2003

Investigation of Matrilineal Relationships via Mitochondrial DNA in the Southeastern Yellowjacket (Vespula squamosa)

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INVESTIGATION OF MATRILINEAL RELATIONSHIPS VIA MITOCHONDRIAL DNA IN THE SOUTHEASTERN YELLOWJACKET (*VESPULA SQUAMOSA)*

BY

ANTHONY DEETS

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY CHARLESTON, ILLINOIS

2003
YEAR

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ABSTRACT

The question of whether and how apparently "altruistic" behaviors can evolve in social animals has received an enormous amount of attention from evolutionary biologists and has been termed "the central theoretical problem of sociobiology". Thus, recent interest is eusocial species with coexisting multiple queens stems from the realization that the genetic relatedness of individuals in such colonies presents additional theoretical challenges beyond those addressed by the Hamiltonian model of kin selection and lengthens the potential list of reproductive conflicts of interest.

The Southeastern Yellowjacket (*Vespula squamosa*) exhibits two social forms sympatrically in the southern parts of its range. These two social forms differ with respect to queen number and are termed monogyne (single queen) and polygyne (multiple queens). Since multiple functional queens within a colony affects offspring relatedness, inclusive fitness theory predicts that polygyne colonies are more likely composed of cooperating sister queens. Therefore, this study tested the Hamiltonian model by investigating matrilineal relationships within polygyne colonies to discern if co-inhabiting queens were sisters or unrelated individuals. Six hundred and twenty-seven base pairs of the Cytochrome B (Cyt B) gene within the mitochondrial DNA (mtDNA) was sequenced for 13 to 15 queens from 10 polygyne colonies and one worker from 17 monogyne colonies. The Cyt B gene of *V. squamosa* has a similar AT content (77%) as that found in other Hymenoptera and other taxa in the Insecta. In addition, the Cyt B gene of *V. squamosa* had the greatest similarity to the Cyt B genes of other insects. The sequence similarity to other vespid Cyt B genes, however, was less than that of the

Honey Bee, *Apis mellifera*. Sequence analysis showed that there was no variation within this region of the mtDNA of *V. squamosa* and this region, therefore, could not resolve relatedness among individuals. Possible reasons for the lack of genetic variation and the unusual sequence divergence are discussed.

INTRODUCTION

Darwin (1859) viewed social insects as particularly problematic to his proposal that differential reproduction of individuals within a species results in organic change through time and explains the properties of extant organisms. The question was, "how could a trait such as reproductive sterility of females in social insects be selected for if individual contributions to future generations were all that mattered in explaining the characteristics we observe in all organisms?" As the mechanism underlying phenotype and inheritance patterns was elucidated in the early 20th century, genetics became the basis for models addressing this issue. The idea that animal behavior is governed entirely by self-interest and that "altruism" is best explained by individual-level functionalism was argued by Hamilton (1964 a, b) and Williams (1966) on the basis of genetic models and has continued to be the dominant tradition in biology. Fitness, in this tradition, is thus viewed as the relative contribution of an individual's particular genotype to future generations relative to that of other conspecifics (Lincoln et al. 1998).

Under the individual-level functionalism, or "selfish gene" model (Dawkins 1989) character states that appear to be altruistic must, in fact, have evolved through "selfishness" by genes that persisted at the expense of others (Hölldobler and Wilson 1990). In other words, no matter how altruistic the event looks, it can be explained evolutionarily to show that the organism providing the action benefits (in the fitness sense) in some way. Most often, that way is by indirect fitness, which was first described by Hamilton, (1964 a, b) who proposed a genetic model of inclusive fitness that modified Darwin's theory of natural selection. Hamilton's theory includes direct fitness, the fitness gained by an individual reproducing, and indirect fitness, which is increased

fitness through helping related individuals reproduce. Inclusive fitness equals the combination of both direct and indirect fitness (Hamilton 1964 a, b, 1972; Brown 1980).

Insect Sociality

Insects are a large class that shows a myriad of life history strategies. Roughly half (50.8 %) of all Eukaryota species and 72.0 % of all animals are insects (Daly et al. 1978). Insects inhabit almost every biome except the extreme polar regions and the highest mountain peaks, and wherever they do occur they tend to be the predominant small fauna. Insect's life histories range from extreme semelparity to iteroparity. One of the most unusual, yet most successful, life histories is that of the social insects. Social systems range from solitary individuals, where interactions among adults are largely limited to sexual behavior and competition, to casteless primitive sociality, and eusociality, where certain castes forego their own reproductive opportunities to help conspecifics reproduce. There are over 12,000 species of social insects with eusociality present in 3 orders: Isoptera, Homoptera, and Hymenoptera (Wilson 1975). Three characteristics are shared by eusocial insects: 1) reproductively inhibited or sterile castes, 2) post-natal care by individuals other than the egg layer, and 3) overlapping generations.

The almost singular and relative abundance of eusocial taxa within the Hymenoptera suggests that members of this order were preadapted to evolve this type of social organization. In the Hymenoptera, there exists well-developed nest building and various levels of pre-social behavior by long-lived female parents that could lead to eusocial colonies. Hymenopterans also have mandibulate mouthparts that are versatile in nest construction and brood care, as well as, useful in tasks of obtaining food. Finally, and most importantly, Hymenopterans have a haplodiploid reproductive system which

creates genetic relationships in a family that favor the evolution of "altruistic" behavior among sisters (Hamilton 1964 a, b; Daly 1978). All of these traits are represented singly within different insect or animal species in the absence of eusociality, but the combination of all three characteristics in the Hymenoptera may have led to repeated evolution of eusociality in this order.

Haplodiploidy, which was the main component used by Hamilton, (1964 a, b) provides the genetic rationale for the evolution of sociality in this order. Females in a haplodiploid system are developed from fertilized eggs and hence diploid, whereas fertile males are developed from unfertilized eggs and are haploid. This system, which Trivers and Hare (1976) termed the ¾ relatedness hypothesis, causes an asymmetry of relatedness among offspring where sister siblings are ¾ related on average to each other, only ¼ related on average to their brother, and ½ related to their offspring. Hence, workers can increase their inclusive fitness by raising sisters ($r = 0.75$) instead of rearing young on their own ($r = 0.5$) (Fig. 1).

Hamilton's (1964 a, b) theory predicts that there will be reproductive conflicts between the members of a haplodiploid colony. In a stereotypical colony that has a single queen who has mated once, a worker's fitness may be increased by producing males whenever possible because sibling workers are related to their male offspring by ½ and only ¼ related to sibling males. Queens however, should favor the production of their own males (Trivers and Hare 1976; Pamilo 1991a, b; Ratnieks and Reeve 1992; Evans 1995; Sundstrom et al. 1996; Arevalo et al. 1998). West-Eberhard (1979, 1981) has expanded this idea showing that worker "altruism" may have been evolved in a selfish manner. This becomes apparent when examining the polyethism or division of

labor in many eusocial colonies. Younger workers, who have a higher fecundity, are reluctant to leave the nest, and tend to help rear the brood providing more opportunities for laying their own eggs. As workers age, they tend to move to the outside of the nest and conduct activities such as foraging and nest defense. When older and less fecund, foraging and defense is the optimum strategy for contributing genes to the next generation because the worker is no longer as efficient in laying eggs and can better benefit the colony and her inclusive fitness through more dangerous occupations (West-Eberhard 1979, 1981).

Monogyny and Polygyny. Although Hamilton's genetic rationale for abundance of eusocial taxa in the Hymenoptera is currently accepted as reasonable, the conditions he specified for his argument are rare. That is, the "typical colony of a single queen who has mated once" seems to be the exception rather than the rule, making the genetic dynamics of colonies much more complicated than previously thought (Queller et al. 1988; Strassmann 1989; Ross and Carpenter 1991; Keller 1993; Herbers and Stuart 1996; Cole and Wiernasz 1999).

Throughout the social Hymenoptera colonies are founded in two ways, i.e. as a single individual or as a group. Current terminology refers to these two founding modes as haplometrotic and pleometrotic colony founding respectively (Wheeler 1933). Hymenopteran colonies can be classified in a variety of ways. Monogyny refers simply to a colony with a single egg-laying queen. "Polygyny" consists of two divisions: 1) functional polygyny, where more than one queen is contributing to reproduction, and 2) functional monogyny, where a colony consists of more than one queen, but the supernumerary queens are not laying eggs either because they are not mated or are being

suppressed by the primary queen (Hölldobler and Wilson 1990). In a functional polygyne nest, two possibilities exist for producing the next generation of queens: 1) all queens lay eggs equally, or 2) there is differential egg production (competition). Unless relatedness between the queens is extremely high, competition between the egg-layers would be expected.

It is reasonable to suppose that properties in colony organization tend to bias species toward monogyny in the course of evolution, and that the tendency to become polygynous only occurs when special ecological constraints are imposed on colonies (Hölldobler and Wilson 1977; Oster and Wilson 1978). In fact, it is actually disadvantageous for colonies to allow supernumerary queens, especially during the early stages of colony founding. This is because extra queens will present a huge burden on a young colony with few workers to provide even for a single queen. In addition, if multiple functional queens are present, as queen number increases colony relatedness inevitably decreases, and therefore, workers should conspire with the single most dominant queen to remove any supernumerary queens so that they can rear closely related individuals. Evolutionarily, it also becomes apparent that natural selection should favor haplometrotic colony founding; in other words, selection should favor genes that enhance single queen success over genes that lead to pleiometrosis. Finally, queens should found colonies in a claustral pattern whenever possible. This is because the highest mortality in workers and queens occurs during foraging, and if enough resources are available, workers and queens should prefer to forage near others due to the dilution effect and a lower chance of predation (Hölldobler and Wilson 1990). In summary, it appears that natural selection should favor monogyny, haplometrosis, and claustral nest

founding. Polygyny, however, has evolved independently in many Hymenopteran taxa, (e.g., the red imported fire ant, *Solenopsis invicta* and *V. squamosa*). Polygyny can arise by one of three means: pleometrosis, with the founding queens remaining together after the first workers appear; adoption of extra inseminated queens after their nuptial flights, and fusion of colonies (Hölldobler and Wilson 1990).

Vespid Wasps. The order Hymenoptera consists of the ants, bees, wasps, ichneumons, sawflies, and chalcids. It is a very interesting and important order from a human standpoint because of the role these insects play in the pollination of plants (Daly et al. 1978; Borror et al. 1976). It is also a very large order falling second only to the Coleoptera (beetles) in species number and diversification.

The family Vespidae includes diverse wasps, which are mostly predatory, usually on soft-bodied insects. The vespids include many solitary forms such as potter wasps (Subfamily Eumeninae), but also comprise more familiar species, such as the primitively social paper wasps (*Polistes*) or the eusocial yellowjackets (*Vespula*) and hornets (*Dolichvespul, Vespa*). Hence, the Vespidae show a graded spectrum of sociality from solitary individuals to advanced eusociality (Wilson 1971). Vespid nests may be suspended from branches, rafters or other aerial structures, may be concealed beneath stones or in hollow logs, or may be constructed in cavities that the wasps excavate in the ground. Nests are commonly constructed from masticated wood or paper, but may be made of mud (Daly et al. 1978).

Within the family Vespidae, there are seven subfamilies, one being the Vespinae. The genus, *Vespula*, within the subfamily Vespinae is commonly referred to as the yellowjackets. The Southeastern yellowjacket, *Vespula squamosa*, is an interesting

member of the genus because of its behavioral plasticity. *V. squamosa* has two social forms that occur sympatrically over the southern portions of this species' range, multiple queen and single queen colonies. Multiple queen colonies are functionally polygyne insofar as more than one queen is a functional egg-layer (Fritz and Stewart unpublished data). Nests can also be perennial or annual. Finally, *V. squamosa* has been observed to facultatively usurp conspecifics or parasitize other species' nests (Akre et al. 1980; MacDonald and Matthews 1984; Carpenter 1991). Because of this social plasticity, *V. squamosa* is a good species for studying the ecological and genetic determinants of nest dynamics within and between two social forms (monogyne and polygyne).

Phylogenetically, *V. squamosa* has been placed as a sister-taxon to *V. rufa* (Aker et al. 1980; MacDonald and Matthews 1984; Carpenter 1987; Carpenter 1991). The range of *V. squamosa* spans from northern Guatemala through Mexico and north throughout most of eastern and mid-western United States (Fig. 2; Akre et al. 1980). The majority of *V. squamosa* nests are found in disturbed habitats such as yards and are constructed subterraneously. These nests may extend up trees and other vegetation, particularly in swampy land (Akre et al. 1980; MacDonald and Matthews 1984; Castner and Fritz 1993). Nests in the southern range that are perennial can become quite large. For example, nests have been collected that are over three meters tall and one meter across (G. Fritz pers. com). As with other vespids, this species commonly feeds on soft-bodied insects, but is also known as a common pest disturbing people in picnic areas, parks, and around garbage.

The life cycle follows that of most vespids. That is, a newly mated queen overwinters in foliage, between the bark of a tree or in any suitable habitat. At the onset

of spring, queens emerge and begin searching for a nest site. After a site is chosen, the queen will construct a small nest of a few cells, lay the first brood of workers, and rear them from her own fat reserve. At maturity, the workers will then begin performing colony tasks such as nest construction and maintenance, foraging, rearing newly laid workers, and feeding the queen. Throughout the rest of spring and summer the nest will continue to grow exponentially. When late summer approaches, the queens will shift her reproductive efforts to laying reproductives, that is, males and daughter queens. Both sexes subsequently take their nuptial flights and mate. Shortly after mating, the males die and the newly-mated queens find refuge for the winter. Finally, the original colony perishes. This is the common life cycle that occurs through the entire range, but in the southern regions the life cycle may be altered. When the environment remains relatively mild throughout the winter months, such as in Florida and other sub-tropical regions, a colony may survive for a number of years. To date, however, perennial colonies have always been found to be polygyne colonies, though it is not clear whether one requires the other (G. Fritz pers. com). In a previous study, it was shown that the two social forms differ significantly with respect to various measures of size and weight (Deets & Fritz 2002), indicating a difference in nest dynamics. Microsatellite analyses also confirm that polygyne colonies contain supernumerary, inseminated, egg-laying queens (Fritz and Stuart unpublished data).

Project Goals

The question of whether and how apparently "altruistic" behaviors can evolve in social animals has received an enormous amount of attention from evolutionary biologists; Wilson (1975) even termed it "the central theoretical problem of

sociobiology". Thus, recent interest is eusocial species with coexisting multiple queens stems from the realization that the genetic relatedness of individuals in such colonies presents additional theoretical challenges beyond those addressed by Hamilton (1964 a, b) and lengthens the potential list of reproductive conflicts of interest (Ross 1986; Queller et al. 1988; Keller 1995; Arevalo et al. 1998; Hastings et al. 1998).

An extension of Hamilton's model (1964 a, b) to eusocial insects with multiple queens predicts that cooperating egg layers are sisters rather than unrelated individuals. By cooperating with sisters instead of unrelated queens, both inclusive fitness, as well as, the probability of nest survival increase. Nest survival rates in social wasps and the risks incurred by foundress queens have generally emphasized mortality associated with the initiation of nests and their maintenance up to the point where numerous workers are present and assume foraging and nest building (Spradberry, 1973a; Akre and Reed 1981; Strassmann 1981; MacDonald and Matthews 1981, 1984; Keeping and Crewe 1983). Nest usurpation in *V. squamosa*, for example, may be interpreted as a strategy that avoids the risks of *de novo* nest initiation.

The purpose of my investigation was to test the Hamiltonian paradigm of sociality by examining the genetic relatedness of queens within polygyne nests of *V. squamosa* and examine patterns of genetic variation throughout a broad range of this species' distribution in order to discern possible dispersal patterns and/or genetic drift events. One way in which relatedness, dispersal and other patterns of genetic variation can be ascertained is by examining the mitochondrial DNA (mtDNA). Because mtDNA is generally maternally inherited and replicated clonally (Avise 1986), all offspring possess the same haplotype as their mother. The mtDNA genome is thus appropriate for

examining maternal lineages through time and space. Although the size, content, and genomic organization of the mitochondrion is highly conserved, its genome evolves at a faster rate than coding regions of the nuclear genome in insects (Avise 1986), probably due to the absence of repair mechanisms. In addition, the mtDNA includes a highly variable, non-coding control region of a few hundred base pairs. Numerous studies have reported substantial intraspecific variation of the mtDNA not only within the control region, but in coding regions (Avis 1986; Avis and Zink 1988; Afonso et al. 1990; Clark et al. 2003). Clark et al. (2003), for example, resolved 20 haplotypes in a 319 bp region of the Cytochrome B gene (Cyt B) in 123 specimens of the timber rattlesnake, *Crotalus horridus*. Goodisman et al. (2001) researched colony and queen relatedness of the German Yellowjacket, *Vespula germanica,* in Australia and New Zealand and reported inter-nest haplotype variation. All queens within a nest, however, most often shared a single haplotype. Goodisman et al. (2001) hypothesized that recruitment of extra queens into a perennial colony is usually reserved to daughter queens returning to their natal nest, which would be consistent with the lack of haplotype differences within a nest (Goodisman et al. 2001).

HAPLODIPLOIDY

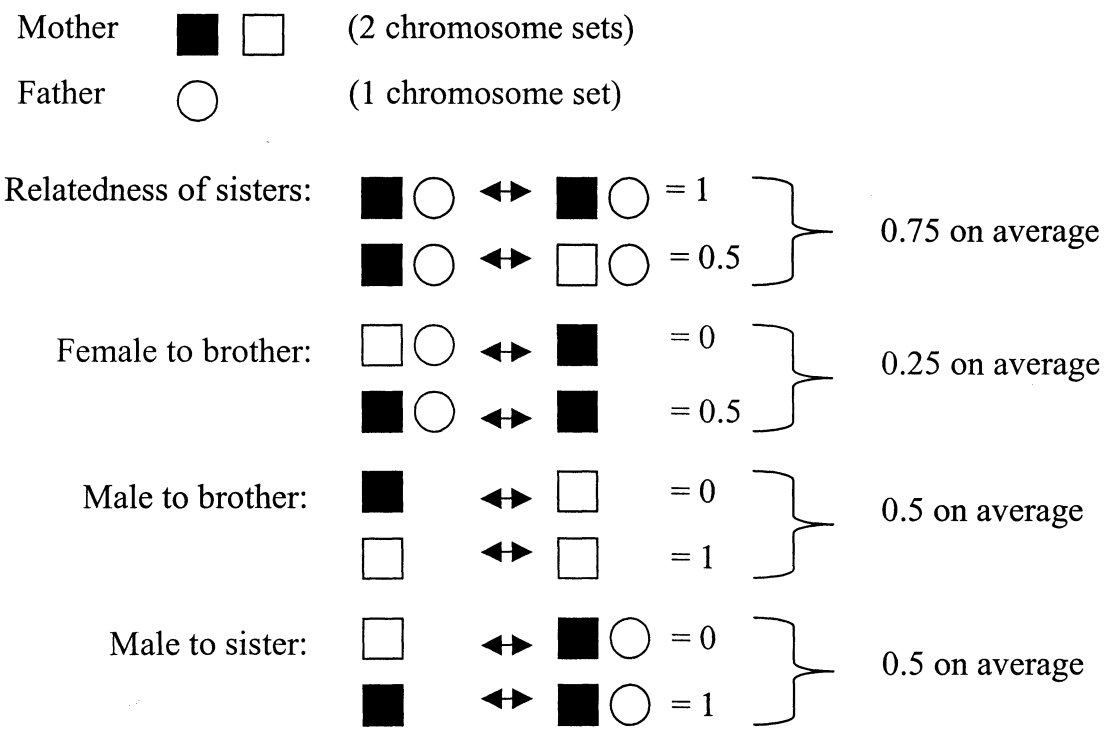

Fig. 1. Relationship between mother, father, and siblings in a haplodiploid colony.

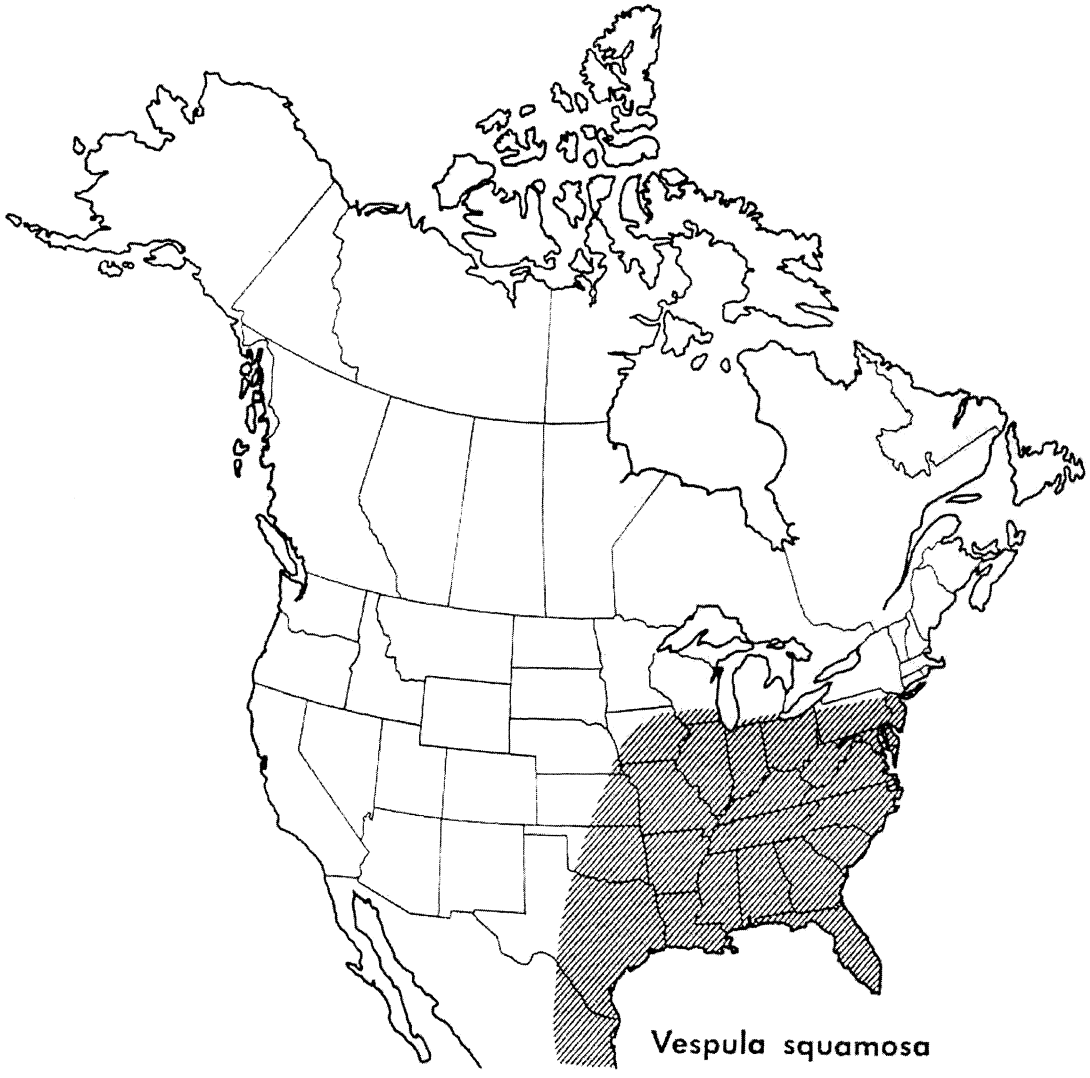

Fig. 2. Range map of *Vespula squamosa* (Castner and Fritz 1993).

MATERIALS AND METHODS

NEST COLLECTIONS

In the summers of 1998 and 1999, 10 polygyne and 17 monogyne colonies of *V. squamosa* were collected from counties in southern Georgia and Florida (Table 1). Collection of wasps was done by manually disturbing the nest entrance(s). A vacuum with a mesh bag attached to the nozzle was used to collect the majority of flying individuals as they were swarming out of the nest entrance(s). After most flying individuals were collected, the nest was unearthed and searched for queens. Specimens were immediately placed on dry ice and subsequently stored in freezer bags at –80°C.

GENETIC ANALYSIS

Since mtDNA is inherited maternally it should be identical between queens that are sisters in a polygyne colony. The initial approach used in this study for estimating genetic relatedness was to amplify 2/3 of the complete mtDNA genome (approximately 10,000 bp) for restriction enzyme fragment polygmorphisms analysis---RFLPS), as explained below. With the subsequent acquisition of an automated sequencer in our Department, the focus changed to sequencing a portion of the mtDNA genome for nucleotide differences between individuals.

DNA Extraction. In order to estimate the population frequencies of haplotypes that may exist in *V. squamosa*, DNA was extracted from one worker from each of the 27 colonies collected in this study. DNA was also isolated from 13-15 queens from five of the polygyne colonies (Table 1) in order to test for the number of matrilineal lines within colonies. For all specimens, DNA was isolated from the thorax. A variety of DNA extraction techniques were tested on yellowjackets including a "squash preparation," a

phenyl-chloroform-isoamyl (PCI) extraction, a Promega Wizard® Genomic DNA Purification kit, a "mini-preparation extraction" suitable for extracting small circular DNA, and a Qiagen DNeasy™ tissue kit. The squash preparation protocol was as follows: A thorax was isolated from the sample, ground in 200 µl squash buffer, (10mM Tris-HCl, 1mM EDTA, 50 mM NaCl, pH 8.2), boiled in a water bath for 5 minutes, and centrifuged at 14,000 RPM in a microcentrifuge for 15 minutes. Portions of the supernatant were then used as the DNA template in a polymerase chain reaction (PCR). The mini-prep. procedure was as follows: A thorax was ground in 200 µl of a homogenization buffer (0.1 M NaCl, 0.2 M sucrose, 0.01 M EDTA, 0.03 M Tris-HCl). Next, a lysis buffer (0.25 EDTA, 2.5% SDS, 0.5 M Tris-HCl pH 9.2) was added and the samples were incubated at 65°C for 30 min. Twenty microliters of 8M KAc was added and the samples were placed on ice for 30 min. The samples were centrifuged for 15 min. and the supernatant was added to 100% ethanol to give a final concentration of approximately 60-70%. The DNA was pelleted in a microcentrifuge for 15 minutes and resuspended in TE buffer (10 mM Tris-HCl pH 8.0 and 1mM EDTA). The Qiagen DNeasy™ tissue kit extraction uses centrifugation through a filtered spin column to bind, clean, and isolate the DNA. Specific procedures were followed according to the instructions provided by Qiagen for DNA extraction of rodent tails (page 19 DNeasy Tissue Kit Handbook (04/99). The PCI extraction and the Wizard kit extraction followed standard procedures for extracting DNA from animal tissues

Primers. PCR primer sequences for amplifying a 10 kb section of the mtDNA genome were developed by obtaining and aligning the complete mtDNA sequence of the honeybee, *Apis mellifera* with that of the mosquito, *Anopheles gambiae,* and the fruit fly,

Drosophila melanogaster. After alignment, these sequences were inspected for conserved regions flanking the target areas. The regions chosen to amplify included: 1) an approximately 10 kb fragment including the control region, 2) the control region between the 12S ribosomal RNA and tRNA Met genes, 3) the region between the cytochrome C oxidase subunit I (COX I) and NADH dehydrogenase 4 (ND4) genes, and 4) the region between the Cyt B and ND4 genes. After choosing prospective priming sites, primer optimization variables were considered in making final choices (e.g., annealing temperatures, GC content, internal primer annealing, etc.).

Polymerase Chain Reaction (PCR) Protocol. The Hybaid-AGS® kit, specifically manufactured to amplify large fragments of DNA, was used in attempts to amplify a 10 kb region of the mtDNA genome. The protocol followed the 100μL reaction mix for high fidelity reactions up to 20 kb using Taq/Pwo mix. Smaller portions of the mtDNA were targeted for amplification by developing additional sets of primers.

Restriction Enzyme Fragment Length Polymorphsims. Primers developed for amplifying the Cyt B and ND4 genes produced an approximately 1.8 kb fragment that was cut with the restriction enzyme AseI in order to discern haplotypes. This restriction enzyme was chosen because it recognizes an AT-rich sequence motif (AT / TAAT). Since the mtDNA of insects is known to be AT-rich (80.6% in the Honey Bee), this enzyme should maximize the number of nucleotides sampled. For example, the entire human mtDNA genome (which is only 37.6% AT) has 11 cut sites for this enzyme whereas that of the Honey Bee has 147 cut sites.

DNA Sequencing. PCR amplification products were purified for subsequent sequencing reactions. Initially, amplification products were cleaned using a PEG purification

protocol. Samples were incubated at 37° C with 95μL of PEG solution, (30% (w/v) PEG 8000 (Sigma P4463), 1.6 M NaCl) centrifuged, alcohol washed and vacuum dried. This method did not successfully produce sequenceable mtDNA template. Next, successful purification was completed using a Qiagen QIAquick™ PCR Purification Kit, which uses centrifugation through filtered spin columns to bind the PCR product and isolate it from any impurities such as excess salts and primers from the original PCR reaction. The specific protocol followed the instructions for microcentrifuge PCR clean up provided by Qiagen. The dye terminator cycle sequence reaction (DTCS) was conducted with Beckman Coulter CEQ™ Dye Terminator Cycle Sequencing Quick Start Kit. Both forward and reverse sequencing reactions were completed for each fragment of DNA from each yellowjacket using primers AJ-F and AJ-R (Table 2) diluted to 1.6 mM. In this manner, each sequence was "double-checked" for sequence reliability. The DTCS master mix consisted of 4.0 μL CEQ™ DTCS Quick Start Master Mix, 1.0 μL of primer, and 4.5 μL of ddH_20. 0.5 μL of PCR template was added to 9.5 μL of DTCS master mix to complete the 10 μL cycle sequence reaction. The tubes were then topped with a drop of mineral oil to ensure no evaporation during the reaction. The thermal cycler temperature protocol consisted of a denaturing step of 96°C for 20 s, an annealing step of 44°C for 20 s, and an extension step of 60°C for 4 min. These steps were repeated for 30 cycles and a final extension step of 60°C for 8 min., and a holding step of 4°C were in the final cycle.

The sequencing products were then cleaned using an alcohol precipitation specified by Beckman Coulter™. Each product was removed and place in a new 0.5 μL ependorph tube taking as little mineral oil as possible. A stop solution consisting of equal

parts 100mM Na_2EDTA (pH 8.0) and 3M NaOAc (pH 5.2) was made and 4 μL of this added to each sample as well as 1 μL of 20 mg/mL glycogen. Finally, 60 μL of cold (-20°C) 95% (v/v) ethanol/ddH_20 was added and the samples were mixed and centrifuged at 14000 rpm at 4°C for 20 min. Next, the supernatant was removed and the samples were washed twice with 200 μL of cold (-20°C) 70% (v/v) ethanol/ddH_20 and centrifuging between washes for 4 min at 14000 rpm and 4°C. The samples were then vacuum dried for approximately 45 min and resuspended with 30 μL of Beckman Coulter™ Sample Loading Solution. Plates were then loaded into the Beckman Coulter CEQ™ 2000XL DNA Sequencer and separated and read according to the LFR-1 DNA sequencing mode.

Sequence Analysis. At the conclusion of the sequencing run the sequences were exported and loaded into the Gene Codes Corporation program Sequencher™ 3.0. This program allows the sequences to be aligned and then edited according to the color chromatogram generated from the individual bases. Each sequence was visually scanned for incongruities. The sequences were subsequently loaded into Paup 4.0 and manually aligned. Paup 4.0 was used instead of Sequencher 3.0 because it is easier to view large data sets and note any differences between individuals of the various colonies. After all individual sequences were aligned; the entire data set was visually scanned for variable sites.

Table 1. Nest identification, collection site, and number of queens collected.

Colony I.D.	Social Form (Monogyne, Polygyny)	No. Queens Collected	Collection Location (State, County, Town)
AL5A	M	1	Florida, Alachua, Gainesville
AL10A	M	1	
AL11A	M	1	
AL1Q	P	-	
AL3Q	P	43	
AL5Q**	P	22	
AL61	M	-	
AP1P	P	-	Florida, Orange, Apopka
AP2P	P	-	
AP3P	P	-	
AP94	M	-	Florida, Highlands, Avon Park
BR1Q**	P	105	Florida, Brevard, Titusville
BR2Q	P	2*	Florida, Brevard, Melbourn
DI1Q	P	2*	Florida, Levy, Old Town
DI2Q	P	31	
EU94	M	-	Florida, Marion, Eureka
GA1P**	P	23*	Georgia, Glynn, Brunswick
KALEY	P	-	Florida, Orange, Orlando
OR1A	M	-	
OR10A	M	-	
PU1P**	P	46	Florida, Putnam, Bostwick
PU1Q	P	-*	Florida, Putnam, Palatka
PU3Q	P	2*	
PU2Q	P	124	Florida, Clay, Keystone Heights
SA94	M	-	Florida, St. Johns, St. Augustine
SL1P**	P	32	Florida, St. Lucie, Port St. Lucie
TR1P	P	27	Florida, Gilchris,Trenton

* Many other queens were seen but unable to be collected due to nest location.
** Fifteen queens from these colonies were used for the experiment.

Table 2. Primers developed and tested for *Vespula squamosa*.

Primer Name	Region	Sequence
AJ-F*		5'- GTC CTC AGG GTA GAA CAT ATC CTA GG -3'
AJ-R*	Cyt B	5'- GAC TAA TTT CAA ATC AAT ATA GG -3'
Cyt B Int-F	Cyt B	5'- AAT GCT ATA TCA ATG GAG GGG CAG -3'
Cyt B Int-R	Internal	5'- CTG CCC CTC CAT TGA TAT AGC ATT -3'
COIND		5'- ACT GTA AAT ATG TGA TGT GCT CA -3'
MND	Cyt B – ND6	5'- GCT AAA TAA AGC TAA CAG GTT CAT -3'
12SAT	12S – tRNA Met	5'- GAA ATT GAC GGG CGA TTT GT -3'
MAT	(includes control region)	5'- CAG GGT ATG AAC CTG TTA GC -3'
COX1		5'- GGA ACT GGA TGA ACA GTA TAT CCA CC -3'
ND4	Cox I – ND4	5'- CCA TAA TAA GGA GCT TCA ACA TGA GC -3'
CYTOB	Cyt B	5'- GCA CCT CAA TAT GAT ATT TGT CCT -3'
Cyt B F	Cyt B	5'- ATA TAG TTA CCT CCC CGA C -3'

* Primers used in final analysis.

RESULTS

DNA Extraction

The DNA of *V. squamosa* proved very difficult to extract by many standard protocols. No DNA was recovered from *V. squamosa* using the Wizard kit, PCI protocol, the mini-prep, and the squash buffer techniques, even though all of these were good for extracting DNA from other organisms (e.g., mosquitoes, fish, snails, etc.). Thus, it appears that yellowjacket DNA must be bound to substances (proteins?) that are not easily stripped during many standard protocols that are used for isolating DNA from animals. The only extraction kit that produced visible DNA on an Agarose gel was the Qiagen DNeasy™ tissue kit. Consequently, all extractions were completed using this kit only.

Primers and PCR

Except for CYTOB and ND4 (Table 2), all primers tested were unsuccessful at amplifying targeted regions of the mtDNA, even when the template DNA used to test each pair of primers was from the Honey Bee itself (to which all primers were 100% homologous). CYTOB and ND4 primers amplified a 1.8 kb region including portions of the genes Cyt B, ND6, ND4L, and ND4, as well as, the intergenic spacers in between (Fig. 3). Both ends of this fragment were sequenced, and, subsequently used to develop internal primers useful for amplifying a region encompassing the Cyt B gene and ND4 genes. Yellowjacket specific primers (Cyt B Int-F, Cyt B Int-R, AJ-F, and AJ-R) were then created by aligning individual yellowjacket worker sequences and choosing a conserved region within the targeted areas. Primers AJ-F and AJ-R, that amplified a 627 bp region of the Cyt B gene, were ultimately used for all subsequent PCR and DCTS

reactions because they produced both the best PCR amplification fragment and DNA sequence data (Table 2).

The PCR reagents used for generating all the sequence data used in this study consisted of a 10x PCR buffer (500 μL of 1M Tris HCl (pH 9.2), 140 μL 1M $(NH_4)_2SO_4$, 19 μL of 1M $MgCl_2$, 341 μL of ddH_20); 1.25 mM dNTPs; and 15 mM primers in a 100 μL reaction. A master mix of 10 μL PCR buffer, 16 μL dNTPs, 2 μL of each primer, 1 μL Taq DNA polymerase, and 64 μL ddH_20 was used with 5 μL of mtDNA template to produce the amplified product. A MJ Research PTC-100™ thermal cycler was used to amplify the mtDNA. The temperature profile consisted of a hotstart step of 94°C for 2 min, a denaturing step of 94°C for 45 s, an annealing step of 51°C for 45 s, and an extension step of 68°C for 3-½ min. This profile was repeated 30 times and ended with a final extension step of 68°C for 8 min. Amplification products were visualized on a 1 % agarose gel using normal gel electrophoresis techniques.

Restriction Enzyme Fragment Length Polymmorphisms

The 1.8 kb amplified region of the mtDNA mentioned above was digested with Ase I for 27 workers, one from each nest collected in this study. For each yellowjacket's digested mtDNA, an eleven-fragment pattern was resolved on a 1.5% agarose gel. All individuals displayed an identical eleven-fragment pattern.

Sequence Analysis

PCR amplification resulted in an 1800 bp product (Fig. 4) that encompassed an area of the genes Cyt B, ND6, ND4L, and ND4. Within this region, a 627 bp section of only the Cyt B gene was subsequently sequenced for 91 individuals including at least one sample from each of the 27 nests collected in this study. These sequences were aligned

with the complete mtDNA and Cyt B sequences of the Honeybee (*Apis mellifera*) for comparison. It was found that the yellowjacket sequence is located around 360 bp from the beginning of the Cyt B gene. The percent AT content of the yellowjacket sequence is 77.0%. Sequences were found to be identical between individuals in all colonies sampled (Fig. 4). This single haplotype was found in all 27 colonies (17 monogyne, 10 polygyne) and was geographically represented from Brunswick, GA south to Port St. Lucie, FL (Fig. 5).

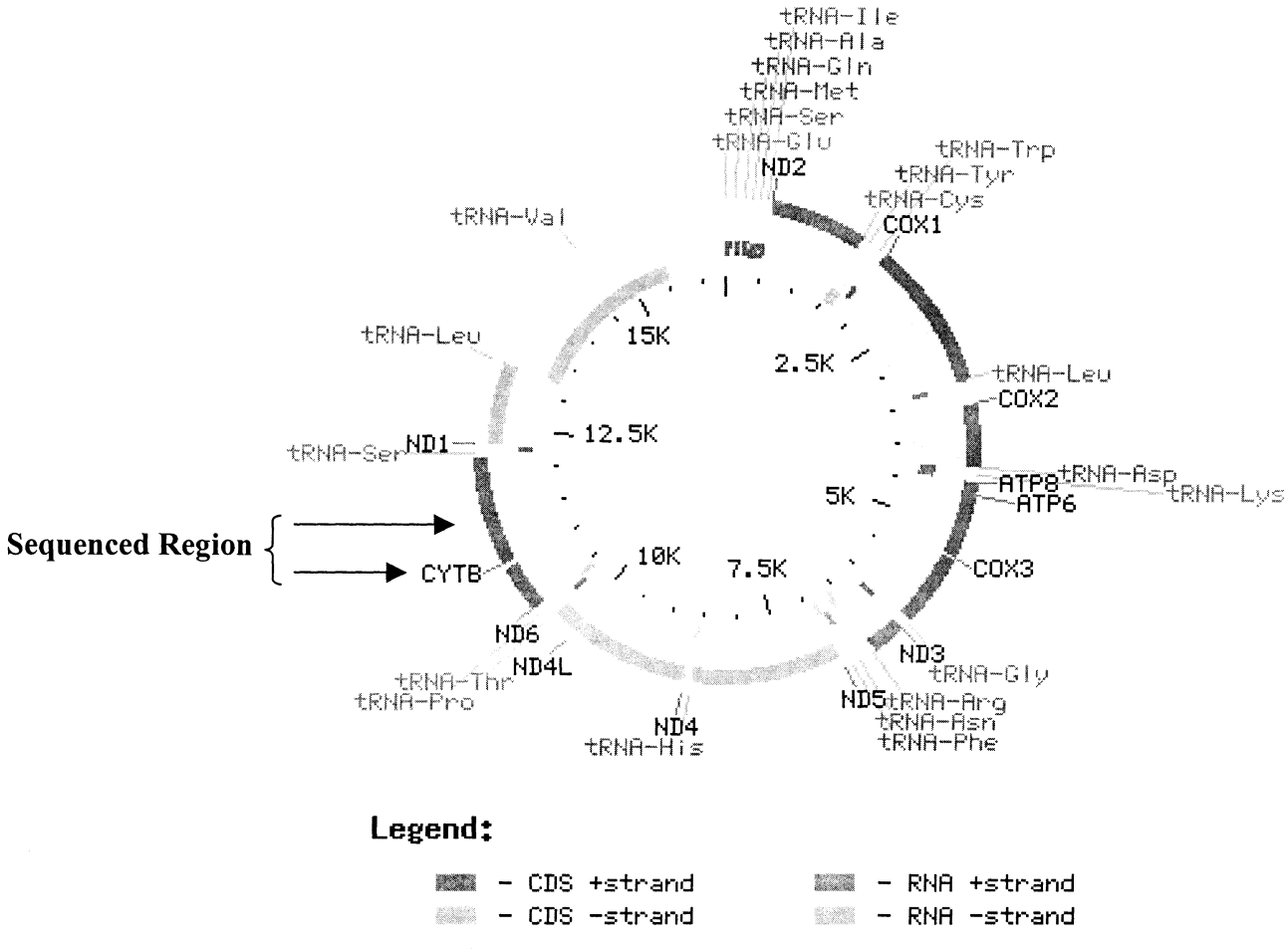

Fig. 3. Mitochondrial DNA of *Drosophila melanogaster* showing sequenced region of Cytochrome B.

```
1     AATATGATAT TTGTCCTCAG GGTAGAACAT ATCCTAGGAA GGCTGTTATT
51    ATAGTGATAA GAAAAATGAT AACTCCAACA ATTCATACAT GAATTAAATT
101   AAATGATTGA TAATAAATTC CTCGTCCAAT ATGAATATAA AGGCAAATGA
151   AAAATAATGA GGCTCCATTA CTATGAAGAA TTCGTATTAT TCATCCATAA
201   TTTATATCTT GTATGATTTG TATAACTCTG TTAAATGCTA TATCAATGGA
251   GGGGCAGTAA TGTATAGATA AAAAAATTCC TGAAATTAAT TGAATTATAA
301   GGCAAAGGCC TAGTAAAGAG CCTAAATTTC ATCAATAATT AATATTAACT
351   GGAGTGGGAA GAAAAACTAA AGAATCTAAG GCAATTTTAA TTAGTGGATT
401   TTTAAATATA AATGTTTTTT TCATAATTAT TTTTTTGTTA TACGAAGGGG
451   TTTAATATTA GTAAGGCAAA TTTTAGTAAT TCTAAATAAA ATAATTAATA
501   AAAATATAAT TAATATGATG ATAAATAAAT TATTAGAATA TATAAATAGT
551   TGGTTAATAT TTATTCATGC GTGTAAATTT TGATTTTGAA TTGATAAATC
601   AATGAAATTA TTTATATTGA TTTGATT
```

Fig. 4. Inclusive mitochondrial DNA haplotype of all colonies sampled.

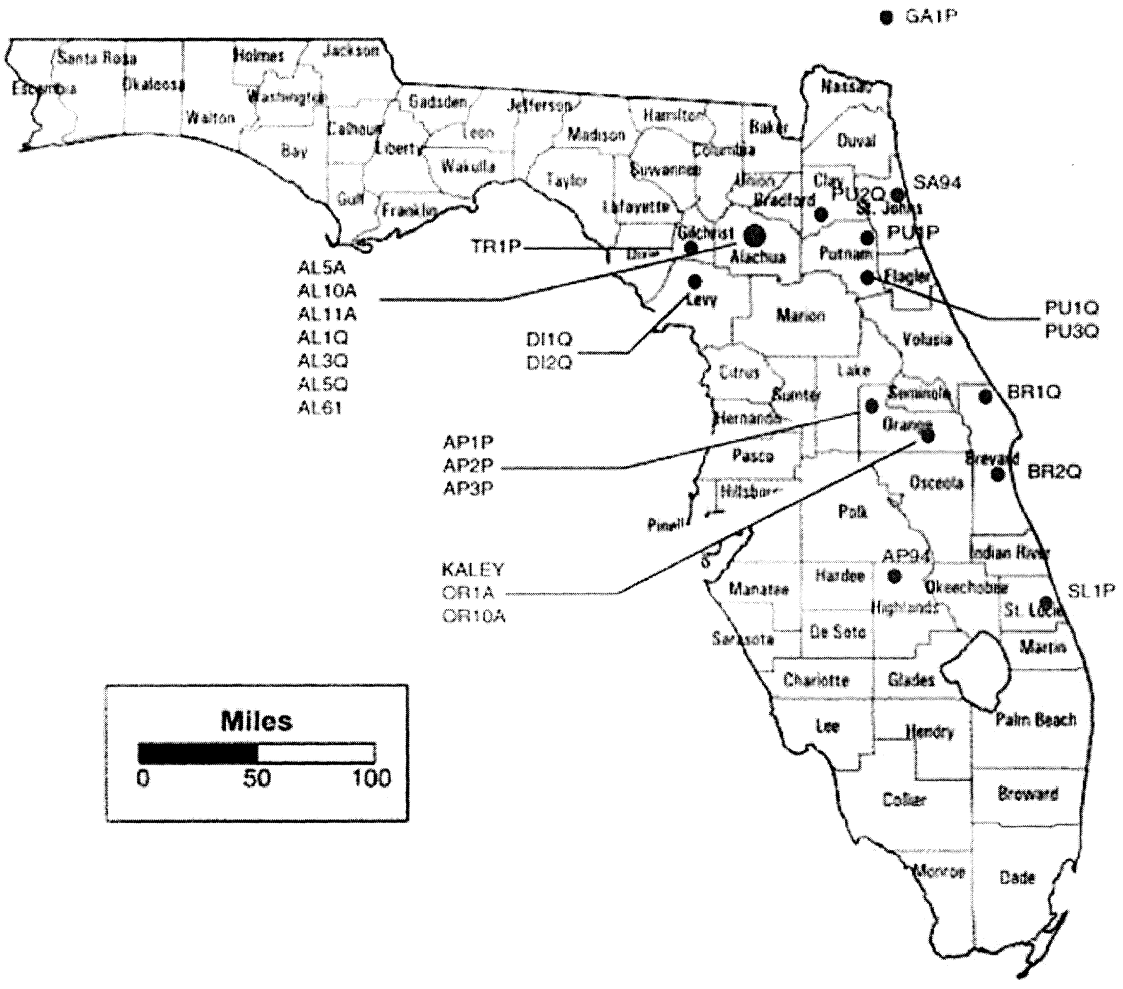

Fig. 5. Geographic locals of all wasp nests sampled.

DISCUSSION

Genetic Analysis

Neither the restriction enzyme analysis of an 1.8 kb fragment of the mtDNA nor the sequence data from the Cyt B gene showed any polymorphism within or between nests. Consequently, these data could not be used to address the matrilineal relatedness composition within nests or any geographic patterns from which to infer haplotype distributions, possible genetic drift events and/or dispersal patterns. In addition, an enzyme electrophoresis survey of *V. squamosa* samples collected throughout Florida also showed absolutely no polymorphism (G. Fritz unpublished data) even though this species in Georgia was shown to be polymorphic at four loci (Ross 1986). Although mitochondrial studies have been published on a variety of insects, many of which report numerous haplotypes (Mitrofanov et al. 2002; Wahlberg et al. 2003; Marquez and Krafsur 2003), no such studies had been published on vespid wasps at the time this study was completed. Recently, however, Goodisman et al. (2001) completed a survey of the German Yellowjacket in New Zealand and Australia for a 450 bp region of the mtDNA and reported two haplotypes. Since the German Yellowjacket is native to Europe, and this study was conducted in Australia and New Zealand (where this species was accidentally introduced), the expectation is that many more haplotypes exist throughout its native range. The lack of genetic polymorphism for both the mtDNA and various nuclear enzyme loci suggests that *V. squamosa* is indeed a very monomorphic species in Florida. Although this species has an extensive range throughout North America, there are no data regarding mtDNA or nuclear variation in any other part of its distribution. If *V. squamosa* is found to be more polymorphic in other parts of its range, the surprising

homogeneity of this species in Florida would suggest a possible bottleneck or founder effect and perhaps a single matrilineage. If this species is in fact more polymorphic in Georgia then this study also suggests a relatively low dispersal rate for *V. squamosa* into more northerly parts of its distribution. Why or when this event may have occurred is purely speculative, but perhaps *V. squamosa* re-colonized Florida after the last glacial period from the southernmost parts of its range (southern Mexico and northern Guatemala). Incremental re-colonization across southern U.S.A. could potentially have led to a lack of variation by eliminating some or many of the mtDNA haplotypes existing in the species.

Queen Relatedness

Queen relatedness plays a crucial role in determining the potential reproductive conflicts among nestmates. It is already known that queens mate multiple times (Hölldobler and Wilson 1990; Keller 1995) and that queens in polygyne nests have the potential to lay eggs because their ovaries are developed and they have been inseminated (Fritz and Stewart unpub. data).

But, are queens in polygyne colonies sisters? There are a variety of factors that may play a role in determining degrees of eusociality and/or polygyny. As previously stated, *V. squamosa* has multiple social forms (monogyny and polygyny). Unfortunately, it is unknown how polygyny arises in this species. Research has been conducted more extensively on this subject in many ant species, and it is here that definitions can be found for ways by which nests become polygyne. For example, primary polygyny can be described as a pleometrotic (multiple-queen) colony foundation that leads to mature colonies in which foundresses coexist (Alloway et al. 1982; Rissing et al. 1989). By

contrast, secondary polygyny arises when colonies that were established by single queens (haplometrosis) become polygynous (Hölldobler and Wilson 1977; Rissing and Pollock 1988; Strassmann and Queller 1989). Yellowjackets seem to follow the latter method of secondary polygyny because founding queens have never been observed cooperating to establish a nest, and quite contrary to cooperating, queens of *V. squamosa* have often been observed to usurp other founding queens of conspecifics and other species (Akre, et al. 1980; MacDonald and Matthews 1984). Also, when this species is found in sub-tropical or tropical habitats it can sometimes survive for multiple years or become perennial. When this happens, *V. squamosa* is effectively outliving its average lifecycle, and it appears that these wasps are then willing to accept newly mated queens. Goodisman et al. (2001) has speculated that these new queens are most likely sisters and microsatelite data from workers also suggest that this is the case for *V. squamosa* (Fritz and Stewart unpub. data). Unfortunately, the relationship of queens accepted into nests is not known for certain, nor do we know the particular requirements for acceptance by the original nest inhabitants.

Perennial Nests

The fact that polygyne nests persist beyond a year (the typical lifespan of yellowjacket species) is very intriguing in consideration of the aforementioned topics. It also generates speculation as to why a nest would be willing to accept newly mated queens. When a nest outlives its average lifespan it is very possible that the original founding queen dies. When this happens, the nest will no longer be under control of the queen and her pheromones, and consequently, workers could lose aggressiveness and potentially be willing to accept a new queen or queens. Another possible explanation for

queen acceptance does not require the original queen to die but, rather, she senesces. The outcome of senescence may be loss of control over her nestmates. Loss of control in concert with an ever increasing nest size may lead to a lack of elicited aggressive response from workers when newly mated queens attempt to enter the colony.

It is also plausible that the majority of queens returning to a nest are daughters of the original queen, and therefore, sisters to the workers. If it is possible for these insects to discriminate between related and unrelated individuals it would become very beneficial to allow newly mated daughter queens entry into an already established nest. That is, workers would be helping their sisters establish the next generation's successful nests. Again, this hypothesis is consistent with the data of Goodisman et al. (2001), who showed that, in *V. germanica*, 20 out of 21 colonies had the same mtDNA haplotype between all individuals sampled. The results of Goodisman et al. (2001) also support the Hamiltonian model which predicts that multiple queens in nests are sisters. It has been shown in a variety of ant and wasp species that nest founding is a very energetically demanding process with a high mortality rate. Thus, newly mated queens that bypass nest founding would have a great advantage over other conspecifics (Strassman 1989; Hölldobler and Wilson 1990; Pamilo and Rosengren 1984). New queens would not have to spend any energy beginning nest construction, rearing the first brood, or nest defense. They could immediately begin egg production and continue with the "exponential growth stage" of a colony lifecycle.

Pseudogenes

Finally, one last item of interest should be addressed. Recent literature has cautioned the use of mtDNA in genetic studies because of the existence of mitochondrial pseudogenes (Numts: nuclear mitochondrial-like sequences). Numts are translocated regions of the mtDNA that have been moved from the mitochondrial genome to the nuclear genome. These translocated regions can be over 8.0 kb in size (half of the mtDNA genome), can include any region of the mtDNA, and can be confused with mtDNA when amplified through PCR, cut with restriction enzymes, or sequenced (Lopez et al. 1994; DeWoody et al. 1999, Bensasson et al. 2000; Petrov 2002). For example, a 7.9 kb region of the mtDNA including the Cyt B gene has been shown to be a Numt in the domestic cat. The potential exists that the region sequenced in this study is a Numt within the nuclear genome of *V. squamosa*. Although not available at the time this study was completed, recent published sequences of the Cyt B gene in the German Yellowjacket (Collins and Gardner 2002) were aligned with that of *V. squamosa* and compared to alignments with the Honeybee (Figs. 6, 7, 8). Disconcertedly, the Cyt B sequence of *V. squamosa* aligned with the Honeybee sequence equally as well as with the German Yellowjacket. And, the German Yellowjacket sequence is quite homologous with respect to the Honeybee Cyt B gene sequence. If a Numt was actually sequenced for *V. squamosa*, it would help explain the poor homology between the Cyt B sequence of this species with that of *V. germanica* to which it is more closely related. Nevertheless, the Cyt B region of *V. squamosa* sequence in this study has the "hallmark" of an insect mtDNA sequence (extremely AT rich), was not repetitive sequence (as would be expected for non-coding AT rich regions in the nuclear genome), and a "Blast" (search

command in the GenBank genetic database used for searching for homologous sequences) of GenBank showed that the Cyt B sequence of *V. squamosa* had the most homology with other insect mtDNA including the Cyt B region. If the region sequenced is truly a pseudogene (which should evolve very quickly) in the nuclear genome, the conclusion remains that *V. squamosa* appears to lack genetic variation in Florida.

Conclusion

Neither the region sequenced of the Cyt B, nor the RFLPS analsysis of the mtDNA showed any variation within or between colonies of *V. squamosa*. Thus, the data could not address the prediction that multiple queens within polygynous colonies are sisters; the data neither validate nor invalidate the prediction. *V. squamosa* in Florida, therefore, appears to be monomorphic in comparison to other insects, including Hymenoptera, that have been studied to date and suggests that genetic drift or some type of founder effect may have played a role in its biogeographical history. In order to establish maternal lineage patterns it would be ideal to amplify and sequence the region of the mtDNA that potentially has the greatest amount of genetic variability, the control region. In addition, one could also answer this question using microsatelite analysis. As previously stated, Fritz and Stewart (unpub. data) have used this technique to analyze worker relatedness. The relatedness estimate they generated from workers within nests (0.13) is consistent with the hypothesis that multiple queens are sisters.

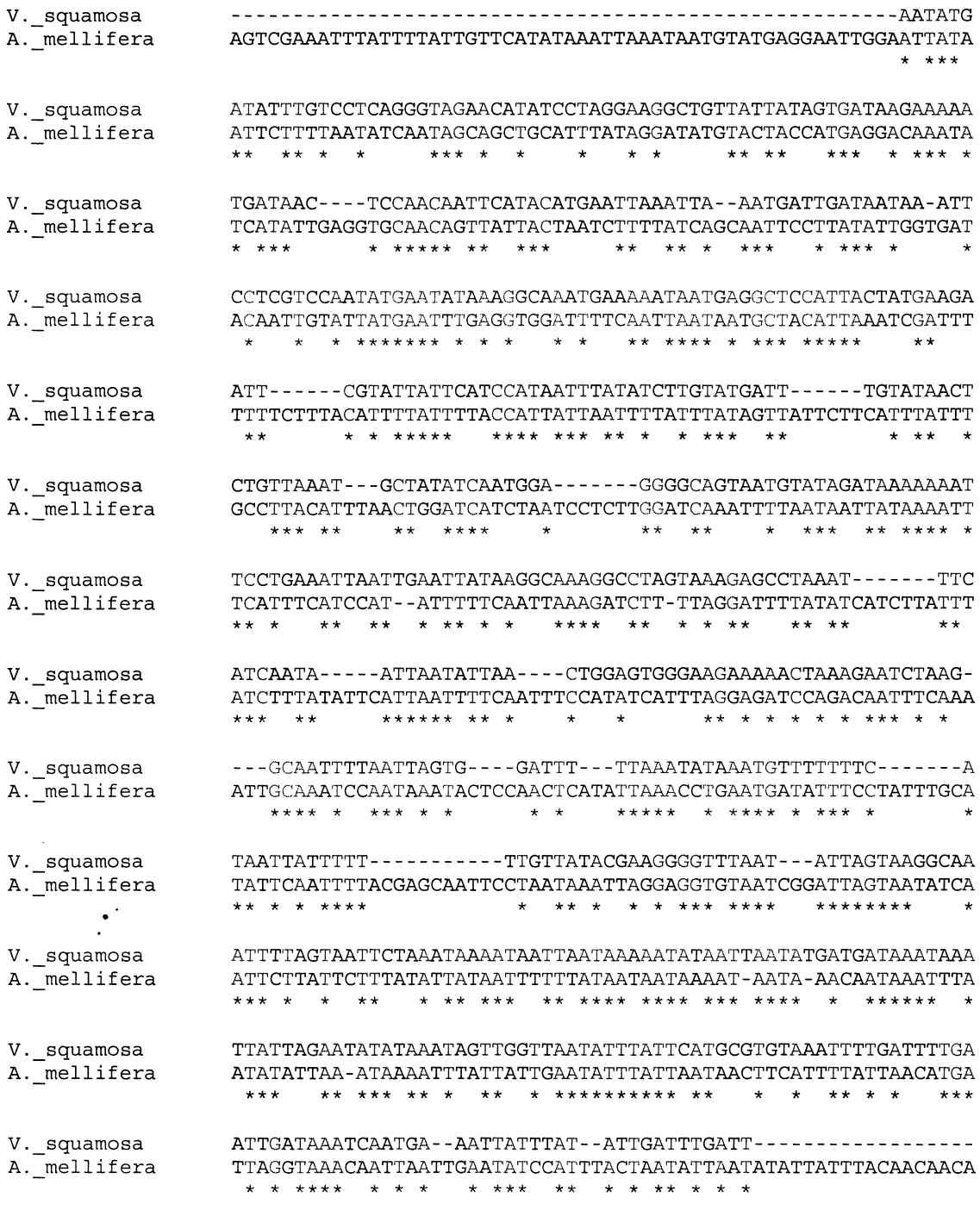

Fig. 6. Sequence alignment for the Honey bee (*A. mellifera*) and the Southeastern yellowjacket (*V. squamosa*).

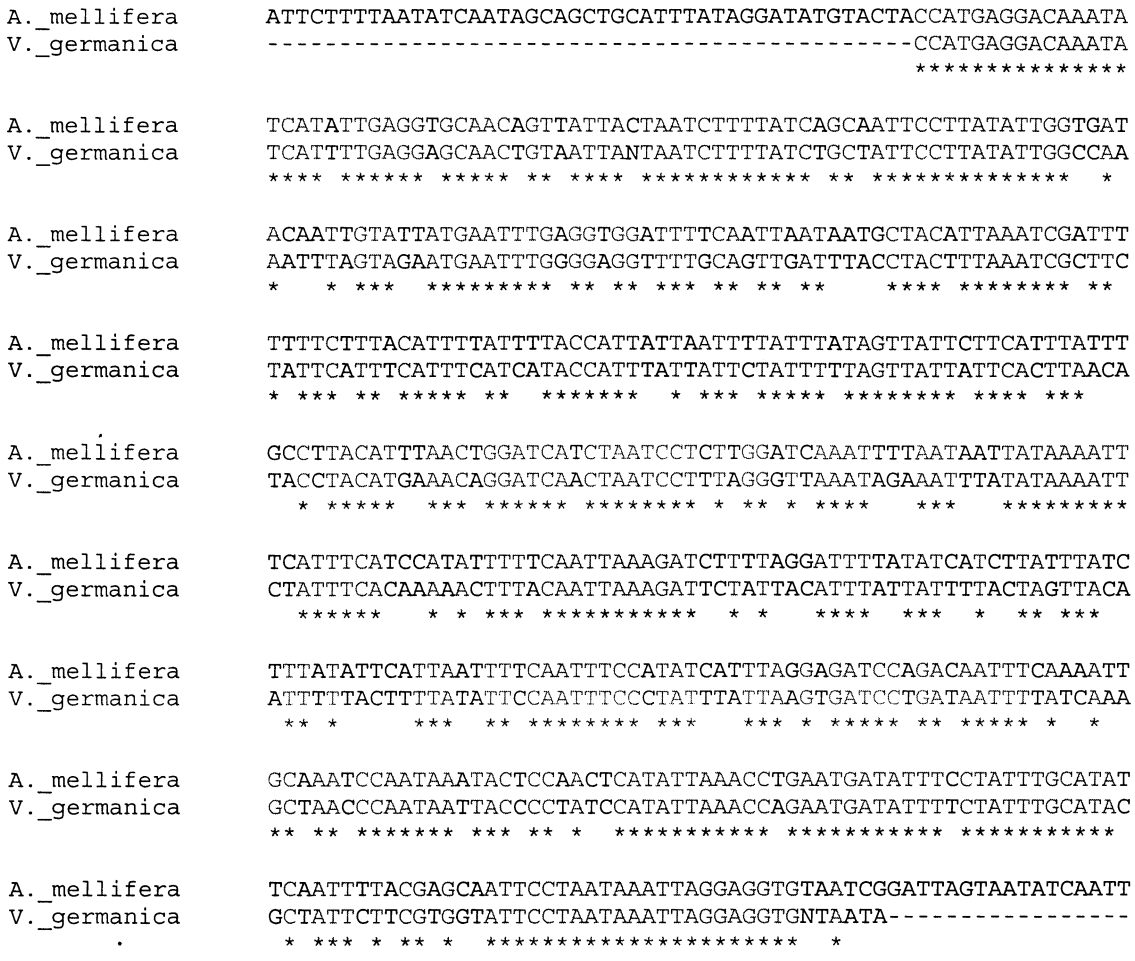

Fig. 7. Sequence alignment for the Honey bee (*A. mellifera*) and the German yellowjacket (*V.germanica*).

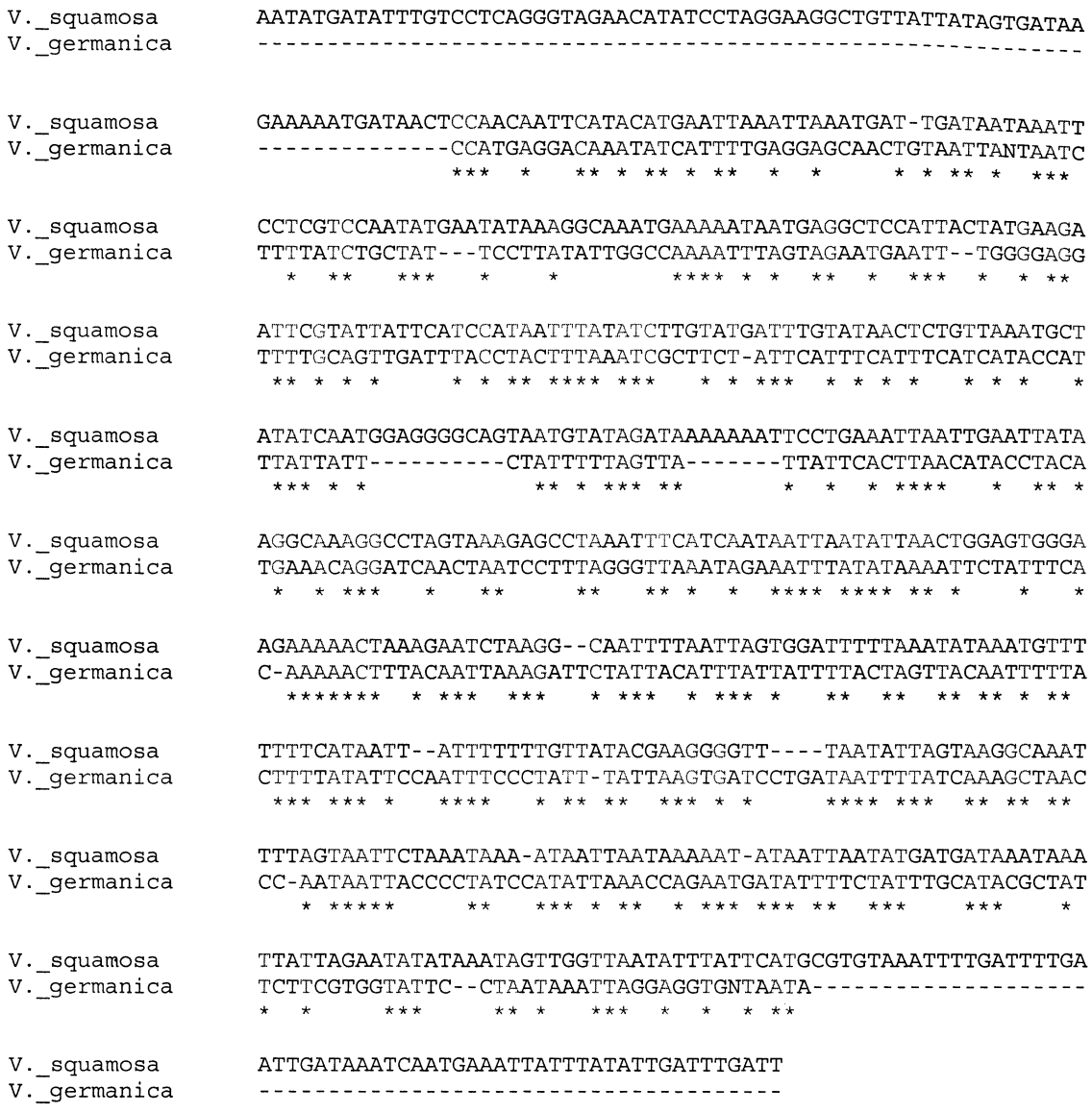

Fig. 8. Sequence alignment for the Southeastern yellowjacket (*V. squamosa*) and the German yellowjacket (*V.germanica*).

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