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Relative Utility of Different Lengths of the Mitochondrial 16S rDNA Gene in Population Genetics of the Tick Species, *Dermacentor andersoni.*

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This thesis for honors recognition has been approved for the

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Director	4/7/14 Date
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Abstract

The hard tick species, *Dermacentor andersoni*, is an important vector of zoonotic disease in the state of Montana. The purpose of this study was to compare different fragments of the mitochondrial 16S rDNA gene to determine which provides the most useful information for the genetic analysis of *D. andersoni* ticks in Montana. Two primer sets were used to PCR amplify a portion of the mitochondrial 16S rDNA gene from DNA obtained from 40 *D. andersoni* ticks from a single population near Helena, Montana. Resulting sequences from PCR products were then analyzed for quality, amount of genetic variability and suitability for use in future population genetic studies. The primer set 16S+1 and D16S5 was found to produce high quality sequences of the 16S rDNA gene that are genetically variable and adequate for preliminary studies of the genetic structure of tick populations as part of the Infectious Disease Ecology project at Carroll College. This study demonstrates the PCR amplification, sequencing and analytic techniques that can be utilized to characterize the genetic diversity in tick populations in the future.

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Introduction

Globally, ticks are considered the second most common vector of human infectious disease after mosquitoes (Parola and Raoult 2001). The impact of tick borne diseases on humans and domestic animals has become more prevalent since the discovery of *Borrelia burgorferi,* the pathogenic agent associated with Lyme disease (borreliosis) in the early 1980s, and the issue is now receiving more attention from veterinary and public health fields (Parola and Raoult 2001, Dantas-Torres *et al.* 2012, Jongejan and Uilenberg 2004). Montana is home to both soft (*Argasidae)* and hard (*Ixodidae)* ticks; for example, the Rocky Mountain wood tick (*Dermacentor andersoni),* winter tick (*D. albipictus),* American dog tick (*D. variabilis)* and soft ticks (*Ornithodoros hermsii)* are among tick species found in Montana that are of interest because of their role as disease vectors (Johnson 2010). Many of these tick species are primary vectors for a variety of zoonotic diseases (Dantas-Torres *et al.* 2012).

The Rocky Mountain wood tick was selected for use in this study due to its high prevalence in the state of Montana, which may correlate with an increased potential for this species to carry disease and be detrimental to the health of humans and animals in the area. This species thrives in the Rocky Mountain region of the northwestern United States, where its role as a disease vector of Rocky Mountain Spotted Fever (RMSF) was first recognized and investigated beginning in the early 20th century in the Bitterroot Valley of Montana, eventually leading to the establishment of Rocky Mountain Laboratories in Hamilton, MT (Honey 2009; Sonenshine and Mather 1994). Montana is home to the highest proportion of *D. andersoni* tick populations per county than any other state according to a study by James *et al*. (2006). Furthermore, *D. andersoni* was

the most common tick species found on human subjects in the northwestern United States including Montana, where a total of 126 ticks were collected including 121 *D. andersoni* (Merten and Durden 2000). *Dermacentor andersoni* has also been associated with pathogens of anaplasmosis, Lyme disease (borreliosis), Colorado Tick Fever (CTF) and tick paralysis in addition to RMSF (Jongejan and Uilenberg 2004; Johnson 2010).

The Infectious Disease Ecology research project has been ongoing at Carroll College since 2009 with the primary goal of identifying viral distribution patterns in the state of Montana in order to develop a model for risk of infection. Geographic Information Systems (GIS) and molecular techniques for detection and characterization are being used to collect information on geographic aspects including climate, elevation and land cover, viral vectors, viral frequency and the genetic diversification of vector populations that will be used in this model (Hokit *et al.* 2008). Currently, a basic model for West Nile Virus (WNV) distribution in the state of Montana has been established (Hokit *et al.* 2013). It was realized that the information gathered from this research protocol might be of value when applied to other infectious diseases and disease vectors found in Montana. Accordingly, the WNV project was expanded to include ticks with the objective of developing a similar model for tick-borne diseases of concern in Montana such as RMSF, Lyme disease (borreliosis), CTF and Tularemia.

The information collected from studying the population genetics of vectors, such as *D. andersoni* ticks, can lend insight into the distribution, size and structure of the populations in question and help determine the best approach to controlling the vectors and ultimately the spread of arthropod-borne diseases (McCoy 2008; Tabachnick and Black 1995). Based on previous studies with various tick species, it has been shown that

mitochondrial (mt) ribosomal DNA (rDNA) sequence analysis is useful in phylogenetic studies (Black and Piesman 1994, Simon *et al.* 1994). Specifically, the mitochondrial 16S rDNA gene is informative of familial and subfamilial phylogenies for all species of ticks (Black and Piesman 1994). This study focused on developing methods of amplifying and sequencing the 16S gene so that it could be used as a basis for determining the most useful genetic analysis methods for only ticks of the species *D. andersoni*. Mitochondrial rDNA genes, though highly conserved at some regions, evolve quickly and are useful for intraspecific studies of vector populations (Simon *et al.* 1994). Norris *et al.* (1996) also showed that the mitochondrial 16S gene was more phylogenetically useful and variable than the 12S gene in a population genetics study of the Blacklegged tick, *Ixodes scapularis.* Sequence variation was also found for *Dermacentor* spp. using the same 16S rDNA gene (Crosbie *et al.*, 1998). Additionally, Patterson *et al.* (2009) utilized a 16S+1 and 16S-1 primer combination to amplify 460bp of the 16S mtDNA gene, while de la Fuente *et al.* (2005) used primers D16S3 and D16S5 for amplification of specific fragments of approximately 360bp of the 16S mtDNA gene. These distinct primer sets were developed and utilized for isolating specific fragments of the mtDNA gene in order to examine the extent of genetic variation within and between populations of *D. andersoni* ticks (Patterson *et al.*, 2009; de la Fuente *et al.,* 2005). With these fragments, both studies determined that variation does exist and is detectable using the selected region of the mitochondrial gene. Both de la Fuente *et al.* (2005) and Patterson *et al.* (2009) performed extensive analysis in order to characterize the genetic diversity of their study populations.

Based on the reported success of Patterson *et al.* and de la Fuente *et al.'s* work, the present study aims to determine which of the primer sets developed by Patterson *et al.* (2009) and de la Fuente *et al.* (2005) will prove most informative for the genetic analysis of the mitochondrial 16S rDNA gene in *D. andersoni* ticks collected in Montana. My hypothesis is that the primer set 16S+1 and 16S-1 described by Patterson *et al.* (2009) will provide the most useful information and be best suited for population genetic studies of *D. andersoni* ticks because these primers amplify a larger, useable portion of the 16S region than do the primers used by de la Fuente *et al.* (2004). These data will ideally contribute to the development of a risk assessment model of infection in the state. DNA extractions from 40 ticks of a population collected from a site near Helena, Montana were amplified with both primer sets, sequenced and hand-edited for analysis and comparison using methods similar to Patterson *et al.* (2009) and de la Fuente *et al.* (2005). This study also attempts to establish a protocol for studying the genetic diversity of this species that can be utilized by students working on the WNV project extension in the future.

Materials and Methods

Tick Collections

Ticks were collected by flagging grassy and shrubby areas on BLM land near York Bridge, near Helena, MT (46.71328˚N, 111.81214˚W) for a total of 60 minutes. Specimens were then identified by species using criteria described by Greg Johnson of Montana State University (pers. comm.). Ticks were sorted into a single population of 40 individual *D. andersoni* and stored at room temperature in 200 proof ethanol.

DNA Extraction

Total DNA was extracted and purified using the protocol provided by QIAGEN DNeasy Blood and Tissue Kit with modifications made for grinding whole *D. andersoni* ticks. Each individual was placed in a 2 mL RNase/DNase-free lysing matrix tube with 180 μL of buffer ATL and homogenized in the FastPrep for 30 sec. at speed 4 (30 oscillations/minute). A volume of 20.0 μL proteinase K was added to each tube and vortexed before being incubated overnight 56˚C. The QIAGEN (2006) protocol was followed as described and collected samples were stored at -20˚C.

PCR Amplification

For each individual, PCR amplification of an approximately 360-bp fragment of the 16S rDNA gene was amplified with a novel combination of primers:

D16S5 (5’-GAATGCTAAGAGAATGGAAT-3’)
16S+1 (5’-CGGTCTGAACTCAGATCAAGT-3’)

as described by de la Fuente, *et al.* (2005) and Patterson *et al.*(2009) respectively using the procedure developed by de la Fuente *et al.* (2001). PCR was performed in 50 μL reactions containing 2.0 μL *Taq* polymerase, 2.0 μL each primer at 10mM concentration, 1.0 μL dNTP at 10 mM concentration, 5.0 μL 10x reaction buffer, 36.0 μL PCR certified water and 2.0 μL sample DNA. The reactions were run on a Bio Rad MyCycler Thermocycler with the following conditions: initial denaturation at 94˚C for 30s; 35 cycles of 94˚C for 30s each, 52˚C for 30s, and 68˚C for 1 min.

PCR amplification of a 460-bp fragment of the mitochondrial 16S rDNA gene was performed for comparison, using primers:

16S+1 (5'-CCGGTCTGAACTCAGATCAAGT-3')
16S-1 (5'-CACAGCAATTTAAAAAATCATTGAGCAG-3')

following the procedure described by Patterson *et al.* (2009) and run on conditions described by de la Fuente *et al.* (2005). PCR was performed in 25.0 μL reactions containing 1.0 μL *Taq* polymerase, 0.5 μL of each dNTP at 10 mM concentration, 2.5 μL 10x Standard *Taq* Reaction Buffer (New England Biolabs), 1.0 μL of each primer, also at 10 mM concentration, 3.0 μL $MgCl_2$ at 10x concentration and 15.0 μL of PCR certified water. Reactions were also run on a Bio Rad MyCycler Thermocycler under the same conditions described above. Negative controls without rDNA were included in each run. Proper amplification of PCR products was verified by gel electrophoresis on 1% agarose gels containing SyBr Safe DNA Gel Stain loaded with 4.0 μL of each sample paired with a 100-bp ladder.

Complications arose when attempting to isolate a single band from amplification of the PCR product using the Patterson *et al.* (2009) primer combination 16S+1 and 16S-1. Due to presence of multiple bands after gel electrophoresis, extra steps were taken in order to isolate enough PCR product with primer combination 16S+1 and 16S-1 to be sent for sequencing. The original product was extracted from agarose gels using QIAGEN MinElute Gel Extraction Kit. The extracted samples were PCR amplified under the same conditions previously described and run on 1% agarose gels. A second gel

extraction was then preformed for the final collection of the Patterson *et al.* (2009) samples.

Once visualized, each product was cleaned by adding 10 μL of ExoSAP-IT (USB Corporation, Cleveland, OH) and thermocycled for 1 cycle at 37°C for 15 min and 80°C for 15 min. The samples were then sent to Macrogen in Gayang-Dong, Seoul, Korea for sequencing.

Analysis and Comparison of Sequences

Forward and reverse sequences were combined using CodonCode Aligner and nucleotide bases were hand-edited in order to develop a single consensus sequence representative of each individual. These consensus sequences for each primer set, for each individual, were then compared directly for size and information quality.

Data Analysis

All sequences produced using the D16S5 and 16S+1 primer combination were aligned in CLUSTALW (Larkin *et al.* 2007). The computer program ARLEQUIN, version 3.5 (Excoffier *et al.* 2010) was used to determine various features of genetic variation within the population of ticks studied, including: the number of haplotypes, respective haplotype frequencies, the total number of bases and the number of those bases that are polymorphic, the number of mutations (transitions, transversions and indels), pairwise differences in distance between the haplotypes (no Gamma correction) and the gene diversity value.

Results

Amplification

Clear PCR amplicons of the approximately 360 bp fragment of the 16S mtDNA gene were obtained from all 40 *D. andersoni* individuals using the novel primer combination of D16S5 and 16S+1 on conditions described by de la Fuente *et al.* (2005). No bands were seen in the negatives controls (Fig. 1). This primer combination of primer D16S5 from de la Fuente *et al.* (2005) and 16S + 1 from Patterson *et al*. (2009), was determined to provide the best PCR products through multiple trials.

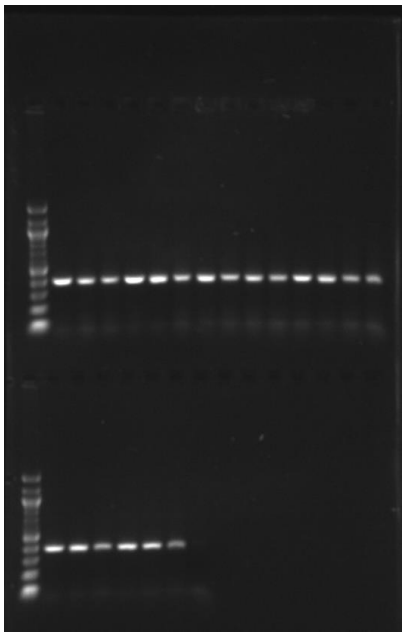

Figure 1. Agarose gel verification of clear banding pattern for 16S+1/D16S5 amplicons of York Bridge D. andersoni ticks #1-20. No banding is detectable in the negative control, which lacked rDNA, in the bottom row, lane eight.

Complete DNA sequences were created for each individual of the York Bridge population (Appendix). A consensus of forward and reverse primer sequences was used to form all final sequences except for that of individual 06 which was created with the

sequence from forward primer D16S5 primer. The average sequence length was 363.6 bp, with a 9.976 standard deviation for the sequence set.

No amplicons of the 460 bp fragment were produced from PCR amplification attempted using the Patterson primers 16S+1 and 16S-1. Though visualization of amplification did occur, clearer banding was not seen until after the original PCR product was isolated by a gel extraction and subjected to a second PCR (Fig. 2). A second gel extraction was performed to collect the product sent for sequencing. No complete DNA sequences were collected for the fragments amplified using the Patterson primer set.

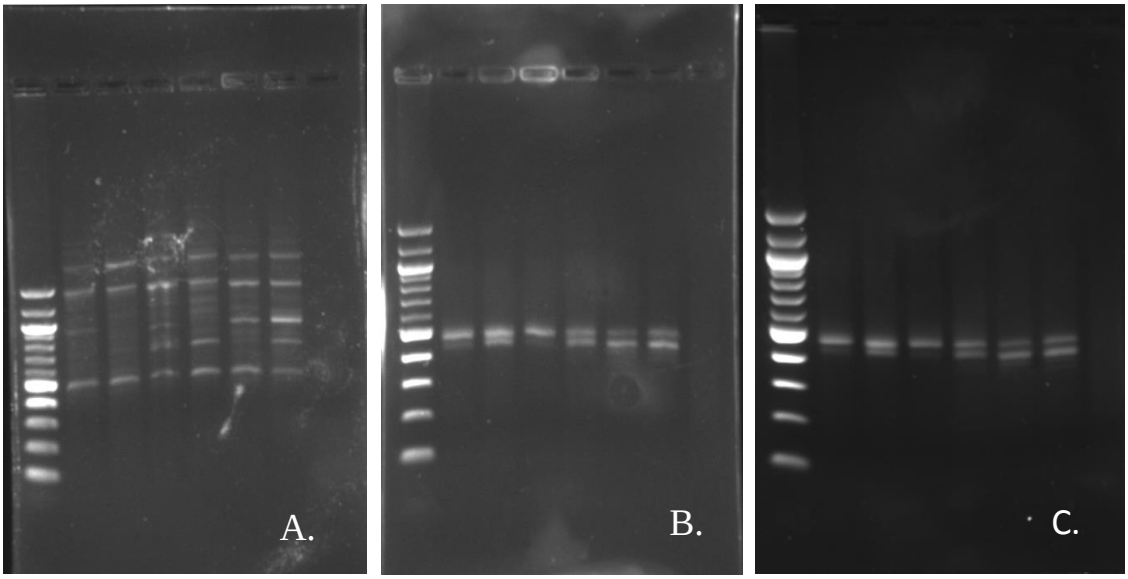

Figure 2. Agarose gel verification of banding pattern for 16S+1/16S-1 amplicons of York Bridge *D.andersoni* ticks #1-6 after initial PCR (A.), gel extraction (B.) and a second PCR on extracted bands (C.).

Genetic Analysis

Sequence alignment produced 387 aligned sites, of which 361 were deemed useable with less than 5% missing data. Four of these useable sites (Sites 237, 266, 293 and 315) were polymorphic for two different alleles. A total of 4 substitutions were found (1.1%), 3 being transitions and 1 being transversion. No indels were identified.

The variability of these sites resulted in five haplotypes of this fragment of the mitochondrial 16S rDNA gene, designated 1-5. These, along with respective frequencies and the number of the samples with that haplotype are shown in Table 1. Haplotype 1 was most common; 65% of the samples within the York Bridge population were designated as this haplotype. Pairwise genetic difference values between haplotypes ranged from 1.0 to 4.0. Overall gene diversity was characterized by haplotypic (*h*) and nucleotide (π) diversity (average over loci) values of 0.5423 ± 0.0792 and 0.002720 ± 0.002091 respectively within this population.

Table 1. Haplotypes, respective frequencies, and the number of ticks with that haplotype

Haplotype	Haplotype Frequency	No. of Ticks
1	0.650	26
2	0.200	8
3	0.025	1
4	0.050	2
5	0.075	3
Total:	1.000	40

Discussion

In this study, two different primer sets were compared for the suitability of each for the amplification and sequencing of fragments of the mitochondrial 16S rDNA gene in *D. andersoni* ticks. Results of repeated trials using different combinations of primers

for the amplification of PCR products suggest that a primer combination of D16S5 and 16S+1, the forward and reverse primers of an approximately 360 bp fragment respectively, will provide the most complete and useful sequences for genetic analysis. This novel primer combination utilizes primers originally described by both de la Fuente *et al.* (2005) and Patterson *et al.* (2009). This finding directly refutes the original hypothesis that the alternative Patterson *et al.* (2009) primer combination evaluated in this study would prove to be more adequate for use.

Unexpectedly, the 16S+1 and 16S-1 primer combination and conditions described by Patterson *et al.* (2009) was unsuccessful in clearly amplifying a 460 bp fragment of the 16S gene (Figure 2A.). Although amplification was visualized when the combination was run on conditions described by de la Fuente *et al.* (2005), it still was not specific enough to be sequenced and utilized for analysis. Repetition of the PCR cycle on the same samples and gel extractions of the band formed by the expected fragment were ineffective and did not provide enough template DNA to allow for the creation of any useable sequences for these samples. All attempts made to resolve the issue were unsuccessful: both the original primers and PCR conditions were verified for accuracy, new primers were made and used, and alternative primer combinations were tested.

No report of having similar difficulties with amplification was reported by Patterson *et al.* (2009). However, in an earlier study by Black and Piesman (1994) amplification of the complete 460 bp fragment did not work consistently when using the same primer combination. Instead, the fragment was amplified and sequenced using a total of six primers to produce the final sequences used for study (Black and Piesman

1994). Additional studies should explore different options for the amplification of this larger-sized fragment, which could potentially prove to be more useful for analytic work.

Alignment of the D16S5 and 16S+1 amplified fragments resulted in useable sequences that were 361 nucleotides long, differing by only one site from the expected 360 bp fragment expected. Analysis revealed that five haplotypes exist within the single population of *D. andersoni* ticks studied, indicating that considerable genetic variation does exist and is detectable using this mitochondrial gene fragment isolated with the primers and conditions described by de la Fuente *et al.* (2005). In their study of 67 *D. andersoni* ticks, acquired from a laboratory-raised colony, 14 haplotypes for the 16S rDNA were detected, indicating extensive genetic variation (de la Fuente *et al.* 2005). Patterson *et al.* (2009) tested 114 *D. andersoni* from two separate, natural populations in Canada and identified results that were more similar to this study in that five haplotypes were found. Other studies have also demonstrated that the 16S rDNA gene can successfully be used for studying the genetic differentiation within populations of *Dermacentor* spp. (Black and Piesman, 1994).

In contrast, it has also been suggested that the 16S rDNA gene is limited due to its level of conservation, whether it can offer any indication of variation and if it can provide any insight to the genetic structure of a population; for example, Crosbie *et al*. (1998) found little genetic variation in the mt16S rDNA gene for representative samples from populations of the species *D. hunteri*. Genetic variation can be influenced by a number of factors, such as tick evolution and the range of hosts that are parasitized, and may explain some of the differences in variability found using this gene (Crosbie *et al.*, 1998).

The degree of diversity within this population of ticks is moderate, as evidenced by a *h* value of 0.5423 (±0.0792) and π value of 0.002720 (±0.002091). These values were both slightly lower than those found by Patterson *et al.* (2009), who reported an average *h* value of 0.695 and π value of 0.008 for three separate localities. This could potentially be attributed to the shorter length of aligned sequences used in this study, which at 361bp were 49 nucleotides shorter than Patterson *et al.*'s (2009) 410 bp sequence. Regardless, these values lend further support for the use of this fragment for analytic work in the future as they reveal that genetic variation does exist and is sufficient enough within a single population to warrant further work and comparison to other populations of *D. andersoni*. Due to the nature of this study, no comparisons were made to determine the genetic relationships between different populations of ticks. However, the examination of genetic distance (F_{st}) and gene flow between tick populations across the state of Montana is pertinent and necessary in order to develop the most complete understanding of the genetic diversification and population structure of these vectors. In addition to the analysis performed by de la Fuente *et al.* (2005) described previously, haplotype and nucleotide diversity were also compared between populations that were associated with differing localities. Future work should look to assess different populations of *D. andersoni* and other tick species in similar ways in order to determine if this gene fragment will truly be suitable for intraspecific population comparisons.

Conclusion

The results of this study identified that the novel primer set (16S+1 and D16S5) produces high quality sequences of the mt 16S rDNA gene that are genetically variable and adequate for preliminary studies of the genetic structure of tick populations as part of

the WNV project extension at Carroll College. The methods used to arrive at this conclusion successfully form a basic protocol detailing the most time and cost effective methods of PCR amplification, sequencing and analytic methods that can be utilized by future students working to characterize the genetic diversity in tick populations. Such analysis will be helpful in developing a risk model for tick-related infectious disease in the state of Montana.

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Appendix

Sequences from PCR Product of novel primer combination, de la Fuente *et al.* (2005) conditions

>Site01-01 363bp
TCAGATCAAGTAGGACTTTAAAAGTTGAACAAACTTCTTTTTTTAACATCTTCATTAAAAAAGT
ATCCTAATCCAACATCGAGGTCGCAAACTATTTTGTCTATATGTTCTATCAAAAATTATTACGC
TGTTATCCCTAGAGTATTTTTATCAAATTATCATTAATAATGGATCATTTTATTAAATAAAAAG
TTTATAATCTTTTTTAGTTGCCCCAACCAAAAGATAATAATTTTAATATTAAAATTATTATTTTT
AAAATTCTTAGGGTCTTCTTGTCCTTAATTTAAATAATTGTTTCTTCACAAATTAAAATAAATT
TAATTTTTAAGTTTAAAACAGTTTTTCCCTGAAATTCCATTC

>Site01-02 380bp
TCCGGTCTGAACTCAGATCAAGTAGGACTTTAAAAGTTGAACAAACTTCTTTTTTTAACATCT
TCATTAAAAAAGTATCCTAATCCAACATCGAGGTCGCAAACTATTTTGTCTATATGTTCTATCA
AAAATTATTACGCTGTTATCCCTAGAGTATTTTTATCAAATTATCATTAATAATGGATCATTTT
ATTAAATAAAAAGTTTATAATCTTTTTTAGTTGCCCCAACCAAAAAATAATAATTTTAATATTA
AAATTATTATTTTTAAAATTCTTAGGGTCTTCTTGTCCTTAATTTAAATAATTGTTTCTTCACAA
ATTAAAATAAATTTAATTTTTAAGTTTAAAACAGTTTTTCCCTGAAATTCCATTCTCTTA

>Site01-03 364 bp
TCAGATCAAGTAGGACTTTAAAAGTTGAACAAACTTCTTTTTTTAACATCTTCATTAAAAAAGT
ATCCTAATCCAACATCGAGGTCGCAAACTATTTTGTCTATATGTTCTATCAAAAATTATTACGC
TGTTATCCCTAGAGTATTTTTATCAAATTATCATTAATAATGGATCATTTTATTAAATAAAAAG
TTTATAATCTTTTTTAGTTGCCCCAACCAAAAGATAATAATTTTAATATTAAAATTATTATCTTT
AAAATTCTTAGGGTCTTCTTGTCCTTAATTTAAATAATTGTTTCTTCACAAATTAAAATAAATT
TAATTTTTAAGTTTAAAACAGTTTTTCCCTGAAATTCCATTCT

>Site01-04 364bp
TCAGATCAAGTAGGACTTTAAAAGTTGAACAAACTTCTTTTTTTTACATCTTCATTAAAAAAGTA
TCCTAATCCAACATCGAGGTCGCAAACTATTTTGTCTATATGTTCTATCAAAAATTATTACGCT
GTTATCCCTAGAGTATTTTTATCAAATTATCATTAATAATGGATCATTTTATTAAATAAAAAGT
TTATAATCTTTTTTAGTTGCCCCAACCAAAAAATAATAATTTTAATATTAAAATTATTATTTTT
AAAATTCTTAGGGTCTTCTTGTCCTTAATTTAAATAATTGTTTCTTCACAAATTAAAATAAATT
TAATTTTTAAGTTTAAAACAGTTTTTCCCTGAAATTCCATTCTC

>Site01-05 363bp
CTCAGATCAAGTAGGACTTTAAAAGTTGAACAAACTTCTTTTTTTTAACATCTTCATTAAAAAA
GTATCCTAATCCAACATCGAGGTCGCAAACTATTTTGTCTATATGTTCTATCAAAAATTATTAC
GCTGTTATCCCTAGAGTATTTTTATCAAATTATCATTAATAATGGATCATTTTATTAAATAAAA
AGTTTATAATCTTTTTTAGTTGCCCCAACCAAAAGATAATAATTTTAATATTAAAATTATTATC
TTTAAAATTCTTAGGGTCTTCTTGTCCTTAATTTAAATAATTGTTTCTTCACAAATTAAAATAA
ATTTAATTTTTAAGTTTAAAACAGTTTTTCCCTGAAATTCCATT

>Site01-06 (using only D16S5 primer) 365bp
TCAGATCAAGTAGGACTTTAAAAGTTGAACAAACTTCTTTTTTTAACATCTTCATTAAAAAAGT
ATCCTAATCCAACATCGAGGTCGCAAACTATTTTGTCTATATGTTCTATCAAAAATTATTACGC
TGTTATCCCTAGAGTATTTTTATCAAATTATCATTAATAATGGATCATTTTATTAAATAAAAAG
TTTATAATCTTTTTTAGTTGCCCCAACCAAAAGATAATAATTTTAATATTAAAATTATTATTTTT
AAAATTCTTAGGGTCTTCTTGTCCTTAATTTAAATAATTGTTTCTTCACAAATTAAAATAAATT
TAATTTTTAAGTTTAAAACAGTTTTTCCCTGAAATTCCATTCTC

>Site01-07 363bp
TCAGATCAAGTAGGACTTTAAAAGTTGAACAAACTTCTTTTTTTAACATCTTCATTAAAAAAGT
ATCCTAATCCAACATCGAGGTCGCAAACTATTTTGTCTATATGTTCTATCAAAAATTATTACGC

TGTTATCCCTAGAGTATTTTTATCAAATTATCATTAATAATGGATCATTTTATTAAATAAAAAG
TTTATAATCTTTTTTAGTTGCCCCAACCAAAAGATAATAATTTTAATATTAAAATTATTATCTTT
AAAATTCTTAGGGTCTTCTTGTCCTTAATTTAAATAATTGTTTCTTCACAAATTAAAATAAATT
TAATTTTTAAGTTTAAAACAGTTTTTCCCTGAAATTCCATTC

>Site01-08 363bp
CTCAGATCAAGTAGGACTTTAAAAGTTGAACAAACTTCTTTTTTTTAACATCTTCATTAAAAAA
GTATCCTAATCCAACATCGAGGTCGCAAACTATTTTGTCTATATGTTCTATCAAAAATTATTAC
GCTGTTATCCCTAGAGTATTTTTATCAAATTATCATTAATAATGGATCATTTTATTAAATAAAA
AGTTTATAATCTTTTTTAGTTGCCCCAACCAAAAAATAATAATTTTAATATTAAAATTATTATT
TTTAAAATTCTTAGGGTCTTCTTGTCCTTAATTTAAATAATTGTTTCTTCACAAATTAAAATAA
ATTTAATTTTTAAGTTTAAAACAGTTTTTCCCTGAAATTCCATT

>Site01-09 365bp
CTCAGATCAAGTAGGACTTTAAAAGTTGAACAAACTTCTTTTTTTTACATCTTCATTAAAAAAGT
ATCCTAATCCAACATCGAGGTCGCAAACTATTTTGTCTATATGTTCTATCAAAAATTATTACGC
TGTTATCCCTAGAGTATTTTTATCAAATTATCATTAATAATGGATCATTTTATTAAATAAAAAG
TTTATAATCTTTTTTAGTTGCCCCAACCAAAAGATAATAATTTTAATATTAAAATTATTATCTTT
AAAATTCTTAGGGTCTTCTTGTCCTTAATTTAAATAATTGTTTCTTCACAAATTAAAATAAATT
TAATTTTTAAGTTTAAAACAGTTTTTCCCTGAAATTCCATTCTC

>Site01-10 365bp
CTCAGATCAAGTAGGACTTTAAAAGTTGAACAAACTTCTTTTTTTTACATCTTCATTAAAAAAGT
ATCCTAATCCAACATCGAGGTCGCAAACTATTTTGTCTATATGTTCTATCAAAAATTATTACGC
TGTTATCCCTAGAGTATTTTTATCAAATTATCATTAATAATGGATCATTTTATTAAATAAAAAG
TTTATAATCTTTTTTAGTTGCCCCAACCAAAAGATAATAATTTTAATATTAAAATTATTATCTTT
AAAATTCTTAGGGTCTTCTTGTCCTTAATTTAAATAATTGTTTCTTCACAAATTAAAATAAATT
TAATTTTTAAGTTTAAAACAGTTTTTCCCTGAAATTCCATTCTC

>Site01-11 375bp
CCGGTCTGAACTCAGATCAAGTAGGACTTTAAAAGTTGAACAAACTTCTTTTTTTTAACATCTTC
ATTAAAAAAGTATCCTAATCCAACATCGAGGTCGCAAACTATTTTGTCTATATGTTCTATCAA
AAATTATTACGCTGTTATCCCTAGAGTATTTTTATCAAATTATCATTAATAATGGATCATTTTA
TTAAATAAAAAGTTTATAATCTTTTTTAGTTGCCCCAACCAAAAGATAATAATTTTAATATTAA
AATTATTATCTTTAAAATTCTTAGGGTCTTCTTGTCCTTAATTTAAATAATTGTTTCTTCACAAA
TTAAAATAAATTAATTTTTAAGTTTAAAACAGTTTTTCCCTGAAATTCCATTCTC

>Site01-12 375bp
TCCGGTCTGAACTCAGATCAAGTAGGACTTTAAAAGTTGAACAAACTTCTTTTTTTTAACATCTT
CATTAAAAAAGTATCCTAATCCAACATCGAGGTCGCAAACTATTTTGTCTATATGTTCTATCAA
AAATTATTACGCTGTTATCCCTAGAGTATTTTTATCAAATTATCATTAATAATGGATCATTTTA
TTAAATAAAAAGTTTATAATCTTTTTTAGTTGCCCCAACCAAAAGATAATAATTTTAATATTAA
AATTATTATCTTTAAAATTCTTAGGGTCTTCTTGTCCTTAATTTAAATAATTGTTTCTTCACAAA
TTAAAATAAATTTAATTTTTAAGTTTAAAACAGTTTTTCCCTGAAATTCCATTC

>Site01-13 377bp
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