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Species Diversity and Host Specificity in Flea Communities from the Two Subspecies of the Gunnison’s Prairie Dog (*Cynomys gunnisoni*)

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Evolutionary Biology and Ecology (EBIO)

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ABSTRACT

In 2008, Gunnison’s prairie dog (*Cynomys gunnisoni*) (GUPD), became a candidate species for listing under the Endangered Species Act (ESA) within the montane (2,300 to 3660m) region of its range due to a higher prevalence of plague and unstable population structures. The geographic division of the GUPD range into the montane and prairie regions also serves to divide the GUPD into two historically defined subspecies: the Gunnison (*Cynomys gunnisoni gunnisoni*) in the montane region and the Zuni (*Cynomys gunnisoni zuniensis*) in the prairie region. To help evaluate why there is a difference in the prevalence of plague, it is important to look at the fleas that serve as vectors of plague. The fleas that persist on GUPD serve as vectors for pathogens, including *Yersinia pestis* (the causative agent of plague) and *Bartonella* spp., among individual prairie dogs and colonies. Analysis of the sequence of a key mitochondrial gene, cytochrome oxidase subunit II (COII), for 63 sampled fleas was used to characterize the number and type of flea species, or flea diversity, occurring on GUPD and whether these fleas tend to specialize on a particular GUPD subspecies. The level to which a parasite, such as a flea, specializes on a host is called host specificity. This evaluation of species diversity indicates the occurrence of a new subspecies of *Oropsylla hirsuta*, three genetically undescribed *Oropsylla* species, and one undescribed *Pulex* species that all occur on GUPD. The evaluation comparing the frequency of the flea species occurring on each GUPD subspecies showed that there is not significant differentiation of fleas species between the two subspecies. This suggests that fleas do not discriminate between the two subspecies. This lack of host specificity may have important implications for the spread of plague between subspecies. An Analysis of
Molecular Variance (AMOVA) revealed that most of the genetic variation in the COII sequences exists among fleas occurs within a colony, and that there is also significant variance among colonies. Thus, there appears to be no genetic break in the fleas that corresponds to the subspecies distinction in GUPD. The colonies differed greatly in the amount of genetic diversity that was estimated to be present and it was found that the number of prairie dog hosts sampled from each colony was the best explanation of the genetic diversity observed. The more prairie dog host sampled from a colony, the more genetic diversity was measured in that colony. The new undescribed flea species and the lack of host specificity of fleas on the two species are important in understanding the apparent differences in susceptibility to plague between the two GUPD subspecies, and the movement of fleas and the pathogenic bacteria between the two subspecies. Subsequent studies that evaluate the presence of pathogenic bacteria in the fleas sampled need to be completed to know the full implications of these results and how they may inform conservation efforts.

INTRODUCTION

Prairie dogs (Cynomys spp.) are an integral part in the functioning North American grassland ecosystem, altering the composition of grasses around burrows and providing an important food source for many other animals, and are thus called keystone species (Davidson and Lightfoot 2008, Garrett and Franklin 1988). In 2008, one of the prairie dog species, Gunnison’s prairie dogs (Cynomys gunnisoni) (GUPD), became a candidate species for listing under the Endangered Species Act (ESA) within a specific section of its range, this decision is still under advisement (USFWS 2008). Currently, the
range of GUPD is divided into two regions called “montane” (2,300 to 3,660m) and “prairie” (1,830 to 2,130m), and GUPD would only be protected within its montane range if listed under the ESA (USFWS 2008). GUPD in the montane range are thought to be in greater danger of significant population declines and extirpation due to a higher prevalence of plague and unstable population structures that have difficulty recovering after an outbreak of plague. The division of the GUPD range into the montane and prairie regions also serves to divide the GUPD into two historically defined subspecies, the Gunnison (C. g. gunnisoni) in the montane range and the Zuni (C. g. zuniensis) in the prairie range (Hollister 1916). The range of the Gunnison subspecies is included in the montane region: the Rocky Mountain region of central and south-central Colorado and northern New Mexico (Hollister 1916). The range of the Zuni subspecies is included in the prairie region: southwestern Utah, northwestern Colorado to the San Francisco Mountain Range, and northeastern Arizona in the Hualapai Indian Reservation (Hollister 1916). These subspecies may have arisen as a result of geographic isolation that prevented migration and gene flow allowing each isolated population to evolve separately.

Many factors threaten the survival of GUPD, including urban expansion and the occurrence of diseases caused by bacteria spread by fleas that live on the prairie dogs (Travis et al. 1997, USFWS 2008). GUPD plays host to a variety of flea species that include Oropsylla hirsuta, Neopsylla inopina, Pulex spp., Oropsylla tuberculatus cynomuris, and Oropsylla labis (Lechleitner et al. 1968, Cully et al. 1997, Cully and Williams 2001, Lewis 2002). These flea species harbor and vector pathogenic bacteria, including Bartonella spp. and Yersinia pestis (De la Cruz and Whiting 2003, Gage and
Several strains of *Bartonella* have been implicated in human diseases, such as cat-scratch fever, and can cause severe disease and death in prairie dogs (Chomel et al. 1996, Higgins et al. 1996, Reeves et al. 2007). *Yersinia pestis* is the causative agent of plague and is often spread between individual prairie dogs by the sharing of fleas within a colony (Perry and Fetherston 1997, Gage and Kosoy 2005). Plague infection in humans is usually acquired through bites from fleas on wild rodents including prairie dogs (Gage and Kosoy 2005).

Plague is extremely deadly to prairie dogs, exhibiting a mortality rate of near 99%, causing extensive colony die-offs, and hindering conservation efforts (Lechleitner et al. 1968, Cully et al. 1997, Cully and Williams 2001, Gage and Kosoy 2005). Particular species of fleas are known to have different competency for vectoring plague and other pathogens, and also have varying degrees of host specificity or the extent to which they specialize on a particular host (Wilder et al. 2008, Jones and Britten 2010). Therefore, it is important to assess what flea species occur on GUPD and whether the two subspecies of Gunnison’s prairie dog, which appear to have different susceptibility to plague, play host to different species of fleas or the same species. In this study, we examined the extent to which the two recognized subspecies of GUPD (*C. g. gunnisoni* and *C. g. zuniensis*) harbor distinct species of fleas that can spread pathogens.

Furthermore, we asked whether there is a genetic break in the fleas that corresponds to the subspecies distinction between the host prairie dogs. Alternatively, fleas may move between prairie dogs without regard to their subspecies status. The particular flea species present on the GUPD subspecies and the level of host specificity are important in understanding the apparent differences in susceptibility to plague between the two GUPD
subspecies and the movement of fleas and the pathogenic bacteria between the two
subspecies. Subsequent studies that evaluate the presence of pathogenic bacteria in the
fleas sampled need to be completed to know the full implications of these results and how
they may inform conservation efforts.

MATERIALS AND METHODS

Sample Collection

Twenty-five prairie dog colonies were sampled in Arizona, New Mexico, and
Utah between 10 May and 30 October 2010 (Figure 1). Prairie dogs were trapped using
16 x 5 x 5 inch Tomahawk live-traps (Tomahawk Live Trap, Inc., Tomahawk, WI, USA)
set throughout the colonies. Captured prairie dogs were sedated using the inhaled
anesthetic isoflurane administrated using oxygen delivery through a plastic cone placed
over the animal’s nose. Once the animal was anesthetized, blood, tissue, and flea samples
were taken. The animal was brushed with a fine-toothed comb, and any visible fleas,
either on the animal’s body or on the tabletop, were collected using forceps. The fleas for
each individual prairie dog were placed in a labeled cryovial filled with a 1% saline
solution and “Tween” (a surfactant, polysorbate 80). The flea samples were labeled with
the three-to-four letter abbreviation for the particular colony and the number for the
individual prairie dog. The samples were then stored at -80°C.

DNA Extractions
DNA extractions were obtained from 96 individual fleas (43 from each subspecies) across the range of each subspecies. The chosen samples were spread out as evenly as possible between the colonies for each subspecies and when possible, no more than one flea was used from each prairie dog. This was not possible for four colonies where only one or two prairie dogs had been sampled for fleas. When this occurred, multiple fleas were used from one prairie dog. The DNA from the fleas was extracted individually using a Qiagen DNeasy® Tissue kit (Qiagen Inc., Valencia, CA, USA) following the recommended protocol for animal tissue. Extracted samples were then stored at -80°C.

**COII Gene Amplification**

Polymerase chain reaction (PCR) was used to amplify the cytochrome oxidase subunit II (COII) gene using the forward primer tLeu (5’-ATGGCAGATTAGTGCAATGG-3’) and the reverse primer TLys (5’-GTTTAAGAGACCAGTACTTG-3’). A GoTaq® Flexi kit was used (Promega Corporation, Madison, WI, USA). 1µl of the extracted DNA from each flea was combined with 10µl of nuclease-free water (NFW), 4µl of 5X Colorless GoTaq® Flexi buffer, 2µl of magnesium chloride (MgCl₂) solution at 25mM, 2µl of deoxynucleosides (dNTPs) at a concentration of 2.5 mM for each nucleotide from Invitrogen (Invitrogen Corporation, Carisbad, CA, USA), 1µl of the forward primer tLeu and 1µl of the reverse primer TLys both at a concentration of 10 µM from Eurofins MWG Operon (Eurofins MWG Operon, Huntsville, AL, USA), and 0.1µl GoTaq® DNA polymerase. The samples were then placed in a GeneAmp® PCR System 9700 Thermocycler (Applied
The samples were first heated to 95°C for one minute to activate the hot-start DNA polymerase. Then the samples were put through a denaturing, annealing, and elongating cycle of 30 seconds at 94°C, 30 seconds at 52°C, and 1 minute at 72°C 35 times. To finish, the samples were kept at 72°C for five minutes and then at 8°C until they were removed from the thermocycler. A ~1% agarose gel was used to evaluate the presence or absence of the crude PCR product. The crude PCR products were stored at -80°C until they were sent to Functional Biosciences (Functional Biosciences, Inc., Madison, WI, USA) for sequencing. Only 66 of the 96 samples were successfully amplified and sequenced due to time constrains.

Alignment of Sequences

The flea COII sequences were edited and aligned using Sequencher ver. 4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). For the first alignment, the parameters were set on dirty data, minimum match percentage at 60%, and minimum overlap at 10. These settings allowed the forward and reverse sequences of 66 of the samples to align easily. One set of sequences did not align and were not included in the analysis. The aligned sequences were edited by hand such that there was a consensus between the forward and reverse sequence. All consensus sequences were then aligned.

BLAST Sequences

Because the species of the fleas in the samples were not determined morphologically before DNA extraction, the COII sequences were used instead. The sequences were entered into the Basic Local Alignment Search Tool (BLAST) on the
National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) in order to determine what possible species of fleas were in the sample. BLAST compares nucleotide sequences to sequence databases to find regions of local similarity between sequences and calculate the statistical significance of the similarity. Based on the BLAST results, COII sequences from the flea species *Oropsylla hirsuta*, *O. tuberculata*, and *Pulex irritans*, found on black-tailed prairie dogs (*Cynomys ludovicianus*) (BTPD), were taken from GeneBank and added as reference sequences. GeneBank® is the National Institutes of Health (NIH) genetic sequence database that contains all available DNA sequences and is also found on the NCBI website (www.ncbi.nlm.nih.gov/genbank/).

**Tree Building and Statistical Analysis**

The tree building software Molecular Evolutionary Genetic Analysis (MEGA) 4 was used to construct a maximum likelihood (see below) tree based on the COII sequence data (Tamura et al. 2007). Maximum likelihood is a tree-building method that estimates the amount of genetic change that has occurred according to an evolutionary model of nucleotide substitutions and then constructs the tree that follows the most likely evolutionary outcome. This phylogeny illustrates how individual fleas are related to each other, and the branch lengths provide a measurement of the amount of genetic change between an ancestral form and an individual flea based on the COII sequences. The flea species present in the sample were identified using the groupings of the sequences in the tree and the branch lengths of each group, a technique called DNA barcoding. A Fisher’s exact test was used to assess the extent to which the particular flea species occurring on a
host are dependent on the GUPD subspecies being sampled. It was preformed using QuickCalcs to analyze a 2x2 contingency table (Motulsky 2007).

An Analysis of Molecular Variance (AMOVA) analysis was performed using Arlequin ver. 3.5 (Excoffier and Lischer 2010) to evaluate the amount of genetic variation among fleas from the two subspecies of GUPD, among the samples for all colonies, and among the individual fleas from one colony. Also, a linear regression was preformed using the theta(s) values (see below) produced by the AMOVA analysis to evaluate which variable, the number of hosts who had their fleas sampled per colony or the number of fleas sampled per host per colony, was the best explanation for the observed genetic variation within a colony. Theta(s) is an estimate of diversity of the COII sequences from each colony. The linear regression analysis was preformed using the computer program R (R Development Core Team 2008).

RESULTS

COII sequence data from 64 fleas were collected from samples taken from GUPD colonies in Utah, Arizona, and New Mexico (30 of the samples failed to yield good sequence data). See Figure 1 for the geographic location of the colonies sampled. All sequences were BLASTed to determine what flea species were possibly present in the sample. All but one sequence was matched with sequences from two known flea species, O. hirsuta and P. irritans, that had been collected from BTPD colonies. The one remaining sequence, DUTS 15 F1, was matched with a sequence from Barypeithes albinae, a species of beetle. This sequence was not included in the tree analysis.
Figure 1. The geographic locations of the sampled Gunnison’s prairie dog (*Cynomys gunnisoni*) colonies in Utah, Arizona, and New Mexico. Each red dot represents a colony and the label specifies what subspecies is located there. White labels indicate that the individuals are part of the Gunnison (*Cynomys gunnisoni gunnisoni*) subspecies and yellow labels mark Zuni (*Cynomys gunnisoni zuniensis*) subspecies colonies.
A maximum likelihood tree was constructed using 54 sampled COII sequences and six reference sequences obtained from GeneBank. This tree is pictured in Figure 2, with the sequences labeled according to the reference sequence they aligned with and the individual sequence names removed. Most of the sample sequences are in the *Oropsylla* spp. with 44 of the sample sequences clustering with the reference *O. hirsuta* sequence. None of the sample sequences aligned with the *O. tuberculata* reference sequence. The remaining three sample sequences are from the *Pulex* spp. and aligned with the *P. irritans* reference sequence. The number of flea sequences in each species for each GUPD subspecies is summarized in Table 1. The branch lengths present in the tree range from 0.095 to 0.174, indicating a 9.5 to 17.4% genetic difference between the sequences and the ancestral form. Figure 3 focuses on the majority of the sampled COII sequences that clustered with the *O. hirsuta* reference sequence and are in the *Oropsylla* spp. The individual sequences names are included and those sequences from the Zuni subspecies are highlighted in blue and the sequences belonging to the Gunnison subspecies are labeled in black. The sequences from the two subspecies are intermixed within the tree.

To conduct the Fisher’s exact test, the flea sequences were divided into two groups: one with the sequences from *O. hirsuta* and one with all the sequences from non-*O. hirsuta* fleas. Figure 4 shows a graph of the number of flea sequences present in each group for each GUPD subspecies. For the Gunnison subspecies, there were 31 flea sequences in the *O. hirsuta* group and nine in the non-*O. hirsuta* group. While, for the Zuni subspecies, there were 13 flea sequences in the *O. hirsuta* group and one in the non-*O. hirsuta* group. The Gunnison subspecies appears to harbor a greater diversity of flea species. The results of the test were not significant (p = 0.26).
Figure 2. Maximum likelihood tree of the 64 sampled flea COII sequences and 6 reference COII sequences obtain from GeneBank. Individual sequence labels have been removed and, instead, the sequences are grouped together by species.
Table 1. The number of flea sequences in each species, as determined from the maximum likelihood tree, for each GUPD subspecies.

<table>
<thead>
<tr>
<th>Flea species</th>
<th># on Gunnisoni</th>
<th># on Zuniensis</th>
<th>Total # in sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oropsylla hirsuta</td>
<td>31</td>
<td>13</td>
<td>44</td>
</tr>
<tr>
<td>O. n. sp. 1</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>O. n. sp. 2</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>O. n. sp. 3</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Pulex n. sp. 1</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>P. irritans</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 3. This maximum likelihood tree contains all the Oropsylla hirsuta sequences, both sampled and reference. The reference sequence is labeled Oropsyllahir. Sequences from the Gunnison subspecies (Cynomys gunnisoni gunnisoni) are colored black and the sequences from the Zuni subspecies (Cynomys gunnisoni zuniensis) are highlighted in blue.
Figure 4. This graph shows the number of fleas COII sequences present in the two groups, *O. hirsuta* in purple and non-*O. hirsuta* in blue, separated by GUPD subspecies. These groups were used in a Fisher's exact test to determine to what extent the flea species found on a prairie dog are dependent on the subspecies of the GUPD being sampled. The results were not significant (*p* = 0.26).

An AMOVA analysis was conducted to evaluate the genetic variation of the sequences between three different groupings of the sampled COII sequences. The results are summarized in Table 2. The percentage of variation values provide an estimate of the proportion of the variance observed within the COII sequences that can be explained by dividing the sequences by subspecies and then by colony. The first test evaluated the variation between the two subspecies. Between the 46 sequences from the Gunnison subspecies and the 19 sequences sampled from the Zuni subspecies, the percentage of variation was 6.29%; this level of genetic divergence was not significant. The second test evaluated the variation between the 15 colonies of both subspecies, nine colonies of the Gunnison subspecies and six colonies of the Zuni subspecies. The observed percentage of
variation among the colonies was 25.18% (p < 0.01). The last test evaluated the variation among flea sequences from a particular colony. Table 3 contains the number of fleas sampled from each colony and the number of prairie dog hosts they were sampled from.

The percentage of variation evaluated among populations of fleas was 68.53%. Thus, most of the variation in flea DNA occurs within colonies, but there is significant variation between colonies.

Table 2. The percentage of genetic variation values obtained from the AMOVA analysis of 64 COII sequences from fleas collected from the two GUPD subspecies. The AMOVA revealed that the percentage of variation among subspecies was 6.29% and among colonies was 25.18%.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Degrees of Freedom (df)</th>
<th>Percentage of variation</th>
<th>Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among subspecies</td>
<td>1</td>
<td>6.29</td>
<td>No</td>
</tr>
<tr>
<td>Among colonies</td>
<td>13</td>
<td>25.18</td>
<td>Yes</td>
</tr>
<tr>
<td>Within colonies</td>
<td>47</td>
<td>68.53</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 3. The number of fleas sampled and the estimate of diversity (theta(s)) of the COII sequences from each colony.

<table>
<thead>
<tr>
<th>Colony</th>
<th># of Fleas</th>
<th># of Prairie Dogs</th>
<th>Theta (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gunnison</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VADO</td>
<td>1</td>
<td>1</td>
<td>0.00</td>
</tr>
<tr>
<td>TPRR</td>
<td>5</td>
<td>1</td>
<td>8.64</td>
</tr>
<tr>
<td>ENSP</td>
<td>6</td>
<td>6</td>
<td>34.16</td>
</tr>
<tr>
<td>FUEN</td>
<td>6</td>
<td>5</td>
<td>1.44</td>
</tr>
<tr>
<td>VNCP</td>
<td>6</td>
<td>6</td>
<td>33.28</td>
</tr>
<tr>
<td>BLFB</td>
<td>6</td>
<td>6</td>
<td>37.44</td>
</tr>
<tr>
<td>SYMS</td>
<td>7</td>
<td>7</td>
<td>106.94</td>
</tr>
<tr>
<td>CBAR</td>
<td>6</td>
<td>6</td>
<td>5.26</td>
</tr>
<tr>
<td>BBM</td>
<td>3</td>
<td>3</td>
<td>0.00</td>
</tr>
<tr>
<td>Total</td>
<td>46</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td><strong>Zuni</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGP</td>
<td>3</td>
<td>1</td>
<td>4.67</td>
</tr>
<tr>
<td>SSLM</td>
<td>4</td>
<td>4</td>
<td>69.82</td>
</tr>
<tr>
<td>ELMA</td>
<td>3</td>
<td>3</td>
<td>7.33</td>
</tr>
<tr>
<td>RSF</td>
<td>3</td>
<td>1</td>
<td>0.00</td>
</tr>
<tr>
<td>DUTS</td>
<td>3</td>
<td>3</td>
<td>2.00</td>
</tr>
<tr>
<td>WSCM</td>
<td>3</td>
<td>3</td>
<td>0.67</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>
The AMOVA analysis also provided an estimate of molecular diversity for each colony called theta(s), which is summarized in Table 3. The theta(s) values ranged from zero to 106.94. Thus, colonies differed in the amount of diversity, with some colonies lacking variation and other harboring multiple distinct lineages. For further analysis, a linear regression was done on these theta(s) values to determine if the number of hosts sampled per colony or the number of fleas sampled per host per colony was a better explanation for the variation seen in these theta(s) values. The number of hosts sampled per colony and the number of fleas sampled per colony is summarized in Table 3. Analysis of the linear models revealed that the best explanation for the difference in genetic diversity of the sampled fleas was the number of prairie dog hosts sampled per colony. The results of the linear regression are given in Table 4. Figure 5 shows a graph of the linear models of the number of hosts sampled per colony in red and the number of fleas sampled per host per colony in blue. There is a positive trend between the number of hosts sampled and the observed flea diversity, while there is a negative trend in diversity the more fleas are sampled per host.

Table 4. Summary of linear models for explaining differences in genetic variation within a colony (theta(s)). Model is defined by the variables, # hosts and # fleas/host and are also the regression coefficients, p is the significance, and AIC is the Akaike Information Criterion, which is a goodness of fit measure. The best model is indicated in bold.

<table>
<thead>
<tr>
<th>Model</th>
<th># Hosts</th>
<th># Fleas/Host</th>
<th>p</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hosts only</td>
<td>8.87</td>
<td></td>
<td>0.016</td>
<td>143.83</td>
</tr>
<tr>
<td>Fleas only</td>
<td></td>
<td>-6.45</td>
<td>0.381</td>
<td>149.90</td>
</tr>
<tr>
<td>Host + Fleas</td>
<td>10.63</td>
<td>5.29</td>
<td>0.048</td>
<td>145.21</td>
</tr>
</tbody>
</table>
Figure 5. This graph shows the linear models for explaining differences in flea diversity between GUPD colonies. The blue line represents the model defined by the variable the number of fleas sampled per host per colony and, with increased flea sampling per host, there is a negative correlation with observed flea diversity. The red line represents the model defined by the variable the number of hosts sampled per colony and, with increasing host sampling, there is a positive correlation with the observed flea diversity.

DISCUSSION

Flea Species

Prairie dogs are parasitized by fleas. In addition, fleas vector pathogens between prairie dogs and other species of mammals, including humans; thus, fleas are potentially a very important influence on the health and well-being of individual prairie dogs. Despite the importance of fleas, few studies have described the diversity of the flea community infecting prairie dog colonies. In this study, we used DNA barcoding techniques and statistical analyses of COII sequences to identify fleas and describe the
diversity of fleas for multiple colonies of the two subspecies of GUPD: the Gunnison
(C. g. gunnisoni) and the Zuni (C. g. zuniensis).

A variety of flea species have been identified from prairie dogs. By far the most
common species is O. hirsuta. COII sequences revealed that the majority (44/54) of fleas
collected from prairie dogs of both subspecies was O. hirsuta. We failed to detect O.
tuberculata, a species that is common in black-tailed prairie dogs. The O. hirsuta fleas
collected from GUPD appear to be in a separate subspecies of O. hirsuta than those found
on BTPD. In addition, three genetically divergent lineages of Oropsylla did not match
any sequences in the public database, suggesting that these three lineages are probably
distinct species of Oropsylla that have either not been subjected to DNA sequencing or
are new to science. Similarly, the DNA data suggest we also have a species of flea for
which the closest relationship to another flea based on DNA sequences was Pulex
irritans. It is likely that this sequence is a new undescribed species of Pulex.
Additionally, to date, the COII sequence has been characterized for only one of the more
than 100 species of Pulex fleas. We infer that these sequences represent distinct species
because, on average, distinct species differ by 6-8% in sequence for another related
mitochondrial gene, cytochrome oxidase subunit I (COI) (Hebert et al. 2003, 2004). The
four branch lengths leading to these four groups range from 0.085 to 0.174, indicating 8.5
to 17.4% differences in base pairs. These branch lengths are greater than the values found
in previous DNA barcoding studies, supporting the assertion that these branches represent
four genetically undescribed flea species.
The Two Subspecies have the Same Fleas

An Analysis of Molecular Variance (AMOVA) revealed that most of the genetic variation in the COII sequences among fleas occurs within a colony, and that there is also significant variance among colonies. There failed to be significant genetic variation between the fleas collected from the two subspecies. Thus, it appears that there is no genetic break that corresponds to the subspecies distinction. Similarly, we failed to discover significant differentiation of fleas between the two subspecies, suggesting that fleas do not discriminate between the two subspecies. Previous studies have demonstrated that fleas tend to be species-specific. For instance, the flea species *Aetheca wagneri* specializes on the deer mice (*Peromyscus maniculatus*) and is only rarely found on prairie dogs (Cully and Williams 2001). It is not clear what factors contribute to the host specificity of fleas; however, what is clear is that the two subspecies of GUPD are not sufficiently distinct to allow species’ specificity. Lack of specificity may have important implication for the spread of plague between the two subspecies.

Our results suggest that the Gunnison subspecies may harbor a greater diversity of flea species. Of the ten total flea sequences that represented non-*O. hirsuta* flea species, nine of them were found on the Gunnison subspecies. This was not found to be significant but further sampling of fleas from the montane region may show that there is indeed more flea diversity in Gunnison colonies. The occurrence of these fleas may help explain the higher prevalence of plague in the montane region. Fleas have been shown to have varying abilities to transmit the plague-causing bacteria and other pathogenic bacteria (Wilder et al. 2008). The most common prairie dog flea, *O. hirsuta*, has been shown to have limited ability to transmit plague, while another common flea, *O.*
*tuberculata*, has been shown to be able to transmit the *Y. pestis* bacteria with about three times more efficiency (Wilder et al. 2008). The transmission efficiency of the new undescribed flea species described in this study needs to be evaluated to assess if these species are important vectors of disease that caused the higher prevalence of plague that threaten GUPD living in the montane region.

**Flea Diversity within a Colony**

Flea diversity increased with the number of hosts sampled per colony but not with the number of fleas sampled per host. This result suggests that each prairie dog can be considered an island hosting a unique assemblage of ectoparasites (parasites living on the surface of hosts). Although we have not exhaustively sampled the diversity of fleas on an individual host prairie dog, it is likely that a single host harbors a single species of flea; however, we detected several exceptions in which multiple species of fleas were sampled from a single host providing evidence of flea sharing between individual prairie dog hosts.

Previous studies have shown a significant positive effect of the number of rodent host species on the species richness (the number of different species present) of fleas (Krasnov et al. 2007). One explanation for the apparent (but not significantly) greater diversity of fleas on the Gunnison subspecies than the Zuni subspecies is that the rodent communities associated with Gunnison colonies is more diverse than the community associated with the Zuni. Most of the colonies with the highest diversity (theta(s)) values were from central New Mexico near the boundary of three biogeographic provinces—the
Sonoran, Chihuahuan, and Southern Rocky Mountains—suggesting that flea diversity may reflect higher rodent species diversity.

**Utility of DNA Barcoding for Describing Fleas**

This study used a technique called DNA barcoding for characterizing the species of fleas living on prairie dogs. DNA barcoding uses DNA sequences from conserved genes for phylogenetic analysis; species are identified by including known species in the analysis (Moritz and Cicero 2004, Hebert et al 2003, Hebert et al. 2004). Species identification using DNA barcoding has its advantages and its limitations. It is an alternative to species identification through morphological characters, which frequently leads to misdiagnosis (Hebert et al. 2003). The use of a mitochondrial gene is good for analysis because mitochondrial genes tend to be easy to isolate, be directly inherited from mother to offspring, have a high level of diversity and evolve faster than nuclear DNA thus allowing the identification of closely related species (Arnold 1993, Hebert et al. 2003, 2004, Neigel 1997). Yet, species identification using mitochondrial genes is limited for several reasons, including the possibility of the retention of ancestral variation and gene duplication (see below) (Hebert et al. 2003, Mortiz and Cicero 2004). The retention of ancestral genetic variation could result because these two populations of fleas have not had enough time to equilibrate and diverge to become genetically distinct, producing an artificially low percentage of variation value between subspecies (Bulgin et al. 2003). Another limitation involves gene duplication that can result from the transfer of mitochondrial DNA (mtDNA) gene copies to the nucleus (Hebert et al. 2003, Mortiz and Cicero 2004). All copies of the gene would be amplified by PCR and could be sequenced.
The amount of variation provided by each gene copy could vary depending on the amount of evolutionary selection pressure on the gene copy and would alter the percentage of variation values. Future analysis of one or more nuclear genes would thus allow a more unequivocal identification of species and help to remove doubts associated with the DNA barcoding method of species identification.

**Limitations of the Study**

Due to time constraints, the sample number was limited and, while 46 fleas were sampled from the Gunnison subspecies, only 19 fleas were sampled from the Zuni subspecies. This inequality in the amount of samples introduces sampling error into the AMOVA analysis and skews the data. Future studies should be conducted with equal and greater sample sizes from each subspecies population. Further, this study included fleas sampled only from the montane region (the Gunnison subspecies) in New Mexico, while the monane region extents up into Colorado. Flea samples from Gunnison colonies occurring in Colorado need to be included to better characterize flea species diversity. Additional analysis needs to be conducted on these sampled fleas. First, to confirm the present of the genetically undescribed species, more reference *O. hirsuta* and *Pulex COII* sequences from BTPD fleas should be included in the construction of the maximum likelihood tree and DNA barcoding should be done with one or two sequenced nuclear gene. Lastly, the presence of pathogens, including *Y. pestis* and *Bartonella* spp., in these fleas should be tested to see if these undescribed species and subspecies are disease vectors and how the fleas might facilitate the movement of these pathogens within the GUPD range. These subsequent studies will provide necessary information to understand
the full implications of these results and how they may inform conservation efforts of the Gunnison’s prairie dog.

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