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Development of a Genetic Marker to Differentiate Between *Pulex irritans* and *Pulex simulans*

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DEVELOPMENT OF A GENETIC MARKER TO DIFFERENTIATE BETWEEN *PULEX IRRITANS* AND *PULEX SIMULANS*

by

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Abstract

Pulex irritans and *Pulex simulans* are zoonotic flea vectors of plague and other infectious diseases. *P. irritans* have historically been known to use carnivores as a host, while *P. simulans* primarily parasitizes omnivores. To fully understand arthropod-borne infectious disease transmissibility and potential for geographical spread, it is important to differentiate between these two flea species. Traditional taxonomy uses the flea's male morphological features to distinguish species. There are no observable morphological differences between the female *P. irritans* and *P. simulans*. Molecular markers of the internal transcribed spacer regions (ITS) have been successfully used in other insect organisms to differentiate species that are difficult to distinguish morphologically. This is due to rapidly diverging repetitive sequences found in the ITS regions. Polymerase chain reaction (PCR) primers were identified for *P. irritans* and *P. simulans* and provide DNA sequence information of the ITS region. With this ITS sequence information, a real time melting curve PCR protocol has been developed. It includes multiple primers for a distinct single nucleotide polymorphisms (SNP) of the ITS region used in melting curve analysis. The development of a real time melting curve PCR identification method of *P. irritans* and *P. simulans* has the potential to aid phylogeographic research and understanding plague transmission in the environment.

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I. Introduction

A. Plague and zoonosis cycles

Originally discovered in 1894 as a bacterial infection, *Yersinia pestis* is more commonly known as the plague, which as the Black Death killed millions worldwide in the 14th century (1). In more recent history, the plague has not yet caused such widespread pandemic. Within the U.S. about 14% of human cases are fatal and the fatality rate is even higher in developing countries (1,2). The majority of human infections (83% from 1957 to 2004) in the U.S. are in the four-corners region (3). The fatalities primarily arise from delayed antibiotic treatment or inadequate treatment (1,2). Global fatalities and the plagues potential for pandemic infection has resulted in extensive research of the *Y. pestis* bacterium. However, limited research has focused on the vector. This may be due to the limited number of cases.

Plague is a zoonotic disease, spread between species, with fleas acting as primary vectors for disease transmission (1,3). Fleas have a wide variety of hosts and are found throughout the world (1). The transmission cycle for *Y. pestis* consists of two cycles: the enzootic and epizootic cycles (1,3,4). The enzootic or maintenance cycle is the transfer of plague from fleas to resistant rodent hosts (1,4). The plague infected rodents act as a reservoir until more susceptible hosts are infected (1,4). The epizootic cycle is characterized by quickly spreading fatalities in new less resistant hosts (1,4). It is during these epizootic cycles that human infection is highest and most widespread (1,2). There is also the possibility for the pneumonic transmission between humans which has the potential to create pandemic outbreaks (1). However, in most cases adequate antibacterial treatment is available before pneumonic transmission is possible (1).

B. *Pulex irritans* and *Pulex simulans*

The fleas *P. irritans* and *P. simulans* have both been found to be capable of becoming infected with *Y. pesti,* and *Bartonella* sp. (7,8). They act as vectors and as reservoirs of infectious diseases (1). Also, as discussed earlier these fleas are part of the transition from enzootic to epizootic plague conditions (1,3,4).

Historically, it has been believed that *P. irritans* and *P. simulans* have different host preferences. It is believed that *P. irritans* host primarily on carnivores such as foxes, coyotes, and dogs. Conversely, it is believed that *P. simulans* host primarily on omnivores such as squirrels, guinea pigs, prairie dogs, and possums. Understanding host preference differences is vital to epidemiological control of enzootic to epizootic spread of infectious diseases. Furthermore, as global climates and habitats change there is greater possibility for even more human-host interactions.

The morphological differences of the two species are minor, which makes classification and differentiation difficult. *P. simulans* was discovered and differentiated second. It closely resembles *P. irritans* and the discoverer Baker (1895) said that *P. simulans* is "distinct, yet it is very closely related to *P. irritans*, and might easily be confused with that species" (10). The significant morphological difference used in differentiation is found only in comparing the male genitalia. *Pulex irritans* have a longer and slender aedeagus and the *P. simulans* is rod-like and small (10). However, as stated the difference is slight and easily overlooked. It is with this plausible error that the species *P. simulans* are easily mistaken for the more common *P. irritans*. This could lead to errors in both phylogeographic data and disease outbreak prevention.

While there are slight morphological differences in the male *P. irritans* and *P. simulans,* there are no observable morphological differences among the female. This presents a problem in determining the species when collected fleas are female.

C. Phylogenetics

The study of phylogenetics is the study of the genetic relationship between species. The importance of understanding the evolutionary history and genetic makeup of fleas can be expressed in three ways. First, they allow for the study of co-evolutionary patterns among the flea and host, as well as the flea and the infectious disease (9). Second, they allow for the study of the adaptation and evolution required to inhabit new regions (9). Third, they aid flea abatement due to better understanding of the geographical distribution of the fleas (9,11). Therefore, the use of correct phylogenetic analysis is important in disease prevention. Phylogenetic research will also aid in many research fields, providing a common base of reference.

D. ITS region as a genetic marker

Genetic markers have been used to differentiate closely related species, when the morphological differences are very minor (12). Such genetic markers have been identified in DNA regions that show evolutionary variability (12). The genetic differences can be used to infer evolutionary history and establish phylogenies (13). The internal transcribed spacer regions (ITS1 and ITS2) found on deoxyribonucleic acid (DNA) of fleas have exhibited qualities for phylogenetic importance (12,13,14). The relatively conserved units of DNA before and after the ITS regions can be used to develop primers for polymerase chain reaction (PCR) (12,14). Isolation of the ITS region provides researchers the ability for species differentiation. This method has been used with other species of fleas such as: *Tunga penetrans, Ctenocephalides felis, Echidnophaga gallinacea, Spilopsyllus cuniculi, Xenopsylla cheopis, Archaeopsylla erinacei,* and *Nosopsyllus fasciatus* (12,14). The ITS regions have been shown to have specific

nucleotide sequences which as a unit repeat within the ITS. Across species the number of repeats and length varies, which provides a genetic marker for differentiation (12).

Gel-electrophoresis of regions such as the ITS can provide useful information regarding the relative size of the PCR amplified region, however, additional specificity is needed. DNA sequence provides the greatest detail of the genetic differences between fleas. However, the use of real time PCR melting curve analysis is a rigorous approach to genetic differentiation (15). It can provide a dichotomous analysis dependent on individual base-pair differences. The technique also has additional benefits as it is less expensive than sequencing and can yield quicker results (15). The method employed in the study utilized differences in single nucleotide polymorphisms (SNPs) found through genetic sequencing.

II. Research Design and Methods

A. Collection of flea samples

Fleas used in this study were collected from two sites; Colorado and Peru. *P. irritans* were collected in the summer of 2009 by a Colorado Division of Wildlife Biologist and transferred to Dr. Bernhardt while a post-doctoral fellow at the Centers for Disease Control and Prevention (Fort Collins, Co). The fleas were collected from multiple coyotes (*Canis latrans*). In the summer of 2011, the *P. simulans* fleas, as well as additional *P. irritans* fleas were collected in Peru by a research scientist in Peru's Ministry of Health. This was done in accordance with local regulations. The fleas were collected from guinea pigs (*Cavia porcellus*), domestic dogs (*Canis lupus familaris*), and bedding. Figure 1 shows the different caging conditions for the guinea pigs in different locations in Peru. All fleas were stored in ethanol.

Figure 1. Living conditions of the guinea pigs. The guinea pigs are raised for food. The guinea pigs in the raised cage (right) hosted fewer fleas than on the ground kept guinea pigs (left).

B. DNA Extraction

The DNA of the flea samples were extracted using an extraction kit (Qiagen DNeasy Extraction Kit) according to kit instructions. However to maintain the identifying morphological

features of the flea specimens, a small incision in the tergite (mid-thoracic region) of each flea was performed (see Figure 2). This was done to access the mid-gut region for potential DNA collection. The cut fleas were placed in a solution of 180 µl PBS, 20 µl Proteinase K, and 200 Buffer AL. The specimens were then incubated for 24 hours at 56° C. After the incubation period 200 µl of ethanol was added and the sample was vortexed. The solution as well as the flea was transferred to a DNeasy spin column and centrifuged at 8,000 rpm for 1 minute. The flow column was discarded and replaced. The flea specimen was collected and stored in ethanol for visualization of the morphological differences. 500 µl AW1 was added and the column was centrifuged at 8,000 rpm for 1 minute. The flow column was discarded and replaced. 500 µl AW2 was added and the column was centrifuged at 14,000 rpm for 2 minutes. The flow through was discarded and the column was centrifuged again at 14,000 rpm for 1 minute. Next, the DNeasy spin column was place in a 1.5 ml micro centrifuge tube. 100 µl Buffer AE was added and the solution was allowed to incubate at room temperature for 1 min. Then the column was centrifuged at 8,000 rpm for 1 minute. The flow through tube which contains DNA was labeled and stored at -80° C.

Figure 2. Image of male *Pulex irritans*. The arrow indicates the incision into the tergite.

C. PCR amplification and Gel Electrophoresis Visualization

The experiment initially tested the effectiveness of PCR primers that were designed and published by Gamerschlag *et al.* (12). In their research, they looked primarily at the differences in the ITS region of the *Tunga penetrans* from different geographic locations (12). They also

compared the ITS region to five other flea species (including *P. irritans*) (12). The ITS1 region was accessed on NCBI Nucleotide Database and can be found under the Accession number: EU169198. The sequences of the six primers (four forward [sen], two reverse [rev]) used can be found in Table 1. The PCR reaction included 9.5 µl Nuclease-free H_2O, 12.5 µl Taq, 1 µl forward primer (20 µM), 1 µl reverse primer (20 µM), and 1 µl specimen DNA. The PCR operating protocol used can be found in Table 2. To visualize the effectiveness of the PCR, gel-electrophoresis was used.

Table 1. PCR primers used in amplification of ITS. From Gamerschlag *et al.* (12)

Primer Name	Primer Sequence	Length
ITS1 sen (1)	GTACACACCGCCCGTGCGTACT	22
ITS1 rev (1)	GCTGCGTTCTTCATCGACCC	20
ITS1 sen (2)	CTTTGTACACACCGCCCGTCGCTAC	25
ITS1 rev (2)	CCAGGTGTGGTCCGGGAACAGTATC	25
ITS1 sen (3)	GTTGCTGGGAAGATGCCCAAACTTG	25
ITS1 sen (4)	CAAGGTTTCCGTAGGTGAACCTGC	24

Table 2. PCR operating protocol used during amplification of the ITS1 region.

	Temperature (Celsius)	Duration (Seconds)
Step 1:	94	300
Step 2:	94	30
Step 3:	57.6	30
Step 4:	72	90
Step 5:	Go to step 2 X 30	
Step 6	72	300
Step 7	4	Indefinite

D. Visualization of Morphological Differences

Both male and female specimens were mounted following the protocol in Table 3. This was performed to lighten the exoskeleton for clear imaging of internal structures of the male genitals as well as to preserve the fleas in mounted form. With an imaging dissection scope, digital images were taken of the all flea specimens. Several images can be seen in Figures 3 and

4. Close up images were also taken of the male genitalia. To determine that the flea samples were in fact *Pulex* genus, the whole body images were compared to a morphological key (16). The male fleas were further differentiated using the same morphological key to the species level; *Pulex irritans* or *Pulex simulans*. The male identification was verified by performing secondary blind confirmation by Dr. Bernhardt.

Table 3. Flea Mounting Protocol.

To Clear Flea	1	750 µl of ddH_2O
	2	3 drops of KOH
To Mount Flea	3	Push on flea to evacuate insides
	4	Place flea in 70% ethanol and let sit for 30 minutes
	5	Place flea in 80% ethanol and let sit for 30 minutes
	6	Place flea in 95% ethanol and let sit for 30 minutes
	7	Place flea in 100% ethanol and let sit for 30 minutes
	8	Place flea in Oil of Wintergreen (methyl salicylate) for 20 minutes
	9	Place flea in Xylene for 60 minutes
	10	Place flea on slide under microscope. Add a drop or two of Canadian Balsam to flea on slide. Cover with slide cover.

Figure 3. Images of Pulex male fleas. Above: *P. simulans* male from Palo Blanco, Peru collected from a guinea pig, (full body right) with enlargement of genitals (left, arrow indicating aedeagus). Below: *P. irritans* male from San Juan de Miraflores, Peru collected from a dog (full body right) with enlargement of genitals (left, arrow indicating aedeagus).

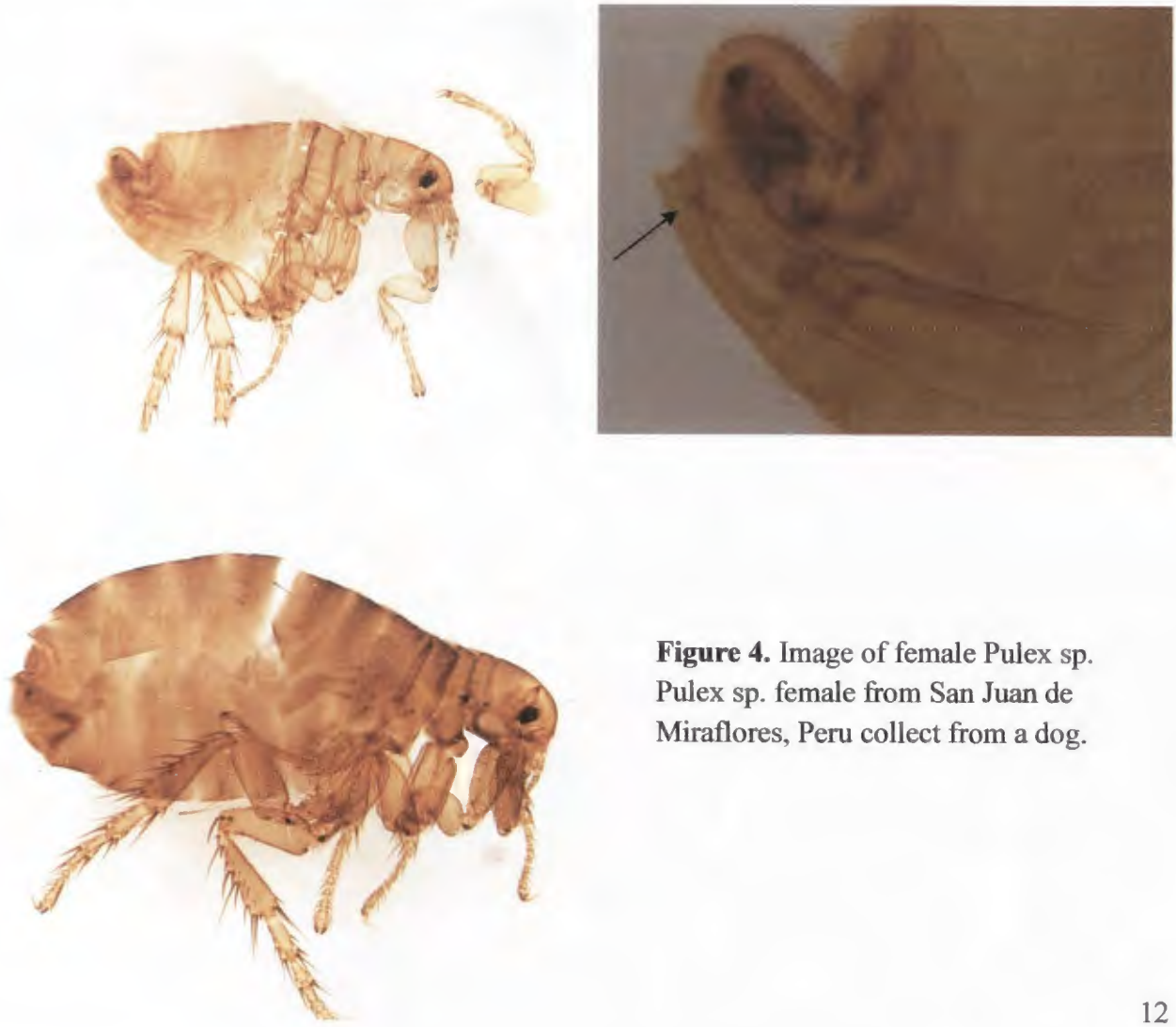

Figure 4. Image of female Pulex sp. Pulex sp. female from San Juan de Miraflores, Peru collect from a dog.

E. Melting curve analysis of SNP regions.

Using the PCR amplified regions of the ITS region, 31 different fleas specimen's DNA was sequenced. Consensus sequences were constructed for each of the 31 fleas and analyzed for SNPs (see Appendix 1). The 18th base pair in the ITS region contained a SNP suitable for melting curve analysis. *P. irritans* from Colorado and Peru contained a cytosine nucleotide. *P. simulans* (only from Peru) contained a thymine nucleotide. Upstream from the SNP the sequence was completely conserved and 19 base pairs downstream provided a conservative region for a reverse primer. Two different forward primers were created (see Table 4) with the final nucleotide differing between a cytosine (C) and thymine (T). The forward primer (Forward A) included 20 additional nucleotides rich in of guanine (G) and cytosine nucleotides. This was done to increase the length of the resulting PCR amplified region relative to the region amplified with Forward B primer. As a result there is additional hydrogen bonding between the DNA of the amplified region. This results in a DNA double-stranded region that requires a greater temperature for the two single strands to disassociate. The differing disassociation temperatures can be measured during the melting curve capture step.

Table 4. Real time PCR primers. The highlighted region shows the conservative regions of the ITS region

Name	Primer Sequence	Length
Forward A	GCGGGCAGGGCGGCGGGGGCGGGGCCTGCGCGGCAGCGTCGTAC	44
Forward B	GCGGGCTGCGCGGCAGCGTCGTAT	24
Reverse	CAAGTTTGGTCATCTTCCC	19

The operating protocol for the real time PCR (see Table 5) included a melting curve capture step. The PCR reaction included 11.25 µl Nuclease-free H_2O, 12.50 µl SSoAdvance™ SYBR® Green Supermix , 0.25 µl forward primer 1 (100 µM), 0.25 µl forward primer 2 (100

μM), 0.25 μl reverse primer (100 μM), and .5 μl specimen DNA. SSoAdvance™ SYBR® Green Supermix contains a chelating agent that binds to the forming double strand during replication. While bound to the double stranded DNA it does not fluoresce. However, when there is sufficient heat to break the hydrogen bonds between the DNA strands the chelating agent is released and fluoresces. The melting curve capture step records the temperature at which the sample begins to fluoresce. The heat was slowly increased (.2° C every 10 seconds) to record the specific temperature in which the DNA strand disassociated. Analysis of melting curve was done using Bio-Rad Precision Melt Analysis™ software.

Table 5. Real time PCR operating protocol.

	Temperature (Celsius)	Duration (Seconds)
Step 1:	95	180
Step 2:	95	10
Step 3:	60	10
Step 4:	72	30
Step 5:	Go to step 2 X 39	
Step 6	Melting Curve: Starting at 65° C increasing .2° C every 10 seconds until 95° C, and plate reading	

III. Results

A. Morphological Identification

The research project included 98 fleas in which DNA was extracted and were mounted. However, 7 male fleas were prepared in such a way as to make morphological identification to the species level impossible, due to the inability to identify the species specific aedeagus. They were excluded from the genetic study. The 91 fleas included 58 females (63.7%) and 33 males (36.3%). Of the 91 fleas, 58 were collected in Peru (69.0%) and 33 were collected in Colorado (31.0%). Within the 58 fleas collected in Peru there were 40 females (70.2%) and 18 males (29.8%). Within the 33 fleas collected in Colorado there were 18 females (54.5%) and 15 males (45.5%). The 33 males included in the genetic study included 12 *P. simulans* (36.4%) and 21 *P. irritans* (63.6%). All 12 *P. simulans* were collected in Peru. All 15 males from Colorado were *P. irritans* and 5 *P. irritans* were collected in Peru.

B. PCR

PCR amplification of the ITS region was successful using primers ITS1 sen (1) and ITS1 rev (1) and an annealing temperature at 57.6° C (see Tables 1 and 2). The ITS region varied in length irrespective of species or collection location (see Figure 5).

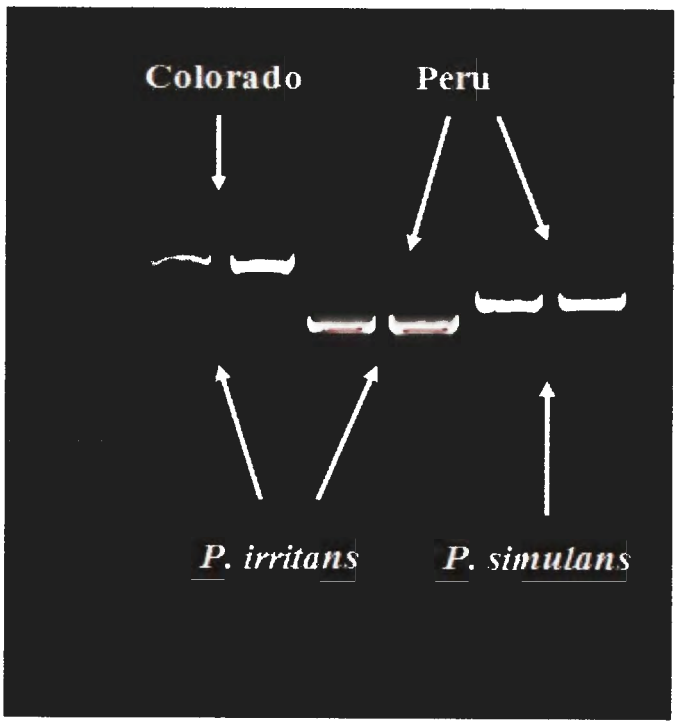

Figure 5. Gel-electrophoresis of PCR from 4 *P. irritans* and 2 *P. simulans*. The variation in the DNA fragment size is independent of species.

C. DNA Sequencing

The isolated ITS region of 31 Pulex male samples were sequenced to identify SNPs for real time PCR melting curve analysis. Three sequences can be viewed in Appendix 1. There were 17 SNPs identified as potential targets for real time PCR melting curve analysis. The DNA sequences also showed variation in the length of the DNA fragment. This was irrespective of species or collection site.

D. Real time PCR melting curve analysis

The real time PCR melting curve analysis of the 18^{th} base pair SNP provided additional information about the genetic structure of the ITS region. The real time PCR primers (see Table 4) provided sufficient amplification for the melting curve capture step (see Figures 6 and 7). The melting curve analysis of the 33 male fleas resulted in the positive grouping of 11 of 12 *P. simulans* and 19 of 21 *P. irritans*. The 3 fleas (2 *P. irritans* and 1 *P. simulans*) cross grouped.

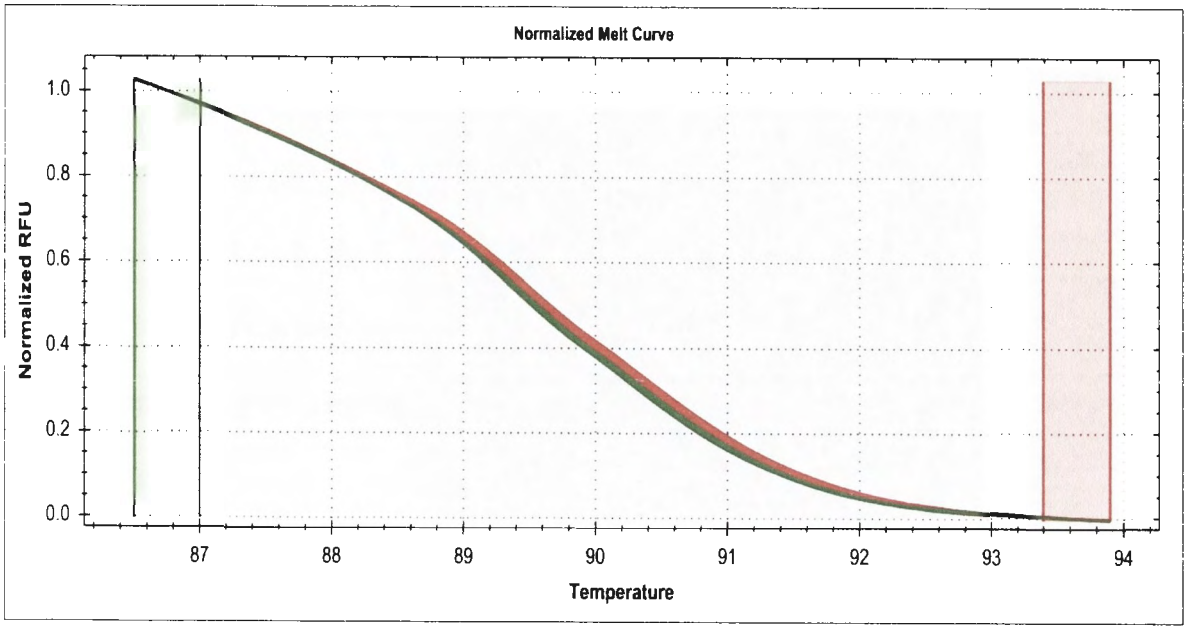

Figure 6. Normalized Melt Curve. The red lines indicate 15 *P. irritans* samples, while the green lines indicate 9 *P. simulans* samples.

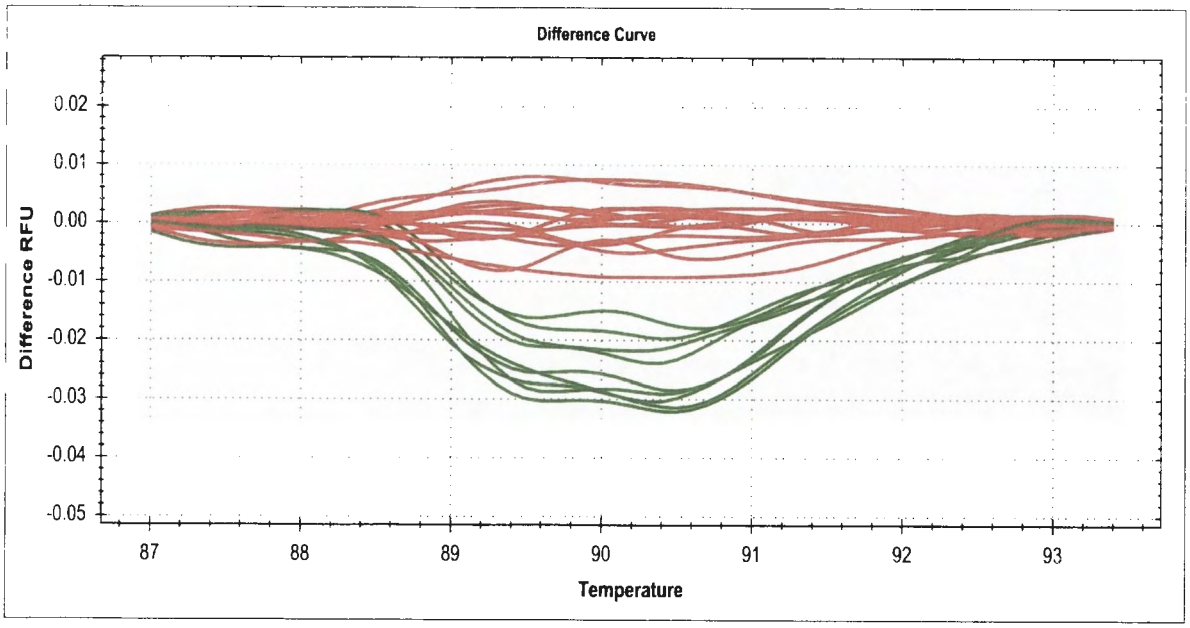

Figure 7. Difference Curve. As in Figure 6 the red lines indicate *P. irritans*, green lines indicate *P. simulans*. The Difference RFU (relative fluorescence units) measures the amount of sample fluorescence relative to others in the study.

IV. Discussion

It was originally thought that there would be a consistent difference between the ITS region of the *P. irritans* and *P. simulans*. However, as the gel-electrophoresis images suggest there was large variations in the ITS fragment size, irrespective of species (see Figure 5). This was also confirmed in the sequence data of the 31 Pulex males. There are large gaps where there is no consensus among the 31 samples, regardless of the species (see Appendix 1 for an example with 3 samples).

This was unexpected and created the need to employ a new and unplanned genetic differentiation technique. The 31 DNA samples that were sequenced show SNPs in regions that are conserved within species (see Appendix 1). The SNP chosen for the study on the 18^{th} base pair provided high reliability (90.9%) in identifying male fleas to the species level.

However, identification was not always correct as 3 of the 33 males in the genetic study cross-grouped. One explanation for this could be that the species specific SNP difference (Thymine for *P. simulans* and Cytosine for *P. irritans*) was not true. This could be the result of the rapid evolutionary nature of the ITS region. It may also be the case that the primer binding affinity is not high enough to bind solely to the correct SNP.

Additional research is needed to provide higher fidelity of the real time PCR melting curve differentiation technique. This may be accomplished by including additional SNP dependent primers in the study to create a multi-SNP identification protocol. It will also be important to include fleas from other geographic sites. This will provide generalizability of the genetic marker for *P. irritans* and *P. simulans*.

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Appendix: CLUSTAL 2.1 multiple sequence alignment of 3 Pulex males. The yellow highlight denotes the 18th nucleotide SNP used in the study. The red highlighted regions denote other potential SNPs for future study. The underlined nucleotides denote the regions targeted by the real time PCR primers.

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P. simulans - Peru      TGCGCGGCAGCGTCGTATGACATTGCCGATGTTGCTGGGAAGATGACCAAACTTGATTAT 60
P. irritans - Colorado  TGCGCGGCAGCGTCGTACGACATTGCCGATGTTGCTGGGAAGATGACCAAACTTGATTAT 60
P. irritans - Peru      TGCGCGGCAGCGTCGTACGACATTGCCGATGTTGCTGGGAAGATGACCAAACTTGATTAT 60
                        ********************* ****************************************

P. simulans - Peru      TTAGAGGAAGTAAAAGTCGTAACAAGGTTTTCGTAGGTGAACCTGCGGAAGGATCATTAA 120
P. irritans - Colorado  TTAGAGGAAGTAAAAGTCGTAACAAGGTTTTCGTAGGTGAACCTGCGGAAGGATCATTAA 120
P. irritans - Peru      TTAGAGGAAGTAAAAGTCGTAACAAGGTTTTCGTAGGTGAACCTGCGGAAGGATCATTAT 120
                        ************************************************************

P. simulans - Peru      CGTGTTACATTT----TGCAATAAATGTAAATAAAACGTTTGCGGACGTTGTCTGAAAAC 176
P. irritans - Colorado  CGTGTTACATTT----TGCAATAAATGTAAATGAAACGTGTGCGGACGTTGTCTGACAAC 176
P. irritans - Peru      CGTA--ACATTTACATCGTAAATAATGTAAATTTAACGTGCATAGACGTTGTCT---TAC 175
                        ***   ******     * **  *********  *****     **********    **

P. simulans - Peru      GACGACGTCGATAAATTTTAAGCGGCGCGCGTCGATGGGCGGCCTATCTCATCGATAGCC 236
P. irritans - Colorado  GACGACGTCGATAAATTTTAAGCGTCGCGCGGCGATGGACGGCCTATCTCATCGATAGCC 236
P. irritans - Peru      GACGACGTCGATAAATCTTCAGCGACGCGCGTCGATGGACGGCCTATCTCATCGATAGCC 235
                        *************** ** **** ****** ****** ********************

P. simulans - Peru      GACCGGAGTGCGTCGCTACAAACCGAGCTCGACAGGGCGCATCGCCGTGTTCTTTCGCGT 296
P. irritans - Colorado  GACCGGAGTGCGTCGCTACAAACAGAGCTCGACAGGGCACATCGCCGTTTTCTTTCGCGT 296
P. irritans - Peru      GACCGGAGTGCGTCGCTACAAACAGAGCTCGACAGGGCGCCTCGCCGTTTTCTTTCGCGT 295
                        ********************** ************** * ******* ***********

P. simulans - Peru      CTCCGTACGACGATCGATCTACGACGATAGAGCGCCCGTCGCT----------------- 339
P. irritans - Colorado  CTCCGTACGACGATCGATCTACGACGATAAAGTGCCCGTCGCGAACAGGTTACCTCGACA 356
P. irritans - Peru      CTCCGTACGACGATCGATCTACGACG--AGAGTGCCCGTCGC------------------- 335
                        ************************  * ** *********

P. simulans - Peru      ------------------------------------CGATCAATTCAGCGATGATTGAGT 363
P. irritans - Colorado  GGGCGCATCGCCGTTTTCTTTCGCGTCTCCGGACGACGATCGATCTA-CGACGATAAAGT 415
P. irritans - Peru      ------------------------------------------------------------

P. simulans - Peru      --------TCGATG------CTCGACAGGGCGCATCGCCGTGTTCTTTCGCGTCTCCGTA 409
P. irritans - Colorado  GCCCGTCGCCAACGGGTTACCTCGACAGGGCGCATCGCCGTTTTCTTTCGCGTCTCCGTA 475
P. irritans - Peru      ----------------------TCGAC----------------------CGTGTTACCCTA 352
                                               *****                    ** **  ** **

P. simulans - Peru      CGACGATCGATCTACGACGATATAGCGCCCGTCGC------------TCGAC-------- 449
P. irritans - Colorado  CGACGATCGATCTACGACGATAAAGTGCCCGTCGCGAACAGGTTACCTCGACAGGGCGCA 535
P. irritans - Peru      CG---------------------------------------------------------- 354
                        **

P. simulans - Peru      ---------------------------------CAATTTA-------------------- 456
P. irritans - Colorado  TCGCCGTTTTCTTTCGCGTCTCCGGACGACGATCGATCTACGACGATAAAGTGCCCGTCG 595
P. irritans - Peru      ------------------------------------------------------------

P. simulans - Peru      ------------------------------------------------------------
P. irritans - Colorado  CCAACGGGTTACCTCGACAGGGCGCATCGCCGTTTTCTTTCGCGTCTCCGGACGACGATC 655
P. irritans - Peru      ------------------------------------------------------------

P. simulans - Peru      -----GCGATGATTTAGT--------TCGATG------CTCGACAGGGCGCATCGCCGTG 497
P. irritans - Colorado  GATCTACGACGATATAGTGCCCGTCGCSAACGGGTTACCTCGACAGGGCGCATCGCCGTT 715
P. irritans - Peru      ----------------------------------------GGG------------ 357
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P. simulans - Peru TTCTTTCGCGTCTCCGTACGACGATCGATCTACGACGATAGAGCGCCCGTCGCTCGATC- 556
P. irritans - Colorado TTCTTTCGCGTCTCCGGACGACGATCGATCTACGACGATATAGTGCCCGTCGCTCGACCG 775
P. irritans - Peru ----TTCAC---------- 362
P. simulans - Peru ----------AATTTAGCGATGATTGAGTTCGATGCGACGATTAG 591
P. irritans - Colorado TGTTAACCCACGGGGTTCAAAACACAATTTAGCGATGATTGAGTTCGATGCGACGATTAG 835
P. irritans - Peru ----------AATTTGGCGATGCTGGAGTTCGATGCGACGATTAG 397
P. simulans - Peru GTCTCGACCGCGGCGCTCGATACTCTGTGTGTGAGACATCTGCTATATAAAATACCTGCT 651
P. irritans - Colorado GTCTCGACCGCGGCGCTCGATACTCTGTGTGTGAGACATCTGCTATATAAAATACCTGCT 895
P. irritans - Peru GTCTCGACCGCGGCGCTCGATACTCTGTGTGTGAGGCATCTGCTATATAAAATACCTGCT 457
P. simulans - Peru CCGTATCATCAGATGCGTGAGCGAATTTCGAAGGCATCGCTCGTTGGCG--TCCTTTTTA 709
P. irritans - Colorado CCGTATCATCCGATGCGTGAGCGAATTTTGAAGGCATCGCTTGATGGCG--TCCTTTTTA 953
P. irritans - Peru CCGTATCATCCGATGCGTGAGCGAATATTAAAGGCATCGCTCGACAGCGCGTCCTTTTTA 517
P. simulans - Peru AATCACTGATGAATGCTGTGTTTGAATCGAATTCGTCGTTC-GTCGACGATTCTTTTCAT 768
P. irritans - Colorado A--CACTGATGAATGCGGTGTTTGAATCGAATTCGTCGTTCCATCGACGATTCTTTTCAT 1011
P. irritans - Peru AATCACTGATGAATGCGGTGCTTGAATCGAATTCGTCGTTCCATCGACAATTCTTTTCAT 577
P. simulans - Peru GTTAATCGCACTCGCATTTCAGTCAAGCTGCTCGATCATGCACTTCGTGCGATCGTGCGA 828
P. irritans - Colorado GTTAATCGCACTCGCATTCCAGTCA----GCTCGATCACGCACTTCGTGCGATCGTGCGA 1067
P. irritans - Peru GATAATCGCTCTCGCATTCCAGTCG----GCTCGATCACGCACTTCGTGCGATCGTGCGA 633
P. simulans - Peru ATGACGGGCGTTCGCGTAACTGCGTCGCTCTAGATTACGGAATATTGCGGCAAGACGACA 888
P. irritans - Colorado ATGACGGGCGCTCGCGTAACTGCGTCGCTCTAGATTACGGAATATTGCGCCAAGACGACA 1127
P. irritans - Peru ATGACGGGCGCTCGCTTAACGGCGTCGCTCTAGATTACGGAATATTGCGCCGAGACGACA 693
P. simulans - Peru GTTCATTGGAAAGTTGTCGAATCGCATTTTCCGCTATCACACAAAATCAATACCGTTTTG 948
P. irritans - Colorado GTTCATTGGAAAGTTGTCGAATCGCATTTTCCACTATCACACAAAATCAATACCGTTTTG 1187
P. irritans - Peru GTTCATTGGAAAGTTGTCGAATCGCATTTTCCACTATCACACAAAATCAATACCGTTTTG 753
P. simulans - Peru ATAAAAACCGAAAGCGTAAAGCTCGAGGTGTACGAATTGTAACTTGAAACATATGCCAAT 1008
P. irritans - Colorado ATAAATACCGAAAGCGTAAAGCTCGAGGTGTACGAATTGTAACTTGAAACATATACCAAT 1247
P. irritans - Peru ATAAAGACCGAAAGCGTAAAGCTCGAGGTGTACGAATTGTAACTTAAAACATATACCAAT 813
P. simulans - Peru TTTCGATAA-CGACTCCCCATCGGTGACGTTGGCGTGCAGTCGATCGAAAGCCGGTAAA 1066
P. irritans - Colorado TTTCGATAAACGAC-CCCCATCGGTGACGTTGGCGTGCAGTCGATCGAAAGCCG----- 1300
P. irritans - Peru TTTCGATAA-CGAC-CCCCATC---------- 833

Author's Biography

Colby Kearl was born in Brigham City, Utah and attended Box Elder High School where he was Valedictorian. He started at Utah State University in the fall semester of 2007. He is engaged in both the Bachelor of Arts degrees of Biology and Economics from the College of Science and Jon M. Huntsman School of Business respectively. He is also pursuing minor certificates in Chemistry and Mathematics and anticipates completing his studies in the spring of 2013. Following completion of his studies at Utah State University, he intends to attend medical school and specialize in a pediatric sub-category.