interest were amplified for two of the clones. One matched insect ubiquitin and another matched the bacteria Bradyrhizobium japonicum (related to termite gut bacteria). For these two clones, oligonucleotide primers were designed and tested against DNA from 2 other mite samples (from samples 7 and 8). DNA from both of these mite samples were amplified using the primer developed from the sequence matching Bradyrhizobium japonicum DNA and produced amplicons with homology to the Bradyrhizobium japonicum sequence. The oligonucleotide primer developed from the sequence matching Bradyrhizobium japonicum DNA also amplified a similar sequence from flea and mosquito DNA. Only sample 7 amplified a

Table 2: *PCR Parameters Tested with Demodex canis Mite DNA*

	Ref Art #	Initiation	Denaturation	Annealing	Extension	Final Elongation	#Cycles
16S rRNA: Scabies	13*	92°, 2min	92°, 2 min	gradient, 30sec	72°, 1.5min	72°, 2min	35
Cyt. Oxidase: Scabies	13*	92°, 2min	92°, 2min	gradient, 30sec	72°, 1.5min	72°, 2min	35
Cyt. Oxidase: blackfly	14	94°, 2min	94°, 30sec	49°, 30sec	72°, 2min	72°, 10min	35
ITS: Scabies	15	92°, 2min **	92°, 1min	64°, 1min	72°, 1.5min	72°, 10min***	45
Chitin Synthase	11****	92°, 2min	92°, 30sec	55°, 30sec	72°, 2min	72°, 10min	35

**No temperatures or times were reported in this paper.*
***No temperature and time were listed in this paper for the initiation step.*
****No temperature and time were listed in this paper for the final elongation step.*
***** No temperature or times were reported in GenBank for this primer. The annealing temperature selected was based on the primer melting temperature and what worked on a flea DNA sample.*

sequence with homology to insect ubiquitin. Dog DNA did not amplify either of these primers.

Mite DNA RAPD Cloning and DNA Sequencing

Random RAPD fragments obtained using the TV1 primer were cloned usinan Invitrogen™ TOPO TA Cloning® Kit according to the manufacturer's directions. Briefly, 0.5 µL of DNA from a recently performed RAPD reaction was added to an equal mixture of 0.5 µL salt solution and 0.5 µL vector. This was then added to 1.5µL sterile deionized water to yield a final volume of 3.0 µL, in a 0.6 mL microcentrifuge tube. Following incubation at room temperature for 5 minutes, 2 µL of this mixture was added to a vial of One Shot® TOP10 chemically competent E.coli, and mixed by gentle tapping. After incubation on ice for 30 minutes, the cells were placed in a 42 °C water bath for 30 seconds. Two hundred fifty µL of SOC medium (room temperature) was added, and the tubes were incubated at 37 °C for 1 hour. Fifty µL of 2% X-gal was spread on LB agar plates containing 50 µg/ml Kanamycin (LB/Kan). All of the transformed cells were spread on LB/Kan plates and incubated overnight at 37 °C. Colonies that contained plasmids with inserted DNA were streaked on fresh LB/Kan plates and incubated overnight at 37 °C.

A Zyppy™ Plasmid Miniprep Kit was used to purify the plasmid DNA. Briefly, bacterial cultures were scraped from the LB/Kan plates and added to 600 µL of TE in 1.5 mL microcentrifuge tubes. The cells were mixed until they were completely resuspended. One hundred µL of 7X lysis buffer was added to each tube and mixed by gentle inversion. After 2 minutes, 350 µL of cold neutralization buffer was added to each tube. The samples were again mixed by gentle inversion. Following centrifugation at 14,000 rpm for 2 minutes, the supernatants were pipetted into the Zymo-Spin™ II columns. The columns were placed into collection tubes provided in the kit and centrifuged for 15 seconds. The flow-through was discarded and the column was placed back into the same collection tube. Two hundred µL of Zyppy™ endo-wash buffer was added to each column and centrifuged for 15 seconds. Four hundred µL of Zyppy™ wash buffer was added to the column and centrifuged for 30 seconds. The columns were then transferred to 1.5 mL microcentrifuge tubes, and 30 µL of Zyppy™ Elution Buffer (room temperature) was added to the column matrix. The tubes were held at room temperature for 5-10 minutes. Plasmid DNA was eluted by centrifugation for 15 seconds.

The plasmid clones were sized by digestion using an EcoR1 restriction enzyme and gel electrophoresis. Random clones from the RAPD reaction, with inserts smaller than 2 kilobases were sequenced. For clones of PCR fragments, a PCR fragment digested with EcoRI was run as a control during the electrophoresis of the candidate plasmid clones. If sufficient clones were available, at least 4 clones to each PCR fragment were sequenced to ensure that artifacts were not introduced by the Taq polymerase.

The DNA sequences obtained from the RAPD amplification were examined for similarity to known DNA sequences using the BLAST similarity search engine program from the NIH/NCBI (National Center for Biological Information) contained in the Macvector ver. 9.0 software package. For these clones, oligonucleotide primers were developed (Primer Brady forward: TGTCGGACTTTGGTTTCTTG, Primer Brady reverse: ATCGCTTCGGCTCATTGTC; Primer Ubiq forward: CTCGGAGTTGAACCAAC, and Primer Ubiq reverse: GTGAATCATAATCTTTTATTTTAC) that were then used in specificity tests of additional mite, dog, and insect DNAs (see PCR with novel primers). These primers have potential for use in PCR tests that compare differences among mite isolates.

RESULTS

DNA Yields and Purity

The DNA concentration and A260/280 ratios from the various mite DNA isolation trials are listed in Table 1. The concentration

and ratios of absorbance from the flea and mosquito samples are also listed in Table 2. For the trials with less than 75 mites, glass beads, chitinase and/or phenol extraction, DNA was either not obtained, or was of insufficient purity. Although the final methods for successful isolation of DNA resulted in reduced yields given the number of mites, it appears that all of the steps are needed to obtain DNA.

RAPD

As can be seen in Figure 1, the banding patterns between *Demodex canis* DNA and dog DNA differed significantly indicating that the results using the various 12 mer primers were not simply canine DNA from the skin scrapings.

Polymerase Chain Reaction (PCR)

Canine DNA contamination test: The gel showed an intense band at MW of 250 bp with the canine sample. Very faint bands were also seen in the mosquito and mite lanes at this MW.

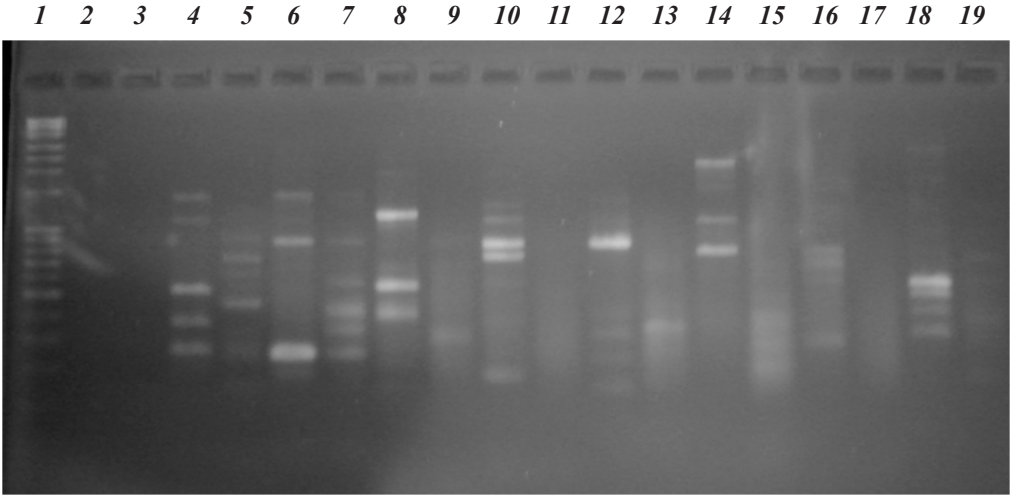
PCR with Known Primers: PCR procedures using protocols shown in Table 1 were attempted with samples 1-6e. These PCR's yielded no positive results.

PCR with Novel Primers: Sequences of interest were amplified for two of the clones. One matched insect ubiquitin and another matched the bacteria *Bradyrhizobium japonicum* (related to termite gut bacteria). For these two clones, oligonucleotide primers were designed and tested against DNA from two other mite samples (from samples 7 and 8). DNA from both of these mite samples were amplified using the primer developed from the sequence matching *Bradyrhizobium japonicum* DNA and produced amplicons with homology to amplicons generated using the primer related to *Bradyrhizobium japonicum* DNA. Only sample 7 showed homology to the primer designed to match insect ubiquitin DNA sequences. Dog DNA did not amplify either of these primers.

DNA sequencing

DNA sequences: The sequences matching insect ubiquitin and Bradyrhizobium japonicum were submitted to GenBank. The accession number for the sequence matching ubiquitin is GU953241 and the accession

Figure 1:



Lane 1, DNA ladder; Lane 2, Negative Control; Lane 3, Negative Control; Lane 4, 1st 12 mer primer and mite DNA; Lane 5, 1st 12 mer primer and canine DNA; Lane 6, 2nd 12 mer primer and mite DNA; Lane 7, 2nd 12 mer primer and canine DNA; Lane 8, 3rd 12 mer primer and mite DNA; Lane 9, 3rd 12 mer primer and canine DNA; Lane 10, 4th 12 mer primer and mite DNA; Lane 11, 4th 12 mer primer and canine DNA; Lane 12, 5th 12 mer primer and mite DNA; Lane 13, 5th 12 mer primer and canine DNA; Lane 14, 6th 12 mer primer and mite DNA; Lane 15, 6th 12 mer primer and canine DNA; Lane 16, 7th 12 mer primer and mite DNA; Lane 17, 7th 12 mer primer and canine DNA; Lane 18, 8th 12 mer primer and mite DNA; Lane 19, 8th 12 mer primer and canine DNA.

number for the sequence matching bradyrhizobium japonicum is GU953242.

DISCUSSION

DNA isolation without the combination of manual separation, chitinase, and manual homogenization did not produce significant amounts of mite DNA as determined by either spectrophometer measurement or in a PCR test. All DNA extracted using methods implemented prior to the use of the standardized protocol in PCR experimentation did not yield positive PCR results. Positive PCR results were also not obtained using known primers with DNA isolated from sample 6e (which was obtained using the standardized mite isolation procedure). Most of the known primers were created from other insects and mites other than *Demodex*. It is possible that these particular primer sequences do not have enough similarity to related *Demodex* mite sequences. The chitin synthase primer that was developed from that listed in GenBank also did not amplify *Demodex canis* DNA. A publication with associated PCR parameters was not available. It is possible that the PCR protocols used with the known primers are not appropriate with the *Demodex canis* DNA. Further work with the known primers is currently being pursued.

In patients with seborrhea, which may occur concurrently with demodicosis, an excessive amount of lipid may be found in the skin. When performing skin scrapings to obtain mites, this material may be present with the mites and other hair debris. For example, with sample 8, following centrifugation an upper layer of lipid was present. Below this layer, many mites with significantly less debris were present. Once this method was performed, the purity increased up to 1.63 (ratio of absorbance at 260 and 280 nm). Lipid present during DNA isolation will impair DNA extraction. Thus, in samples from patients with concurrent seborrhea, centrifugation prior to mite isolation provides for a better sample.

As the known primers were not yielding positive results with the various DNA isolates, RAPD was utilized. The gel obtained from the RAPD technique with sample 6 demonstrated that the DNA isolated from the mites was different than canine DNA. Therefore, the technique developed for isolation of mites is sufficient to eliminate contamination of mite samples with canine DNA. This was further verified by running the 2 primers on a 1.5% agarose gel developed from the RAPD technique against the canine DNA. No bands were produced with canine DNA. Further verification is that the partial genes that were sequenced from the mite DNA with primer 4 was consistent with insect ubiquitin. Finally, the canine specific primer DLA-DRA was run against canine and mite DNA. This primer amplifies canine DNA at 250 bp. Very faint bands at 250 bp were observed in the lanes that contained mite and mosquito DNA. This was not surprising since the mosquitoes had fed on canine blood and the mites had fed on canine samples. Consequently, some canine DNA was expected to be present in these samples.

It is not known whether the three morphologic types of canine *Demodex* mites are genetically different. We are hopeful that the results of the techniques described here will encourage additional research on the genetics of the proposed species and morphologic variants. Many aspects of the pathogenesis of canine demodicosis remain poorly understood. Among them are why certain dogs develop demodicosis and others do not.[1] Also, why does the disease differ in severity among infested dogs? Do these differences reflect the different pathogenetic potential of the different mite species, different host factors such as immune responses or dermal environments, intercurrent agents, or stress? Not all dogs with clinical demodicosis can be cured using available therapeutic agents.[1] Does this represent variations in distribution, metabolism and elimination of the therapeutic agent, or a combination of treatment and host factors? These are questions that may be answered in part by additional study of the *Demodex* genome. Perhaps the methods of isolation of *Demodex canis* DNA summa-

rized here will encourage additional molecular analysis of *Demodex* mites. A better understanding of genetic inter-relationships of the different *Demodex* species may help us to understand the disease process and to devise more effective therapeutic strategies.

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