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## A Phylogenetic Analysis of The Population Dynamics of a Captive Colony of *Diptera punctata*.

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**A Phylogenetic Analysis of The Population Dynamics  
of a Captive Colony of *Diploptera punctata*.**

*A capstone research project*

*By Nick Peterson*

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## Abstract

The unique milk production of *Diploptera punctata* makes this species a promising model species in entomology for studying the evolution of milk production and for insect endocrinology. Despite this, the genetic diversity of captive populations of the species is not well studied. To better evaluate the captive diversity and population dynamics of *Diploptera punctata* at UNO we extracted the DNA of 20 random individuals and amplified their DNA using 4 different primers before cleaning the amplified products and sequencing the genes of the samples for use in both individual and concatenated phylogenetic trees. Our results indicate the UNO colony of *Diploptera punctata* has a relatively low diversity in comparison to other members of their species. Additionally, we found that the LCO gene in *Diploptera punctata* varies widely and is closer to *Diploptera minor* than other members of the same species in some instances, possibly indicating that the genes of the *Diploptera punctata* samples have been evolving in a different manner than the species.

## Introduction

*Diploptera punctata* is a blaberid cockroach known for being the only cockroach to produce milk for its young (Banerjee 2016, Schal 1984). As a result, this cockroach is a promising model organism for both insect endocrinology and evolutionary biology (Marchal 2013, Li 2017). This species ranges throughout Asia and the Pacific, with various genetically diverse populations in the wild. Many captive populations of the species exist, though their population genetics have not been analyzed in-depth.

Accounting for possible genetic variation in any trait (at the population level especially) is an important factor when using a model organism in research, as it could impact the repeatability of results. Captive populations often have low genetic diversity as a result of a phenomenon called the founder effect, where the only alleles present in the new population are the relatively few alleles found in the initial founding population (the initial collected roaches). Additionally, captive populations are often small, leaving them vulnerable to random fixation or loss of alleles caused by genetic drift. All of these operating within captive populations in laboratories serves to further decrease their genetic diversity (Lacy 1997, O'Connell 2020).

One way to assess the genetic diversity between individuals in a captive population is with a "molecular clock". This occurs when a gene is found to have a very stable mutation rate over time, allowing one to calculate the amount of time between 2 genetic sequences based on the number of differences in their genetic code. Molecular clock genes are especially prevalent in the Mitochondrial genome, as their functional importance and lack of recombination result in

more steady mutation rates. The CO1 gene is among these mitochondrial genes and is useful for the analysis of closer related individuals due to its relatively high mutation rate (Andújar 2018).

Mitochondrial genes have been successfully used for both quick identification of common pest roaches and phylogenetic analysis of multiple cockroach species in the past (Hashemi 2017, Marquina 2019). Using multiple mitochondrial genes in a single analysis, a highly accurate phylogeny can be created. The method found to have the highest accuracy is to concatenate multiple mitochondrial genes together to generate a phylogenetic tree (Gadagkar 2005). This study seeks to examine the genetic diversity in a captive population of the lactating pacific beetle cockroach, *Diploptera punctata*, using the molecular clock genes CO1 and 12S to better understand the population dynamics and genetic diversity of UNO's captive colony of *Diploptera punctata*.

## **Materials and Methods**

### **Colony maintenance**

The UNO captive population of *Diploptera punctata* contains nearly 150 individuals of random ages spread throughout 2 separate colonies. Both colonies were kept within a smooth plastic container containing egg crates for surface area and breeding space. All individuals are raised on blended purina dog food as a staple diet, with food being scattered around the container for easy and constant access. The colony containers are kept within an incubator to maintain a constant temperature of 27°C. Hydration is maintained through a container of water placed within the same incubator to result in a higher humidity and through spraying water in their

container every 2 days to allow the roaches to drink the droplets. Each container is cleaned every month to remove food, exuvia, and other waste to prevent the growth of mold.

### **DNA Extraction**

A random set of 20 large cockroaches was chosen from the UNO colony and killed through freezing. Wings were removed on each cockroach before each was sterilized through a minute long soak in a 10% detergent solution before two rounds of soakings in distilled water for a minute each. Once washed, the prothorax, legs, and head of the cockroaches were separated and stored at  $-19^{\circ}\text{C}$  to be used in the extraction.

The 20 samples were placed into a minispin powerbead column containing 750  $\mu\text{l}$  of powerbead solution and 60  $\mu\text{l}$  of C1 solution. The Samples were suspended in solution by mashing for 2 minutes prior to 15 minutes of vortexing and 10 minutes of thermal vortexing at  $55^{\circ}\text{C}$ . Each sample was then centrifuged at 10,000xG (10,000 times the force of gravity) for 30 seconds and the supernatant transferred to a clean tube. 250  $\mu\text{l}$  of C2 was then added to each sample and the samples were vortexed for 5 seconds to mix. Each sample was then centrifuged at 10,000xG for 1 minute to remove cellular debris and 600  $\mu\text{l}$  of the supernatant was transferred into a clean 2 ml tube. 200  $\mu\text{l}$  C3 was then added to the tube and vortexed for 5 seconds before another 1-minute centrifuge at 10,000xG. 750  $\mu\text{l}$  of the resulting supernatant from each was then transferred into a clean tube containing 1200  $\mu\text{l}$  of C4 and vortexed 5 seconds.

The entire solution was then filtered through a minispin spin column, with each aliquot being centrifuged through the filter for 1 minute at 10,000xG to separate the desired DNA and other material from the liquid solution. Once complete, each filter was filled with 500  $\mu\text{l}$  C5 and centrifuged at 10,000xG to remove any non-DNA impurities through resuspension. This was

done twice, with the first being centrifuged for 30 seconds and the second for a minute. Finally, the DNA on the filter column was resuspended through the addition of 100  $\mu$ l C6 and centrifuged at 10,000xG for 30 seconds.

The purity and concentration of the extracted DNA was measured using a nanodrop device. The device was blanked using C6 before 2  $\mu$ l aliquots of each sample were measured. Purity was determined through the 260/280 ratio, and samples with less than 0.15 difference between 1.8 and their own ratio were considered pure. Samples with a concentration above 50 ng/ $\mu$ l were considered concentrated enough for use.

### **PCR Amplification**

To confirm the presence of the COI genes in the extracted DNA samples we made use of 4 different primer sets to amplify different target genes. The primers used include 12S, HCO1, CO1, and LCO1 primers. For each forward and reverse primer, a stock solution of 0.1 nM was created before an additional dilution to 0.01 nM to create the working solutions.

Gradient PCR reactions were carried out to find the optimal annealing temperatures for each primer. PCR solutions were prepared using 2  $\mu$ l/25  $\mu$ l of the extracted DNA, 1  $\mu$ l/25  $\mu$ l working forward primer, 1  $\mu$ l/25  $\mu$ l working reverse primer, 12.5  $\mu$ l/25  $\mu$ l Gotaq, and 8.5  $\mu$ l/25  $\mu$ l nuclease free water. Each primer set was tested at a gradient of  $\pm 5^{\circ}$ C. Gel electrophoresis was done on the resulting PCR products in the presence of DNA ladders. The products and ladders were run in a 1.2% agarose gel diluted in 10% TAE buffer containing EtBr for 70 minutes at 110 volts and 400 amps within a solution of 10% TAE buffer. The resulting gel was then examined under UV light to find the quality of the samples through their expected size and the quantity



through the intensity of their fluorescence. PCR was determined to be successful if the bars matched the expected product size of the primer set and the bars were clearly visible.

Successful primers sets were then used in a full-scale normal PCR with a 5 minute 98°C denaturation step before 35 cycles of the following: 50 seconds at 98°C, 1 minute at the recorded optimal melting temperature, and a 1 minute elongation step at 72°C. Once the cycles are complete a final 10-minute 72°C extension period is done. The PCR products were then checked for quality through Gel electrophoresis in the same way as the gradient PCR results.

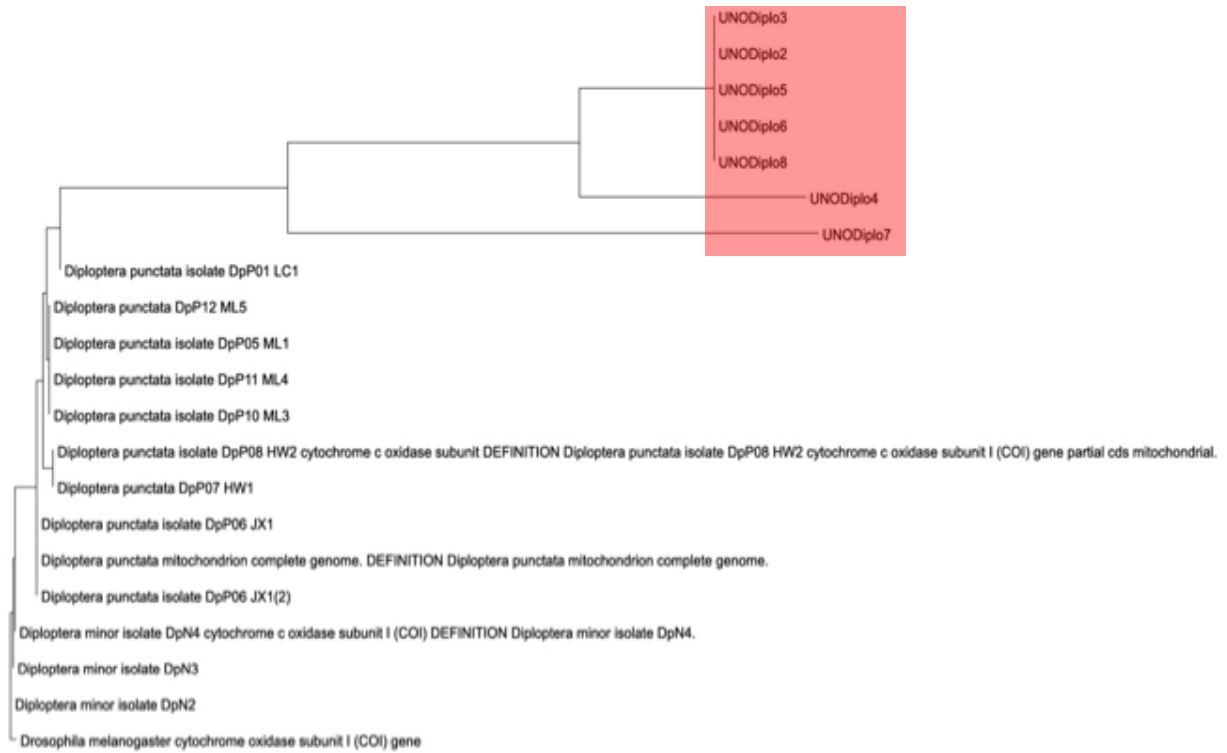
### **PCR Product clean up, Sequencing, and analysis**

Once amplified, PCR solutions containing 10 µl /14 µl PCR sample and 4 µl /14 µl ExoSap were created for each before being ran through a PCR machine for 15 minutes at 37°C prior to another 15 minutes at 80°C. The product was then diluted within distilled water at a ratio of 14 µl /100 µl. For each sample, a new solution of 8 µl/12 µl sample and 4 µl/12 µl 2 µM primer was created and then sequenced at Eurofins genomics company.

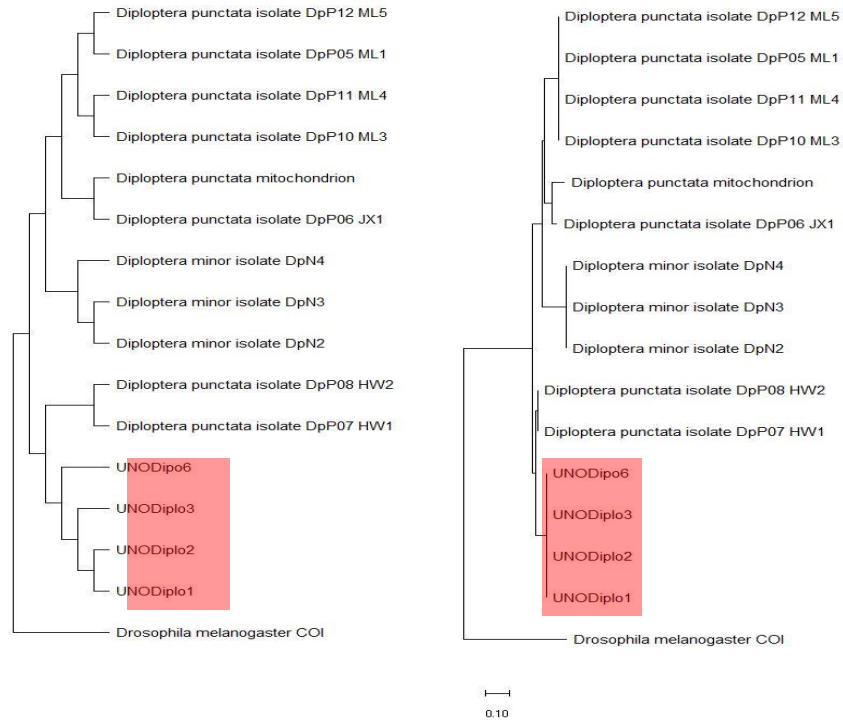
The resulting sequence data was then cleaned using finchTV to remove areas that were not of interest or of low quality. Only nucleotides 100 and 360 were kept for CO1 samples while 100-540 were kept for LCO1 samples. Sequences were aligned and concatenated in MEGA. Non-UNO *Diploptera punctata* samples were obtained from NCBI for comparison, and the target regions were separated for analysis using MEGA. Once prepared, the sequences of each sample were phylogenetically compared both individually and concatenated together through the neighbor joining algorithm in MEGA to generate the phylogenetic trees.

## Results

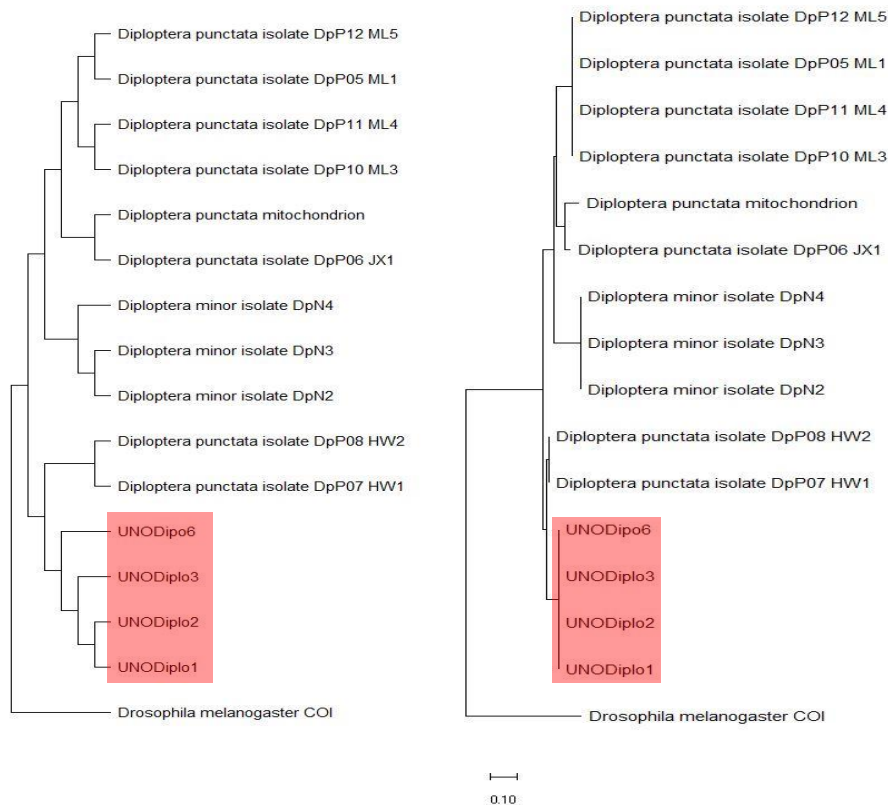
In all 3 phylogenies the UNO colony samples were found to be more similar to one another than they were to other members of the same species (figures 1, 2 and 3). Additionally, evidence of separate lineages were not seen within the UNO colony samples. The results from the CO1 gene phylogeny show the UNO samples being most similar to the other publicly obtained samples of *Diploptera punctata* when compared to the CO1 gene of *Diploptera minor* or of *Drosophila melanogaster* (figure 1). The LCO primer set phylogeny however showed the UNO *Diploptera* samples as being closest related to only 2 other *Diploptera punctata* samples from Hawaii, DpP08 and DpP07 (figure 2). All other *Diploptera punctata* and *Diploptera minor* samples were found to be closer related to one another than they were to the UNO *Diploptera* samples and the Hawaii samples (DpP08, and DpP07). The concatenated sequence shows the same results as the LCO primer set, with UNO *Diploptera* samples, DpP08, and DpP07 forming one branch and all other *Diploptera* forming another (Figure 3). Multiple other possible lineages within the 2 main branches can be seen in both the LCO gene and concatenation phylogenies, with the other groupings consisting of the *Diploptera minor* samples, the DpP ML samples from the China-Laos border (5, 10, 11, and 12), and the complete mitochondrial genome with DpP06 from the China-Vietnam border.



**Figure 1.** The generated phylogeny of the CO1 gene displayed in distance-based form. The phylogeny was generated through the neighbor joining algorithm on MEGA and includes the corresponding sequence from non-UNO *D. punctata* samples, *D. minor* samples, and *Drosophila melanogaster* as a control. UNO *Diploptera punctata* samples are highlighted in red.



**Figure 2.** The generated phylogeny of the LCO gene displayed in both topological (left) and distance based (right) form. The phylogeny was generated through the neighbor joining algorithm on MEGA and includes the corresponding sequence from non-UNO *D. punctata* samples, *D. minor* samples, and *Drosophila melanogaster* as a control. UNO *Diploptera punctata* samples are highlighted in red.



**Figure 3.** The generated phylogeny of the concatenated CO1 and LCO genes displayed in both topological (left) and distance based (right) form. The phylogeny was generated through the neighbor joining algorithm on MEGA and includes the corresponding sequence from non-UNO *D. punctata* samples, *D. minor* samples, and *Drosophila melanogaster* as a control. UNO *Diploptera punctata* samples are highlighted in red.

## Discussion

These results support the hypothesis that UNO's *Diploptera* colony has a relatively low genetic diversity and a homogenous population. In all tested samples, the UNO colony individuals were found to be closest related to one another when compared to other members of their species. Additionally, all high-quality UNO samples show no distance between one another when a phylogeny was created. The small size of the UNO colony along with the small starting

population of other captive roaches supports that the founder effect and genetic drift were likely factors in the low genetic diversity of the UNO colony. The two most distant UNO colony samples within the CO1 phylogeny are also the lowest quality sequences, possessing many ambiguous nucleotides. Despite this, these 2 lower quality samples were still found to be closest to the other UNO samples, further supporting the hypothesis.

One interesting thing to note is that the results from the LCO primer set and the concatenation show 2 distinct monophyletic groups in the *Diploptera* samples. The first contains the UNO samples, DpP08, and DpP07 as their own monophyletic lineage while the second shows all other *Diploptera* samples as closer related to one another despite also containing samples of *Diploptera minor*. These results may be due to alignment methods, genes can evolve separately from their parent species, or even the 2 main *Diploptera punctata* branches possibly representing different species to one another, though much more research would be needed to find the true cause.

Much of the non-UNO *Diploptera* samples were collected from various locations from the wild, and as a result are able to represent some of the species diversity in the wild (Li 2017). Within the 2 main phylogenetic branches, multiple other possible lineages can be seen in addition to the UNO lineage. These different lineages were found to contain samples that share a geographical location, indicating that these populations may be showing different population genetics to one another due to geographic isolation (Figure 3). The UNO samples themselves were grouped most closely with the *Diploptera punctata* samples from Hawaii, though more research would likely be needed to find the original collection location our colony began from (Figure 3).

## Limitations

The analysis however faced a few limitations. Due to the relatively small amount of research this species has received few sequences are publicly available for this specie's mitochondrial genes and all but one *Diploptera* sample originates from a single study (Li 2017). As a result, any skew in the non-Uno data may not be obvious and could interfere with the accuracy of the results. Only 2 primers were able to be used in the comparison, as only the CO1 and LCO primer sets were able to be successfully amplified. While 8 samples were sent out for sequencing from both primer sets, the LCO primer samples had only 4 readings of a high enough quality for use in the individual phylogeny and the concatenated phylogeny. The small sample size of both roach sequences and primer sets used leave the results prone to inaccuracy or being unreliable.

In all, while not enough data is present to make a confident conclusion, this study still provides a good starting point for future phylogenetic research into *Diploptera*. Additionally, enough evidence is present to conclude that UNO's *Diploptera punctata* colony has low genetic diversity and is relatively homogenous, allowing for use as a reliable model organism in research.

## Further research

If taken further, we intend to make use of more primers in the future with the samples we possess to create a more comprehensive and accurate phylogeny through concatenation. Additionally, all 20 amplified samples would be cleaned and sent out for sequencing for each primer set to maximize the number of samples to be used in the concatenation. Lastly, we would make use of more non-UNO samples and species from the *Diploptera* genus and other less related species for comparison in the phylogenies. This will give us a better view of both the

reliability of our own phylogeny and allow for better comparison of the true evolutionary distance our own samples show from both one another and the non-UNO *Diploptera*.

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