# GENETIC ANALYSIS AND MITOTYPE DETERMINATION OF A SURVIVOR POPULATION OF HONEY BEES (APIS MELLIFERA) IN NEW YORK

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Honors Bachelor of Science in Entomology and Wildlife Ecology with Distinction

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## ABSTRACT

The 'western' honey bee, *Apis mellifera* L., was established in the United States over 400 years ago as a result of European settlement and today exists as two groups: managed and non-managed (feral). Beekeepers manage colonies selecting for a few traits and phenotypes associated with Italian and Carniolan subspecies. The arrival of a parasitic brood mite, *Varroa destructor*, caused a major decimation to both managed and feral honey bee populations. The current status of feral bees is relatively unknown although managed populations continue to decline across the country. My study examines the genetic structure of non-managed "survivor stock" bee colonies in the Arnot Forest, located in Ithaca, NY, and compares it to bees found in two surrounding apiaries. Nuclear DNA allele frequency comparisons found genetic differentiation (3 distinct clusters) between the feral bees and bees from the two managed apiaries. I found that 1) the Arnot tree bees are feral as opposed to escaped managed bees filling old nest cavities, 2) these feral bees are persisting in the wild despite treatment for agents such as Varroa and other human manipulations, and 3) there are barriers to gene flow between feral and managed populations. I uncovered two mtDNA haplotypes among my samples—*Apis mellifera carnica* and *Apis mellifera ligustica*, both subspecies of the western European honey bee. Based on these findings, I suggest more sustainable management practices of our managed honey bee populations in the hopes that they too well develop a more stable co-existence with the agents causing their decline. Strategies include reducing hive size, discontinuing the selection for low-swarming bees, slowing or discontinuing the use of

mite-control treatments, spacing hives further apart, and decreasing transportation-induced stress of bees. I believe that by working to ensure bees can foster a balanced relationship with their disease agents, the population decimations across the globe can be reduced.

# Chapter 1

# INTRODUCTION

Honey bees are eusocial, haplodiploid insects characterized, as their name suggests, by their production of honey and the creation of perennial, colonial wax nests. The species *Apis mellifera* L. represents just one of the approximately 20,000 known species of bee, and has been the most abundant and least specialized pollinator species across various global ecosystems (Aizen & Harder 2009).

As the primary pollinator in the United States, the honey bee is an important asset to the agricultural industry. The USDA and The Entomological Society of America state that pollination services account for an estimated $15-20 billion annually and crops utilizing those services encompass approximately 1/3$^{rd}$ of the U.S diet (McGregor 1976; Morse and Calderone 2000, Johnson 2007). The sale of honey, beeswax, and other hive products also contribute an estimated $140 million every year. The sale of honey bees is also becoming increasingly important in the medical field, as their honey is known to have healing and nutritional properties and their venom provides pain relief therapy for many people (Lee et. al 2005). As such a utilitarian and beneficial species, the status and health of honey bee populations is a field of ever-increasing importance in the United States' growing society.

## History

*Apis mellifera* L., is native to the Middle East, Europe and Africa and until around AD 1600, only occupied these regions. Humans have since introduced *A.*

*mellifera* to the Americas, Australia, eastern Asia, and many of the Pacific islands (Crane 1999).

Humans have served a major role in the distribution of honey bees across the North American continent. Eight strains, or subspecies, were originally imported to North America between 1622 and 1891 (Delaney 2008). Today, honey bees in North America exist as both unmanaged colonies inhabiting natural nest cavities as well as managed colonies inhabiting hives maintained by beekeepers. Beekeepers typically select for three strains in their managed populations, particularly those with traits and phenotypes associated with Italians and Carniolans. The three strains favored among commercial breeders and sellers include *Apis mellifera ligustica* (Italian honey bees), *Apis mellifera carnica* (Carniolan honey bees) and *Apis mellifera caucasica* (Caucasian honey bees) (Delaney, 2008). Studies have shown that feral populations are genetically different and more diverse, containing the genetic vestiges of subspecies imported during early North American colonization but not selected for by the beekeeping industry (Schiff et al. 1994).

Rreports beginning in 2006 from the the USDA Agricultural Research Service showed that populations of the few selected strains of managed honey bees in the U.S. are declining. Factors such as parasites (notably *Varroa destructor*, a parasitic brood mite), viruses and pathogens, poor nutrition, lack of genetic diversity, physiologic stress (caused from transportation, confinement, and biological and environmental stressors), chemical residue including pesticide exposure and contamination of wax, and food stores are all thought to play a role in this decline. The USDA's National Agricultural Statistics Service (NASS) has been tabulating the number of honey bee colonies since 1943, and reports indicate a 61% decline –from 5.9 million managed in

1947 to about 4 million in 1970, to 3 million in 1990, and finally to 2.3 million in 2008. Managed colony strength as of 2012 was said to stand at about 2.5 million (Epstein et al. 2012), with 60% of those colonies required for the pollination of single crop in California—almonds. NASS also concludes that over-winter percentage loss for beekeepers in recent years surpass the average historical rate by about double, although numbers vary. A recent preliminary survey conducted by the Bee Informed Partnership indicated that 31.1% of managed honey bee colonies in the United States were lost during the 2012/2013 winter, which is a 42% increase over the 2011/2012 total losses which were estimated at 21.9% (vanEnglesdorp et al. 2013).

"Colony Collapse Syndrome" (CCS) or "Colony Collapse Disorder" (CCD), is the term coined to describe the rapid and large scale loss of adult managed colony workers. Declines were first recognized in 2006, and have since been studied and documented annually by beekeepers and researchers throughout the country (vanEngelsdorp, et al. 2010). However, a census of feral populations is still needed as their current numbers are unknown.

## Objectives and Analysis

A population of unmanaged honey bees in the Arnot Forest were originally censused in 1978, then again in 2002. The 2002 census revealed as many colonies as before despite the introduction of *Varroa destructor* to North America in between those time frames (Seeley, 2007). Because of the evident persistence of bees in this forest, we chose to focus on this study site for our research. The first objective of the study is to determine, through microsatellite analysis, if the colonies living in the Arnot Forest are genetically distinct from bees living in the closest managed apiaries. The study also seeks to assess maternal lineage by uncovering the mitotypes (or

haplotypes) of the sampled bees. Molecular analysis allows for determination of the genetic diversity and differentiation among feral and managed hives, and therefore assists in determining the extent of gene flow among these two groups. This information will ultimately contribute to the larger-scale investigation of whether or not feral populations are persisting on a regional level (and truly self-sustaining) or if natural nests cavities are being repopulated by escaped, managed swarms. This study also uncovers haplotype information among the sampled bees in order to compare maternal ancestry and lineage information between commercial and feral populations

Microsatellites are repeating sequences of 2-6 base pairs of DNA and are considered neutral markers, not coding for any particular function. Variability in these repeat sequences allows us to distinguish differences between populations and identify substructure. Mitotypes are based on multiple single nucleotide polymorphisms and are often determined using Restriction Fragment Length Polymorphisms or sequencing a particular informative region of the mitochondrial DNA. In honey bees the COI-COII intergenic region of the mitochondrial DNA is a representative genetic marker for determining lineage and diversity.

This study also seeks to understand the mechanisms allowing these located tree bees to persist despite vast documented managed bee decline, and provide recommendations for commercial beekeepers concerning better management practices.

# Chapter 2

# METHODS

## Study Site

Samples for this study were collected by Dr. Thomas Seeley in and around the Arnot Forest in Ithaca, NY. The 1651-ha research preserve, located in Tompkins County and northern Schuyler County (42° 17'N, 76° 39 W), encompasses 17-$km^2$ of forest owned by Cornell University (Fig. 1). The area is sparsely populated and at a high elevation (350-600 m), with vegetative cover that includes old-field successional forest as well as mature hardwood and softwood forests. Surrounding land includes state forests protected by New York State or abandoned by agriculture over the past 100 years.

## Collection, Bee-lining

One hundred$^{+}$ individual bee specimens from each of 10 feral colonies in the Arnot Forest were located and sampled via bee-lining techniques and 10 colonies from each of 2 manage apiaries outside the Arnot Forest were also sampled. Bee-lining began in late July and ended in late August 2011. The technique began by capturing bees on flowers in a forest clearing using a "beebox" filled with sugar syrup. The bees were marked and released, and the timing and direction of their flight to their hive and back to the beebox was measured. Recruits were also captured and marked with paint, and the vanishing bearings and round-trip times continued to be recorded. Eventually, select bees were trapped and moved to a clearing 100-200 m away, then

released with bearings and roundtrip times again being recorded. Distance to the nest was estimated by minimum roundtrip times of individually marked bees. This process continued until Dr. Seeley made his way close enough to the nests and hundreds of bees were arriving at the beebox, at which time he collected a sample of 100 bees in 95% ethanol (see Table 1). This technique was used to locate all unmanaged colonies in this study.

A search for nearby managed apiaries was also conducted within a 6-km-wide band of the reserve. Swarms rarely disperse over 3 km (Seeley, 1977) and so the likelihood of swarms entering the Arnot Forest beyond this band is small. Three methods for uncovering managed hives were employed: 1) contacting Finger Lakes Beekeeping Club members that live within 10 miles of the Arnot Forest, 2) bee-lining in flower patches just outside the Arnot Forest, and 3) driving slowly down all roads within 4 miles of the forest looking for hives of bees. Only two apiaries were located, the first .5 miles from Arnot and the second 2.5 miles from Arnot. With permission from the beekeeper, 100 specimens were collected from each of 10 colonies in both apiaries (see Table 2).

## DNA Analysis

The hind leg of 50 random worker specimens from each colony was pulled, cut, and given an extraction code. Nuclear DNA was extracted from each specimen following the protocol from Qiagen DNeasy Blood and Tissue Kit, which utilizes a series of buffers and centrifuge techniques to lyse the cell tissue and release DNA. The extracted samples were stored at -80˚C.

Using a thermocycler, samples were screened at 10 variable microsatellite loci for two separate multiplex PCR amplications (Plex 1 and Plex 3). The loci in Plex 1

consisted of A107, A113, HB-The-03, A024, A006. Plex 3 loci included A28, A88, AP66, AP81, and B124 (Shaibi et al. 2008; Delaney et al. 2009).

A 10 µl final reaction volume per sample contained 5 µl of PCR Master Mix (Promega, Madison, WI), 1.0-2.5 µl of fluorescent dye-labeled primer, 0.9 µl of nuclease-free water and 2 µl of DNA extract. All reactions were amplified for one cycle at 95˚C for 7 min, 30 cycles of 95˚C for 30 sec, 54˚C for 30 sec, 72˚C for 30 sec, and a final extension at 72˚C for 60 min.

Samples were sent for sequencing on an Applied Biosystems (ABI) 3730 automatic sequencer. Allele sizes were scored using GeneMapper® software. Allele frequency data and factorial correspondence analysis were generated by the software program GENETIX, version 4.04 (Belkhir et al. 2002). The software program HP-Rare (Kalinowski 2005) was used to calculate the average number of alleles per locus, allelic richness and expected heterozygosity. Converted files generated from GENETIX were used to determine any departures from Hardy-Weinberg equilibrium, genotypic disequilibrium and genic differentiation. The Bayesian based software program STRUCTURE, version 2.2.3 (Pritchard et al. 2000), was used to determine K or the number of clusters/populations based on Marchov Chain simulations. We estimated K by assessing the values of ln(p(*X*/K)) and the value of α with values of K ranging from 1-4. We evaluated higher values of K to identify any possible substructure that might exist. A 50000 burnin period was used with 250,000 replications to ensure accurate estimates of K from the simulations. We used the admixture model with allele frequencies correlated and we used the ΔK method (Evanno et al. 2005) to assign the most appropriate value of K. We also used STRUCTURE HARVESTER (Earl et al. 2012) to confirm the value of K based on

ΔK, the rate of change of the likelihood distribution, and the mean of ln(p(*X*/K)). We determined the allele frequencies and single locus frequencies for each population using GENETIX (Belkhir et al. 2002) and we used *FSTAT* (Goudet 2001) to determine the $F_{st}$ for each pair of populations.

## Mitotyping

In order to determine each colony's maternal ancestry, the mtDNA COI-COII intergenic region of the DNA extracts was amplified using PCR and the primers E2 (5'-GGCAGAATA-AGTGCATTG-3') and H2 (5'-CAATATCATTGAT-GACC-3'), following the protocol developed by Garnery et al. 1992. The final reaction volume for each sample was 25 µl and included: 12.5 µl of PCR Master Mix (Promega, Madison, WI), 2.5 µl of each primer (E2 and H2), 5.5 µl of distilled water and 2 µl of DNA. PCR amplification took place at 30 cycles at 92˚C for 30 s, 1.5 min at 47˚C, and 2 min at 63°C. PCR product for each sample was purified using the Qiagen® MiElute Purification Kit and then sequenced using an Applied Bisosystems 3730 automatic sequencer.

DNA chromatograms were uploaded into the software program FinchTV (Geospiza, Seattle, WA) for viewing and editing. Sequences were aligned in GeneBank using the Basic Alignment Search Tool (BLAST) which allowed us to determine the mitotype identity.

Table 1 Sample amounts and Tree Types for Forest Bees

| Arnot Tree; Sample Amount | Tree Type | Arnot Tree; Sample Amount | Tree Type |
|---|---|---|---|
| #1: n=99 | Red Oak | #6: n= 283 | Sugar Maple |
| #2: n=140 | Red Oak | #7: n= 265 | - |
| #3: n=100 | Hemlock | #8: n= 142 | Quaking Aspen |
| #4: n= 136 | Hemlock | #9: n= 150 | - |
| #5: n= 94 | White Pine | #10: n= 100 | - |

Table 2 Sample amounts and Tree Types for Managed Bees

| Managed Bees | |
|---|---|
| **Apiary 1 (.5 miles from Arnot)** | Hives 1-10 n=80-165 from each hive |
| **Apiary 2 (2.5 miles from Arnot)** | Hives 1-10 n=120-150 from each hive |

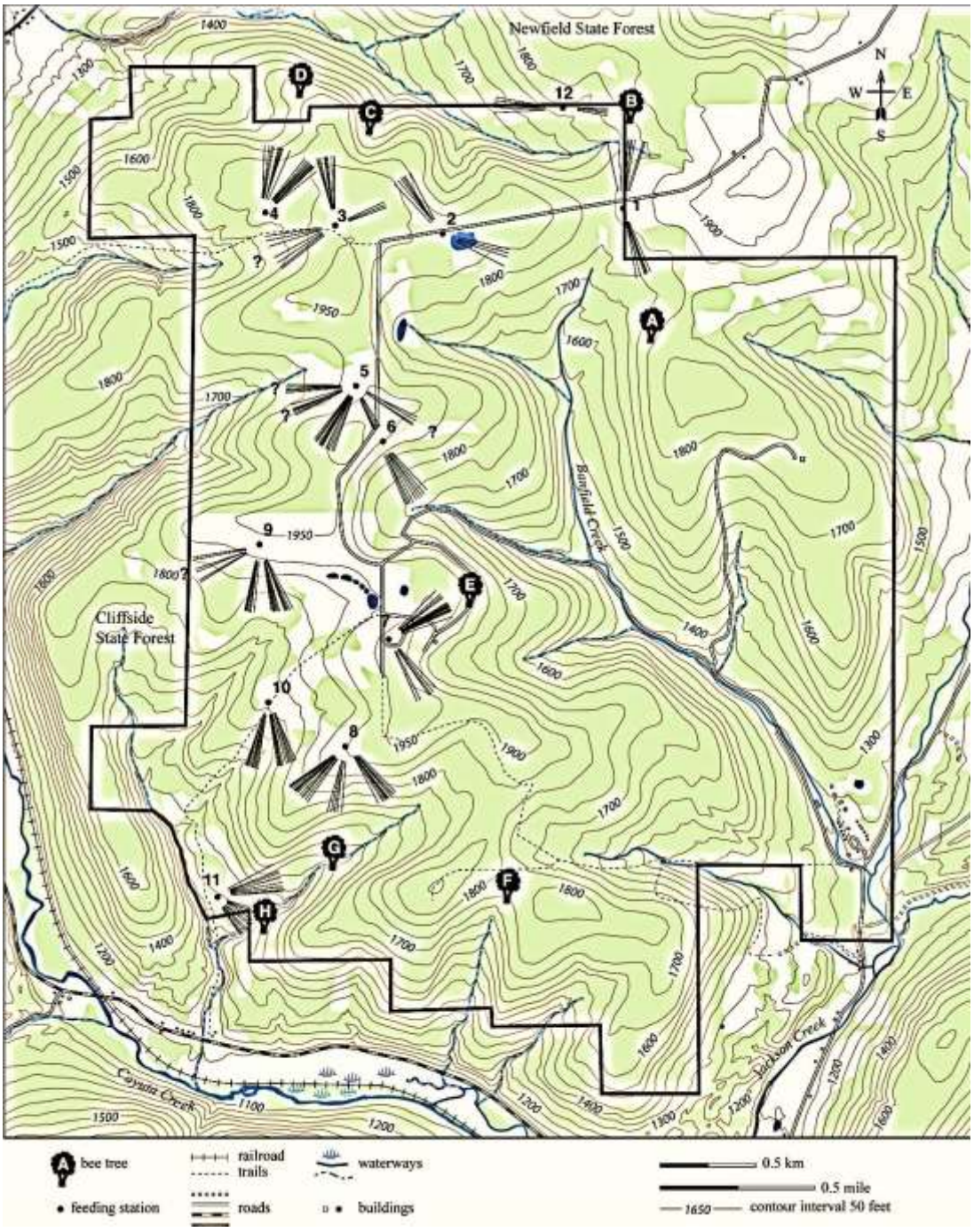
Newfield State Forest
Cliffside State Forest
Bayfield Creek
Jackson Creek
Coyote Creek
N
W
E
S
bee tree
feeding station
railroad
trails
roads
waterways
buildings
0.5 km
0.5 mile
contour interval 50 feet

Figure 1 **Map of the Arnot Forest** 16.5-km2, surrounded by state forests. Feral colonies mapped in 1978 and 2002. Map depicts locations of the bee trees (8) and of the feeding stations (12) used to create beelines that led to the bee trees. Lines radiating from feeding stations portray disappearing bearings of bees leaving each station. Most lines occur in clusters and point toward located bee trees; the clusters that do not are marked with question marks and point to unidentified bee trees. Green areas represent forested land and white areas represent cleared land. Most of the eastern half of the forest was not surveyed for feral colonies, so the total number of feral colonies within the forest boundary is probably considerably higher than the 8 shown here.

# Chapter 3

# RESULTS

## Wild and Managed Colonies

Approximately 50% of the area of the Arnot Forest was utilized in this study's search for unmanaged honey bee colonies, with ten colonies being effectively located. Nine of those colonies resided inside the forest's boundaries and only one colony was located just outside (Fig. 1). Nine is therefore the minimum estimate for amount of colonies persisting in the Arnot, because we cannot be sure every colony was located.

The search for nearby managed colonies revealed two apiaries, both owned by Tremblay Apiaries. Apiary 1, established in April 2011 with 22 colonies, was located 1.0 km off the southwest boundary of the Arnot Forest (Fig. 1). Each colony's queen was purchased from Wooten's Golden Queens in Palo Cedro, California and had been mated there in the spring of 2011. Apiary 2, established in 2001 with 24 colonies, was located 5.2 km off the northeast boundary of the Arnot Forest. This apiary's colonies have been given new queens sporadically all purchased from Wooten's Golden Queens.

## DNA Analysis

**Microsatellites.** Formal Concept Analysis (FCA) allowed us to determine the amount of genetic difference between colonies. Pairwise multi-locus $F_{st}$ estimates showed Arnot Forest colonies were significantly different from Apiary 1 and Apiary 2 colonies ($P < 0.016$, both comparisons), with $F_{st}$ values of 0.387 and 0.403 respectively. The two apiaries showed a substantial level of allele sharing (.0681) with each other (Table 4).

The average number of alleles per locus varied from 3.68 ± 1.47 (Arnot Forest) to 3.02 ± 1.32 (Apiary 1) and 3.08 ± 1.31 (Apiary 2). Arnot forest bees contained more total alleles (69) than either of the managed colonies (53 and 44). Table 6 provides values for allelic richness and number of private alleles for the three groups. The Arnot Forest colonies displayed a significantly higher average number of private alleles per locus than colonies from either apiary (2.85 ± 1.83 vs. 0.83 ± 0.78 and 0.68 ± 0.63), containing more private alleles at 10 of 12 loci.

The STRUCTURE analysis revealed that the microsatellite alleles from these three groups of colonies form three distinct genetic clusters (K = 3). This conclusion is supported by three separate estimations of K: ΔK, rate of change of the likelihood distribution, and the mean of ln(p(*X*/K)). Based on factorial correspondence analysis, Fig. 2 depicts 40 individual bees from each of the three groups (Arnot, Apiary 1 and Apiary 2) and what portion of each bee's marker set falls into a cluster that is most strongly associated with her group or with one of the other two groups. Table 5 summarizes the proportions of the alleles in each group that are associated with each of the three clusters. The alleles in the Arnot Forest bees and the Apiary 1 bees fall almost entirely into two separate clusters. The bees in the Apiary 2 show some overlap with the alleles associated with the Arnot Forest bees as well as those associated with the bees in Apiary 1. Little to no genetic overlap was shown between unmanaged Arnot colonies and the managed colonies in Apiary 1 or Apiary 2, although some genetic overlap exists between the colonies in these two apiaries (Fig. 2).

**Mitochondrial DNA**. Formal Concept Analysis (FCA) allowed us to determine the amount of genetic difference between colonies. Pairwise multi-locus $F_{st}$

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Table 3 Numbers Of Alleles Observed In Each Sample At Specified Loci

| Loci | A024 | A107 | AC 006 | HB-THE-3 | A79 | AP 43 | HB-THE4 | A28 | A88 | AP 66 | AP 81 | B124 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tree | 5 | 10 | 5 | 8 | 7 | 6 | 4 | 2 | 4 | 4 | 4 | 10 | **69** |
| Apiary 1 | 5 | 8 | 0 | 5 | 6 | 6 | 7 | 3 | 2 | 4 | 4 | 3 | **53** |
| Apiary 2 | 4 | 7 | 2 | 3 | 6 | 7 | 3 | 3 | 2 | 2 | 3 | 2 | **44** |

Table 4 This table provides the pairwise Fst values between the feral tree bees and the managed apiary bees. The Fst values use allele frequency distributions to determine the degree of genetic differentiation between population samples. The closer the value is to 1, the more genetic isolation and less allele sharing.

| | Tree | Apiary 1 | Apiary 2 |
|---|---|---|---|
| **Tree** | 0 | 0.3873 | 0.4037 |
| **Apiary 1** | 0.3873 | 0 | 0.0681 |
| **Apiary 2** | 0.4037 | 0.0681 | 0 |

**P-values obtained after : 300 permutations**
**Indicative adjusted nominal level (5%) for multiple comparisons is : 0.016667**

Table 5 Average portions of the bees' marker sets that belong to each of the 3 genetic clusters identified by the STRUCTURE analysis, for each of the three groups of bees.

| Group | Tree | Apiary 1 | Apiary 2 |
|---|---|---|---|
| **Tree** | 0.984 | 0.011 | 0.004 |
| **Apiary 1** | 0.018 | 0.978 | 0.004 |
| **Apiary 2** | 0.091 | 0.035 | 0.873 |

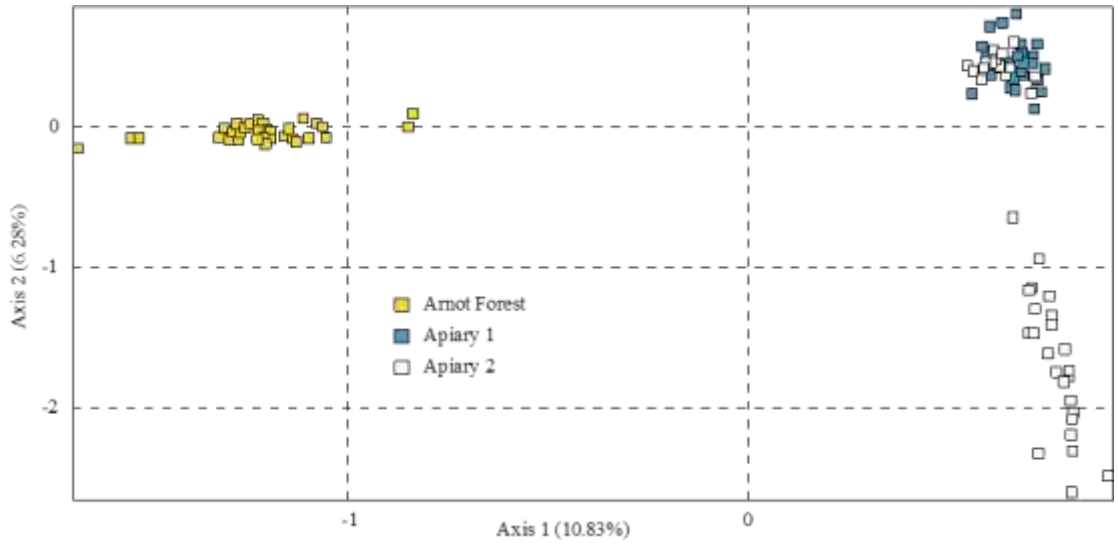


Figure 2 Two-dimensional representation of the results of a factorial correspondence analysis based on the allele frequencies of 12 variable microsatellites for 40 individuals in each of the three groups: Arnot Forest, Apiary 1 and Apiary 2. A third axis (not shown) represents another 4.63% of the variation.

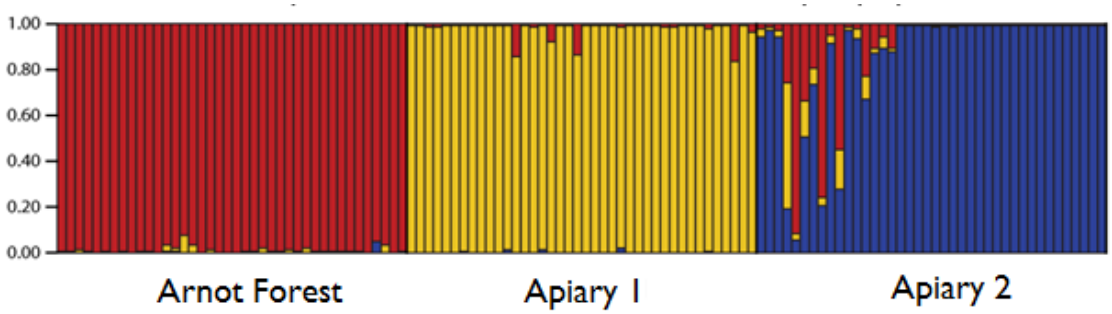


Figure 3 Population structure based on allele frequency data: results of the Bayesian-based cluster analysis performed with STRUCTURE. The bar plots show for each worker bee (40 bees per population) the portions of her marker set that fall into each genetic cluster.

Table 6 Average allelic richness and private alleles after rarefaction

| | Tree | Apiary 1 | Apiary 2 |
|---|---|---|---|
| Allelic richness | 3.7 | 4.2 | 3.0 |
| Private alleles | 2.8 | 0.8 | 0.7 |

Table 7 Haplotype determination by colony ( C1- ligustica, C2- carnica)

| Arnot Forest | Lineage | Apiary 1 | Lineage | Apiary 2 | Lineage |
|---|---|---|---|---|---|
| AFB 1 | C1 | Apiary1-1 | C2 | Apiary2-1 | C1 |
| AFB 2 | C1 | Apiary1-2 | C2 | Apiary2-2 | C1 |
| AFB 3 | C2 | Apiary1-3 | C2 | Apiary2-3 | C2 |
| AFB 4 | C2 | Apiary1-4 | C2 | Apiary2-4 | C2 |
| AFB 5 | C2 | Apiary1-5 | C2 | Apiary2-5 | C2 |
| AFB 6 | C1 | Apiary1-6 | C2 | Apiary2-6 | C2 |
| AFB 7 | ? | Apiary1-7 | C2 | Apiary2-7 | C2 |
| AFB 8 | C2 | Apiary1-8 | C1 | Apiary2-8 | C2 |
| AFB 9 | C1 | Apiary1-9 | C2 | Apiary2-9 | C2 |
| AFB 10 | C1 | Apiary1-10 | C2 | Apiary2-10 | C2 |

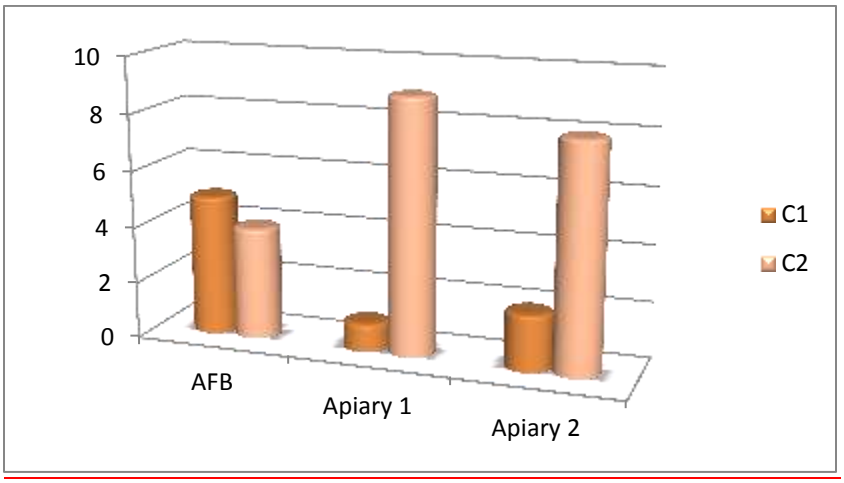


Figure 4 Percentage of bees in Arnot, Apiary 1 and Apiary 2 samples that belong to C1 lineage (*ligustica*) and C2 lineage (*carnica*)

# Chapter 4

# DISCUSSION

The rapid colonization and global dispersal of the ectoparasitic brood mite *Varroa destructor* has increased both the occurrence and virulence of many honey bee viruses, which it acts as an incubator and vector for (Gisder et al. 2009). These viruses, including Deformed Wing Virus, serve to decrease the resilience of bees while making them more susceptible to other lethal agents, and therefore act as major contributors to the negative health and death of millions of managed honey bee colonies across North America and Europe (Martin et al. 2012). These mites are speculated to be one of the leading causes of reported "Colony Collapse Disorder" among managed bee colonies. However, the full impact, including the degree of genetic loss, these mites and associated viruses have on unmanaged colonies, remains unknown.

Bee-lining techniques employed in this study have revealed that there are at least as many unmanaged colonies persisting in the Arnot Forest as there were before Varroa was introduced to New York State in the mid 1990's. This indicates that these bees are coexisting with the mites, although the mechanism allowing them to persist is unknown.

$F_{st}$ calues (Table 4) represent allele frequencies to determine the degree of genetic differentiation between population samples. Figures 2 and 3 visually represent the results of Bayesian-based cluster analysis, which used a factorial correspondence based on the allele frequencies of 12 variable microsatellites for a subset of 40 individuals in each of the three groups (Apiary 1, Apiary 2 and Arnot Forest bees). Results indicate the colonies are separated into 3 distinct clusters, or populations, and

pairwise $F_{st}$ values in Table 4 indicate that there has been little gene flow between the Arnot Forest cluster and the two apiary clusters. The $F_{st}$ values and the cluster analysis suggest that the Arnot-tree bees are genetically distinct from the managed apiary samples and that these feral nest cavities are not simply being filled by escaped managed swarms. Although we have determined that the managed and unmanaged groups are not interbreeding on a significant level, despite a small degree of introgression, the low $F_{st}$ estimate (0.068) found between Apiaries 1 and 2 indicates that all of the colonies in these two clusters share a similar genetic background. The genetic overlap is likely because one commercial queen producer produced the queens heading either all the colonies (Apiary 1) or some of colonies (Apiary 2) in these two apiaries. The small introgression of genes from the unmanaged Arnot colonies into the managed colonies in Apiary 2 is likely a result of managed queens mating with feral drones.

Our results bring into question what barriers are preventing gene flow among the sampled Arnot and managed populations. Distance or familiarity between bees in the three clusters could be a factor. We do see (Table 4) more allele sharing among the Arnot tree bees and bees from the first managed apiary, as opposed to the second managed apiary. These findings are intuitive when you consider the distance of both apiaries from the forest, with Apiary 1 located only 1.0 km from the southwest boundary of the forest and Apiary 2 located 2.5 km from the northeast boundary. However, it is interesting to see that although Apiary 2 was established 10 years prior (2001) to Apiary 1 (2011), there is more genetic isolation between them and the forest bees when compared to Apiary 1.

## Mitotyping

In most animal mtDNA, size variation is typically present within a single region of the molecule, the control region. Most regulatory sequences are found in this region, and the length variation has to do with the existence and abundance of repeat sequences. The honey bee mitochondrial genome is between 16.5-17 kb in length, with the variable range coming from two regions—the control region and the COI-COII junction region. (Smith and Brown 1988). The honey bees' mitochondrial genome encompasses a long intergenic sequence between the COI and COII genes, a large and noncoding region that is thought to have arisen through tandem duplication (Cornet et al. 1991). Because the length of this sequence varies between and within subspecies, we are able to measure these sequences in order to uncover mitotype information. The length categories of this region (of which there are four- 200, 250, 450 and 650 bp) contain two units, an optional P sequence (54 bp, 100% A + T) and a varying number of Q sequences (196 bp, 93.4% A + T) between tRNA"" and COII genes. Lengths of sequences are grouped into the following combinations: Q, PQ, PQQ, and PQQQ (Cornuet et al. 1991).

It is understandable that since their introduction to the Americas, and after years of selection and breeding, the natural distribution of honey bee subspecies in United States' wild lands has changed. It is relatively unknown what subspecies rule the wild today. Through E2H2 amplification of the COI-COII intergenic region, we were able to obtain information about evolutionary lineages of both our managed and forest samples to see which mitotypes dominated. Two mtDNA haplotypes were detected— *Apis mellifera carnica* and *Apis mellifera ligustica*— both subspecies of the western European honey bee. Carnolian bees are originally native to Slovenia, whereas *Apis mellifera ligustica* was originally from Italy. When breeders select for

subspecies, they look for characteristics that include passive or gentle behavior, limited swarming tendency, over-wintering strength, disease resistance, honey-ripening efficiency, decreased propolis production, among other traits. However, singling out these traits could prove detrimental as we narrow the genetic pool of our commercial stock.

## Management Implications

Without question, honey bees are ecologically and economically vital. Understanding how to increase the fitness of bees both in managed and wild environments, by assessing unmanaged, survivor stock, is a topic of increasing importance and relevance in a society whose ever-growing population relies on them to provide much of the food consumed.

Our findings that the unmanaged bees of the Arnot Forest are a true "survivor population", meaning that they are not relying on genetic input from managed swarms, and that they are persisting despite human interference or treatment, bring into question what strategies beekeepers might employ in order to see similar stable-co-existence rates among bees and the parasites and pathogens affecting them. Currently, hive managers in both North America and Europe rely heavily on pesticide and various antibiotic treatments, but this approach is only increasing the risk of honey crop contamination, evolutionary resistance by the pathogens and parasites to the treatments, along with other risks associated with the bees themselves.

The Arnot bee survival and persistence found in this study may indicate either a heightened selection for disease resistance among these colonies or a heightened selection for mite/virus avirulence. In order to understand these potential mechanisms one must evaluate the differences among colonies living in the wild and

colonies that are managed in apiaries. First, pesticide treatments applied in colony management dull selection for already-resistant bees, whereas wild colonies rely on their own resistance and pass those genes down. Secondly, the spatial distribution of bees differs substantially amongst wild and managed colonies, with wild bees dispersed widely over the landscape and managed bees cramped in apiaries. This crowding permits more horizontal transmission of pests and parasites among unrelated colonies through drift and robbing behaviors, while the more widely spaced colonies of unmanaged bees are most likely to transmit pathogens vertically, or only to offspring through swarming behaviors. In the latter scenario, parasites and pathogens are more likely to be avirulent since they would rely on healthy hosts to transmit them to new colonies.

Another potential mechanism driving unmanaged bee persistence could be the size of the hives and therefore number of bees occupying it. The nest cavities that unmanaged colonies occupy are on average 50-75% smaller than the hives kept by beekeepers. This means that number of worker brood, drone brood, and adult worker bees in unmanaged colonies are smaller than in managed apiaries. Not only does this mean fewer bees for parasites to attack, but the smaller nest cavities also mean a potential increase in swarming rates once colonies do get too large. Swarming causes the loss of approximately 70% of the worker bee population, about 35% of adult mites, and creates a broodless period that can last from two to four weeks. At this time, mites and other pathogens have no host to reproduce on. These affects are likely to lead to a decreased population of mites in a colony post-swarming.

It is our belief that current apiculture practices are a driving force behind Colony Collapse Disorder, and basic management strategies may help to

prevent these losses. Strategies include discontinuing the selection for low-swarming bees, slowing or discontinuing the use of mite-control treatments as to allow natural selection to favor the more disease-resistance bees, spacing hives further apart, keeping hives that are smaller in size to encourage healthy levels of swarming (even though honey production might be smaller), as well as decreasing transportation of colonies which not only puts physiological stress on the bees but also hinders natural selection in closed populations. Other possible mechanisms that would be interesting to test for could be comparing propolis production or hygienic behavior between the Arnot and Apiary colonies and examining potential effects on survival rates. Our lab also did some work to examine intra-colonial genetic diversity among hives as a mechanism for persistence, but results so far have been inconclusive. With further studies and consideration for our proposed management recommendations, we believe bees in managed populations can begin to show rates of persistence consistent with the unmanaged Arnot colonies. We believe that by working to ensure bees can foster a balanced relationship with their disease agents, the population decimations across the globe can be reduced.

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