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Population genetics study of the imported fire ants (Formicidae: Solenopsis spp.)

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POPULATION GENETICS STUDY OF THE IMPORTED FIRE ANTS

(FORMICIDAE: *SOLENOPSIS SPP*.)

By

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The study was divided into three main sections. In the first section, temporal stability of genetic structure of red imported fire ant populations and effective population sizes were assessed with the help of microsatellites. The second part of the study was focused on the development of new microsatellite markers for the population genetics study of fire ants. The third part of the study was aimed at describing the association of phenotypic and genotypic characters of polygyne hybrid (*Solenopsis invicta* x *richteri*) fire ant in a colony and identifying the origin of polygyny in hybrid imported fire ant colony.

Meridian, Yazoo City and Crystal Springs in Mississippi and Mobile, Alabama were selected for conducting the study. The overall F_{ST} estimates and the pairwise F_{ST} estimates between the populations of study, indicated little genetic differentiation and low

spatial variation in the nuclear genetic structure of red imported fire ant. Gene flow estimates indicated that there is extensive movement between these populations. From 2006 to 2008 the F_{ST} estimates decreased and gene flow estimates increased, indicating that there is no equilibrium between genetic drift and migration in the populations studied. Effective population sizes ranged from 10 -17 in these populations.

We have isolated and characterized an additional 11 polymorphic microsatellite loci in the invasive ant *S. invicta* from a population in Lauderdale County, Mississippi. The observed and effective number of alleles ranged from two to six (average 3.1818 $\pm$ S. E. 0.0486). Probability tests indicated significant deviations from the Hardy-Weinberg equilibrium at three loci. The polymorphism information content of the microsatellites ranged from 0.1482 to 0.6208.

Identifying multiple inseminated queens in a colony and sequencing the critical region of *Gp-9* locus (1600 base pairs) in individuals with the polygyne allele strongly indicate the association of phenotypic and genotypic characters of polygyny in a hybrid imported fire ant colony. A 100% match of $Gp\text{-}9^{B}$ and $Gp\text{-}9^{b}$ alleles and concordance in the critical amino acid substitutions of the *Gp-9* locus in the hybrid fire ant with *S. invicta* Buren provides the evidence that polygyny in the hybrid imported fire ant colony is derived from *S. invicta*.

DEDICATION

To GOD: for helping throughout my journey from my childhood to present in all the ways, making my life happy and making all my wishes possible with his gracious blessing and affection.

To Friend: Sujan an unforgettable person in the world who believed in my passion and caliber and helped me as a god's messenger to reach my goals. Without his guidance and encouragement, I would have not reached my goals. This is as much his accomplishment as it is mine.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

History of imported fire ants

Fire ants are considered as New World species primarily occurring in tropical and subtropical areas, belonging to the genus *Solenopsis*, family Formicidae and order Hymenoptera (Buren 1972). The genus *Solenopsis* was derived from the Greek, solen, a pipe or channel, and opsis, appearance or sight. Imported fire ants are a complex of non-native ants in the genus *Solenopsis* which were introduced into United States in the early 20th century. Loding (1929) made the first official collections of imported fire ants in the vicinity of Mobile, Alabama and later identified as *Solenopsis saevissima* var. *richteri*, or black imported fire ant by Creighton (1930).

The history of imported fire ants is short and yet controversial in the United States. Due to their rapid expansion, high reproductive capacity and aggressive stinging behavior they cause considerable damage in both rural and urban environments (Lofgren 1986). The imported fire ants were initially identified as single species, *Solenopsis saevissima richteri* Forel (Wilson 1951), later they were recognized as two species *S. invicta* Buren, the red imported fire ant (RIFA) and *S. richteri* Forel, the black imported fire ant (BIFA) with separate introductions. RIFA are native to the Pantanal region of

southern Brazil and BIFA is native to Uruguay and Argentina (Buren et al. 1974, Ross and Trager 1990 and Tschinkel 1993a).

According to Loding (1929) imported fire ants were possibly introduced in soil ballast from South America into the port of Mobile around 1918 (Vinson 1997). According to George's (1958) hypothesis BIFA arrived with fruit and Trager (1991) thought that RIFA were introduced with cattle shipments which created a controversial history to the introduction of imported fire ants. BIFA were introduced earlier than RIFA in Mobile and it was believed that the spread of BIFA was hindered by another introduced ant occurring in the same place, the Argentine ant, *Linepithema humile* (Loding 1929).

The red imported fire ants were noticed in the late 1930's or early 1940's and were described by Smith (1949) and Wilson (1951, 1959). According to these scientists they were introduced into Mobile by discarded soil ballast and invaded United States and dominated black forms due to their greater adaptability to environmental conditions. Schmidt (1995) suggested from the genetic evidence, between five and fifteen mated RIFA queens made the initial colonization, there may have been multiple invasions (Ross et al. 1985 and Taber 2000).

Buren (1972) studied both forms and concluded they were two different species, based upon the lack of evidence for hybridization and phenotypic variability (Lofgren 1986). Buren assigned the current scientific names to the red and black forms. As Buren believed that the control of RIFA is difficult, he named the red form invicta which means "invincible". Some researchers believe that BIFA had laid path for RIFA's success by competing with the native southeastern United States ants before RIFA's arrival (Lofgren

et al. 1975, Taber 2000). Hybridization between the two species was recently discovered based on chemical data on venom, hydrocarbons and trail pheromones in areas of Mississippi and Alabama (Vander Meer et al. 1985) and parts of Georgia (Vinson 1997).

RIFA has spread throughout the southern region of United States from North Carolina to Florida and westward to Texas, Arkansas, Arizona, New Mexico and California. RIFA were found in Oklahoma, Tennessee, Puerto Rico and Hawaii (Vinson 1997 and Taber 2000). RIFA have infested over 106 million hectares in the United States (Vinson 1997). Natural mating flights can result in long distance dispersal of mated queens when they land on pickup trucks, railroad cars and trailers. The spread of RIFA is also facilitated by movement of sod, nursery stock, soil, electrical and telecommunications lines. The spread of RIFA northward may be slowed down by the colder temperatures (Allen et al. 1995). Areas with seasonal flooding are prone to be rapidly invaded with RIFA invasions, while other ant species are killed by seasonal flooding. Currently BIFA is only found in small areas of northeastern Mississippi, along the central and northern borders of Mississippi, Alabama and Tennessee (Taber 2000).

Biology of imported fire ants

The biology of RIFA and BIFA are similar unless otherwise noted. The main difference between these two species is their aggressive behavior and color. RIFA are more aggressive than BIFA and are small and reddish brown to dark brown (Buren 1972, Lofgren et al. 1975), whereas BIFA are dark brown to black (Buren 1972, Taber 2000). RIFA and BIFA are polymorphic, which means ants occur in more than two sizes and live in colonies, the polymorphic forms include alate males, alate females, dealated

females (queens), major workers and minor workers (Green 1952). Males have highest metabolic rate in the colony and they participate in highly energetic mating flights which will take place outside the nest (Vogt and Appel 1999, Taber 2000). The size of worker ants ranges from 2.5 to 4.5 mm in length (Freeman 1993). Queens can reproduce for five to seven years, laying up to 5,000 eggs/ day and at maturity each colony may consist of 200,000 workers (Tschinkel 1993a) and produce an estimated 4,500 new queens/year. The dispersal of fire ants to new places is by flight or on foot (Maxwell et al. 1982). Newly mated queens take a period of 15 – 18 weeks to produce sexual alates after the establishment of a new colony.

The establishment of colonies in ants takes place in one of the following ways: 1) unassisted by the workers independently, 2) assisted by the workers known as dependently and 3) assisted by workers of other species known as temporary social parasitism (Holldobler and Wilson 1990). A fourth method was also found in RIFA by Tschinkel et al. (1995). He found that sexuals produced in the late summer to fall, over-wintered and mated in the early spring. The mated individuals enter orphaned colonies and exploit them to produce a new colony by intra-specific social parasitism.

Among all the ways mentioned above, the most common method is via independent formation. In this method workers bore numerous exit holes one hour before the nuptial flight in the mound which helps the males and female sexual alates escape in the late spring or early summer and participate in nuptial flights (Markin et al. 1971, Tschinkel 1993a). Workers move around the nest by fanning out to form a moving circle and protect the sexual alates. On an average each mound produces about seven hundred males, and virgin queens and may leave the nest at rates of up to one hundred per minute

(Tschinkel 1993a). The dispersal of queens takes place by flights up to a kilometer or more, and up to 11- 19 kilometers with the help of wind currents (Markin et al. 1971, Lofgren et al. 1975). The newly mated queens shed their wings and start to establish a colony by digging chambers and rearing the first workers by themselves or in cooperation with other newly mated queens (Markin et al. 1972, Tschinkel and Howard 1983, Tschinkel 1993a). Males die shortly after mating and do not help in colony founding. The mated queens prefer to land near reflective surfaces which gives them a chance of landing near water (Bhatkar 1990). Vinson and Sorrenson (1986) found in their study that the queens may fly or carried by wind as far as 19.3 km from their original nests searching for a suitable landing site.

Two forms of fire ant colonies occur. One is monogynous with a single queen and the other is polygynous with more than one queen. The monogynous forms are territorial, aggressive, and disperse by flight (Vinson 1994). The polygynous forms are less aggressive and typically disperse on foot. The density of monogynous colonies range up to 200 mounds/ha, whereas the polygynous colonies density is six times greater than monogynous with numbers up to a 1000 mounds/ha (Vinson 1994).

RIFA usually prefer developed forested areas which include state parks, commercial orchards and mostly open pasture land (Taber 2000). They are less common in the woods. Mounds are also found in open sunny areas such as: clear cuts, replanted forests and margins of highways, seasonal pond margins, highway tracks, along vehicle tracks and frequently occur in and around human constructs. According to Buren (1972) fire ants dominates in altered habitats. This is supported by many cases of their unique ability to withstand disturbances and able to mitigate adverse conditions. An example of

this kind was described by Rhoades (1977); he discovered a RIFA colony on a Florida sandbar which was covered by salt water tides twice a day.

RIFA can be described as weedy species with R-selected reproductive strategy (Tschinkel 1986), having high rates of reproduction, efficient methods of mass dispersal and their ability to specialize at colonizing and dominating disturbed habitats (Taber 2000).

Mounds are the first sign of fire ants which we notice, in which their brood is reared. The colony is initiated when a queen or queens land after copulation. The queen digs a vertical tunnel in the soil and then constructs a chamber. One day after, the entrance will be closed from the inside and the queen starts laying eggs which is known as claustral founding (Markin et al. 1972). The shape and size of the mound tends to change according to the season and the weather conditions. In Brazil, in the native range of fire ants, the sizes of mounds are small in dry season and large in the wet season. The mound size in Brazil is smaller through out the year when compared to the mounds in the United States (Wojcik 1986). Mounds found in Tallahassee, Florida tend to be oval and oriented in north-south direction allowing them to warm up efficiently (Hubbard and Cunningham 1977). Substantial amounts of precipitation after a dry hot season or changing seasons generally signal the return of mound-building activity (Barlett and Lofgren 1961).

The dimensions of the mound depend upon the soil type and may reach up to 30-40 cm in height (Vinson 1997). The brood is reared in the above-ground portion of the mound. The workers play an important role in identifying the changes in temperature and humidity in the above-ground portion of mound and help in moving the brood to the ideal

environment for development. Each mound consists of several brood rearing chambers and foraging tunnels radiating from the center of the mound (Wilson et al. 1971). More than half the volume of the mound consists of galleries and tunnels (Tschinkel 1993a). Fire ants also construct lateral foraging tunnels which allow workers to cover greater distances underground, when soil surface temperatures are lethal for their movement (Porter and Tschinkel 1987). The critical thermal limits for the cessation of foraging in RIFA average between 40.7 ^{o}C maximum and a minimum of 3.6 ^{o}C (Cokendolpher and Phillips 1990). RIFA colonies are regulated naturally by competition between neighboring colonies. The total ant biomass should be used to describe whole colony, rather than mound density (Adams and Tschinkel 2001).

Behavior of imported fire ants

Imported fire ants feed on invertebrates, vertebrates and plants (Vinson 1994, Macom and Porter 1996). They are considered as omnivorous and opportunistic feeders (Hays and Hays 1959, Wilson and Oliver 1969, Morill 1978, Tschinkel 1982). The foraging ants are more scavengers than predators (Young 1984). RIFA even utilize fungi and bacteria for food (Vinson 1992 and Jouvenaz et al. 1996). The diet sources include tree leaves, nectar, bark, sap, fruit, roots, herbaceous plants, insects and other arthropods (Reagan 1986), reptiles (Whiting 1994), birds (Adams 1986), insect excretions (Tennant and Porter 1991) and mammals (Mitchell et al. 1996, Scott et al. 1987).

In the communal activities of fire ants, soil temperature plays an important role. In the spring, brood production begins when the soil temperature rises above 10 ^{o}C at five centimeters from the top of mound, worker and reproductive pupae appear at 20 ^{o}C, and

alates appears at 22.5 ^{o}C (Markin et al. 1974). An average temperature of 24 ^{o}C is required for successful colony founding by newly mated queens (Markin et al. 1974). The period of colony founding was determined to range from 83 days in northern Mississippi to 198 days in Florida with the help of average temperature prevalent in these areas. The ambient air temperature ranged between 23.9 ^{o}C and 32.2 ^{o}C and relative humidity readings were 80 per cent or higher for the occurrence of most of the mating flights observed by Rhoades and Davis (1967).

Problems and control measures for red imported fire ant

RIFA's aggressive behavior combined with high densities has multiple impacts on human life. An estimated 2.5 million people were stung monthly by RIFA in highly infested areas of Texas (Brown 1982), hindering their normal outdoor activities. This affects the tourism (Davidson and Stone 1994) and real estate industries and reduces property values (Lofgren et al. 1975, URL a.). Each year they cause an estimated six billion dollars worth of damage in the United States (ARS 2003). RIFA feed on many crop species and they cause problems to cultivated crops (Vinson 1994). They not only feed on crops but also cause damage to farm machinery, irrigation systems and influence harvesting efficiency. They attack and occasionally kill young calves and other domestic animals (Lofgren et al. 1975, URL a.). RIFA attack roots, tubers of potatoes, sunflowers and cucumbers (Adams et al. 1988, Stewart and Vinson 1991). They feed on plants and fruits of soybean, eggplants and okra (Lofgren et al. 1975) and they cause damage to citrus and pecan orchards (Tedders et al. 1990, Banks et al. 1991).

Reports suggested that RIFA impacted natural ecosystem by disturbing the plant assemblage and wildlife. They attack eggs of several species such as wood duck (Ridlehuber 1982), colonial water birds, turtles, lizards (Vinson 1994) and crested caracara (Dickensen 1995). The aggressive nature of RIFA played an important role in declining populations of northern bobwhite quail in the southeastern United States and native invertebrate communities (Allen et al. 1995).

Different control measures have been developed to manage growth and spread of RIFA including quarantine, bio-control and chemical methods. Insecticide control was most effective (Williams 1994). However, if the treatments are not 100 percent effective RIFA colonies relocate to another mound (Green 1952, Banks 1994). For long term control, toxic baits were found more effective when compared to contact insecticide treatments. Insect growth regulators also provide effective long term control (Banks 1994).

USDA has initiated a quarantine in all fire ant infested areas restricting the movement of queens and colonies in soil, potted plants, sod and hay to fire ant free areas. In spite of quarantine measures implemented by USDA, RIFA has continued to invade new areas. Insecticides also play an important role in RIFA spread as they are not always species specific eliminating beneficial invertebrates (Apperson and Adams 1983). According to Tschinkel (1993b) reduction in native fauna helped in removal of natural impediments to the fire ant spread and increased the rate of fire ant colonization. Showler and Reagan (1987) also supported the above theory; insecticides simultaneously suppressed many ant species like *Conomyrma flavopecta*, *Diplorhoptrum* sp., *Iridomyrmex pruinosum, Monomorium minimum, Pheidole dentata, Solenopsis geminata*

and *S. xyloni*. After environmental disruptions and insecticide applications RIFA have natural resurgence power (Buren 1983). Whitcomb et al. (1972) and Reagan et al. (1972) found a reduction in ant faunal diversity in the areas infested by RIFA in soybean and sugarcane fields which were regularly treated with insecticides.

Currently research is focused on biological control due to ineffective results of chemical treatment. *Pseudacteon* genus (Diptera: Phoridae) are specialized *Solenopsis* parasitoids. These flies are species specific and appear to reduce and maintain RIFA colonies below the economic threshold (Gilbert 1998). Greenhouse reared phorids were released in Texas and Florida with partial success (Taber 2000). It will take few years of research for consideration on a wide spread scale.

Importance of population genetics

Population genetics deals with Mendel's laws and other genetic principles which affect entire populations of organisms. These populations may be natural, agricultural or experimental and includes human beings, animals, plants, insects and microbes. Studies of population genetic structure provide ways to examine the roles of evolutionary forces such as selection, gene flow and genetic drift play in processes such as local adaptation and speciation (Barton and Clark 1990, Avise 1994, Slatkin 1994, Foster et al. 1998). The fundamental goal of population genetics is to understand the relative importance of microevolutionary forces such as gene flow, selection and genetic drift in determining the existence of genetic variation within a species (Black et al. 2001).

Knowledge about adaptation plays an important role in entomology. The genetics of adaptation is mainly about the genotypes favored by natural selection in a population

and replace other genotypes which lead to change the traits within populations. Previously the genetics of adaptation has been approached in different ways: by quantitative genetic analysis of phenotypic variation, by marker based population genetics and by single-gene enzyme polymorphism analysis. Some entomologists are interested in phenotypic evolution in which molecular techniques are applied to know genetic causes for the phenotypic changes. Population genetic theories and statistical tools help to interpret the microevolutionary forces acting on genes. In combination the above strategies lead to a description of the genetic basis for phenotypic variation and the evolution of genes that lead to increased adaptation and helps in probing into problems in evolutionary entomology such as insecticide resistance, plant-herbivore interactions, pathogen transmission, sex determination, pheromone use and the genetics of speciation (Black et al. 2001).

'Gene' refers to a region of DNA that code for RNA, whether the RNA is modified and translated into enzymes and protein, or transforms into ribosomal RNA or transfer RNA. 'Locus' refers to a particular region of DNA sequence whether it codes for RNA or not. Locus specific effects include mutation, recombination, selection and assortative mating which affect one or a few genes at a time (Black et al. 2001).

Allozymes are the most commonly used protein markers which are separated on starch, polyacrylamide or cellulose acetate media. Allozymes of a given enzyme are products of different alleles at a specific locus. These show heterozygous banding patterns of increasing complexity which depends on the number of enzyme sub-units present, e.g. monomers, dimers, trimers etc. In most of the cases the interpretation of allozyme data should be done carefully, in some instances the molecular markers

indicated more restricted gene flow in the populations where as allozyme markers indicated extensive movement across a geographic region (Hale and Singh 1991, Karl and Avise 1992, Krafsur 2002). Generally estimates of gene flow from DNA markers and allozymes differ substantially (Baruffi et al. 1995, Martel et al. 2003).

We have seen a paradigm shift from population genetics to population genomics during the last decade with the discovery of genetic markers which can be analyzed simultaneously in a single organism. The important technologies in developing population genomics of insects include first, the polymerase chain reaction (PCR) which helped to amplify many loci from the small amounts of DNA. Second, a variety of highly polymorphic DNA markers has been developed which include microsatellites, random amplified polymorphic DNAs (RAPD's), and amplified fragment length polymorphisms (AFLP's) and finally statistical algorithms were developed to analyze the data. Locus specific and genome wide effects can be distinguished by examining the variation at many loci or segregating nucleotides within a locus. The impact of the above technologies on genetic studies of adaptation in insects is profound. Entomologists can now choose genetic markers to analyze the whole genome and draw inferences regarding multilocus disequilibrium, selection, random mating, gene flow, effective population sizes and relatedness among insect populations. Population genetic analysis can be conducted by using variable loci dispersed throughout the genome and among polymorphic nucleotide sites within individual genes. Locus-specific effects include selection, mutation, recombination, and nonrandom mating and these loci also influence genome wide effects that include genetic drift, migration and inbreeding (Black et al. 2001).

Several studies have been conducted in insects using highly variable restriction fragment length polymorphisms (RFLP's) and AFLP's to describe the geographic origin and dispersal of insect populations (Scataglini et al. 2000, Katiyar et al. 2000, Kim and Sappington 2004a). These markers can be developed without the prior knowledge of the DNA sequences of the species of interest. These are highly polymorphic and dominant. These are useful for differentiating closely related populations. Homozygous and heterozygous individuals are indistinguishable. This makes it difficult to describe the genetic structure and allele frequencies. They do not help in estimating the deviations of Hardy Weinberg equilibrium directly. RAPD, RFLP and AFLP data may give insufficient information in evaluating the genetic structure of populations (Kim et al. 2002, Kim and Sappington 2004b).

Microsatellites are short tandem repeats of DNA sequences which are highly polymorphic with 10^{-3} to 10^{-5} estimated mutation rate per generation in mammals (Dallas 1992 and Ellengren 1995) and 10^{-6} in *Drosophila* (Schug et al. 1997). These have been successfully utilized for population studies in many animal species. Microsatellites are increasingly applied in population studies of insects (Hufbauer et al. 2004, Brouat et al. 2004, Kim and Sappington 2004 c, 2005). Microsatellites are co-dominant markers which help to assess deviation from Hardy-Weinberg equilibrium directly, genetic structure within populations and gene flow among populations. These are valuable for inferring values of genetic variation and patterns of population structure among closely related or recently diverged populations like invasive RIFA in the United States. Hence we believe they are the best choice for assessing population genetic structure, temporal stability, effective population sizes and gene flow.

The major applications of population genetics theory have been in the estimation of genetic and demographic properties of populations. Migration is the movement of genes within a population or between two populations which causes genetic admixture. Gene flow or migration creates new combination of genes or alleles in the individuals between two different populations.

Genetic drift is the alteration of allele frequencies due to sampling variation from one generation to the next and is a chance process. Genetic drift leads to loss of polymorphism within subpopulations and increased genetic differentiation between subpopulations.

Gene frequencies drift in local populations, but alleles lost locally may be restored by migration from other populations. High migration homogenizes variation in allele frequencies between populations; low migration allows differentiation by genetic drift. The balance between genetic drift and migration is reflected as population structure across the species. In population genetics the concept of effective population size N_e was introduced by the American geneticist Sewall Wright, (Wright 1931, 1938). He defined it as the number of breeding individuals in an idealized population that would show the same amount of dispersion of allele frequencies under random genetic drift or the same amount of inbreeding as the population and it is a basic parameter in many models in population genetics. The effective population size is usually smaller than the absolute population size.

From population genetic data, quantities such as mutation rate, migration rate and recombination rate can be estimated, at least as products with the effective population size. The variance effective population size (N_e) is an important quantity in evolutionary

biology, which helps in describing the rate at which the genetic variance changes due to genetic drift. The possibility of estimating effective population size from temporal changes in allele frequencies is known as the temporal method (Krimbas and Tsakas 1971, Begon et al. 1980, Nei and Tajima 1981, Pollak 1983, Tajima and Nei 1984). A disadvantage of the temporal method is the effect of migration is ignored which bias the estimates of N_e.

The estimation of N_e can be affected by migration in two ways. In the short term, migration can change allele frequencies quite quickly. In the long term, constant migration and drift would cause the population to approach an equilibrium level of genetic differentiation. A deme often refers to any isolated subpopulation subjected to selection as a unit rather than as individuals. The rate of change of allele frequencies in a deme would slow down the approach which is predicted by the effective size of the whole metapopulation. The effective population size of the deme would be substantially over estimated in the long term. This can be avoided by simultaneously estimating N_e and the migration rate jointly (Wang and Whitlock 2003).

Another approach of estimating N_e is by assessing the change in allele frequencies measured in a population sampled at different times, assuming that the observed change in allele frequencies is caused by genetic drift (Krimbas and Tsakas 1971). This temporal method has been applied using moment estimators (Nei and Tajima 1981, Waples 1989, Jorde and Ryman 1995).

The RIFA has emerged as a case for investigating the consequences of variation in patterns of gene flow and routes to possible speciation (Ross and Keller 1995). The fire ant represents an excellent model system to conduct detailed studies of genetic structure

using many markers of multiple classes. The RIFA which was introduced into U.S.A from South America in the 1930s (Lofgren 1986), was the subject of a vast literature concerning the natural history, social biology and breeding biology in the introduced range. A considerable amount of information was available on the basic population genetics of both native and introduced fire ants (Ross 1993, Ross et al. 1993, 1997, Ross and Shoemaker 1997).

Ross et al. (1999) studied the genetic structure of RIFA between Georgia and Louisiana with multiple classes of molecular markers. F_{ST} (0.06 to 0.07) estimates with microsatellites revealed that significant site differentiation was observed between the sites of study. This differentiation may be due to isolation by distance, socially induced barriers and due to genetic drift.

Studies of population genetic variation in different locations such as Texas, Louisiana, Mississippi, Georgia, Florida and Mobile Bay Alabama revealed that there is significant nuclear regional differentiation. The regional differentiation was due to isolation by distance due to spread of fire ants by long distance, human mediated dispersal, with subfounder populations in two different forms of fire ants (Shoemaker et al. 2006).

By using data from several genetic markers surveyed in the source population and the populations in the U.S.A, numbers of founders were estimated in the introduced population of RIFA. The results obtained from different markers like microsatellites, allozymes, sex-determination locus and mitochondrial markers suggested that 9-20 unrelated mated queens comprised the initial founder group which colonized USA at Mobile, Alabama (Ross and Shoemaker 2008).

RIFA was recently introduced into USA and local populations may have not had sufficient time to get into equilibrium between migration and drift. RIFA queens have a long generation time which may extend the time required to attain equilibrium between drift and migration. The differentiation between populations may therefore be due to a recent introduction rather than a balance between drift and migration. We hypothesize that there is substantial gene flow between these populations which decreases variation among these populations making them relatively uniform. An alternate hypothesis is that the differentiation is due to recent introduction.

With this rationale, a study was initiated to assess the following

- To assess genetic differences of RIFA among different places and to estimate effective population sizes in the presence of gene flow.
- To assess the temporal stability of population structure and genetic diversity of RIFA.

Polymorphism in microsatellites helps in finding more variation within and between populations. This plays a key role in population genetics study of fire ants. Earlier reports suggest a fewer number of polymorphic microsatellites in RIFA. In this regard an objective was designed

- To develop additional microsatellites in RIFA for studying the population genetics of fire ant populations.

During our collections, we found some hybrid fire ant polygyne mounds in Lauderdale County, Mississippi. Earlier studies in hybrid fire ants described phenotypic and genotypic characters separately. A study was initiated with the following objectives.

- To describe the association of both phenotypic and genotypic characters of polygyne hybrid imported fire ant colony.
- To identify the origin of polygyny in hybrid imported fire ant colony.

All the objectives of the study will be described in detail in the upcoming chapters.

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CHAPTER II

EFFECTIVE POPULATION SIZES AND TEMPORAL STABILITY OF GENETIC STRUCTURE IN THE INVASIVE SPECIES *SOLENOPSIS INVICTA* BUREN, THE RED IMPORTED FIRE ANT

Abstract

Earlier population genetics studies of the red imported fire ant (RIFA) did not investigate the temporal stability of the genetic structure of RIFA. Understanding the temporal stability of genetic structure in the introduced species would help to determine the extent of genetic equilibrium between genetic drift and migration. RIFA was introduced in the late 1930's or early 1940's near Mobile, Alabama and their queens can reproduce up to 5-7 years extending the time required for these populations to approach equilibrium between genetic drift and migration. Meridian, Yazoo City and Crystal Springs in Mississippi and Mobile, Alabama were selected for conducting the study. The overall F_{ST} estimates and the pairwise F_{ST} estimates between the populations of study, indicated little genetic differentiation and low spatial variation in the nuclear genetic structure of red imported fire ant. We also estimated the effective population size of the three Mississippi populations from the Mobile, Alabama population. The overall F_{ST}

values (0.06-0.03) indicated a decreasing trend over time (2006 to 2008). Pair wise F_{ST} values between these populations also indicated a decreasing trend over time. The gene flow estimates (2.1 - 2.4) are high and are increasing over time. Effective population sizes ranged from 10 -17 in Mississippi populations. The results indicated that the populations in the study are not in equilibrium between genetic drift and migration. The overall F_{ST} values and pairwise F_{ST} values indicated low spatial variation. Gene flow estimates indicated extensive movement among these populations.

Introduction

Red imported fire ant (RIFA) *Solenopsis invicta* Buren (Hymenoptera: Formicidae) was believed to be first introduced into USA in late 1930's or early 1940's. The first recorded invasion occurred in Mobile, Alabama, believed to have been in soil used as ballast or dunnage in ships. By 1953 they had spread to ten surrounding states. At present RIFA is found in Alabama, Arkansas, California, Florida, Georgia, Louisiana, Mississippi, North Carolina, Oklahoma, Arizona, New Mexico, California, South Carolina, Tennessee, Texas, and Puerto Rico. The spread of RIFA northward may be limited by the colder temperatures (Allen et al. 1995). Areas with seasonal flooding were prone to be rapidly invaded with RIFA. The other ant species are killed by seasonal flooding, whereas the RIFA form floating mats of ants which can survive for several weeks. *Solenopsis invicta* is reported to be native to the Pantanal region of southern Brazil in South America (Allen et al. 1995).

A mature colony of RIFA consists of 100,000 to 500,000 polymorphic workers, several hundred reproductive winged males and females, one or more reproductive

queens, and brood that includes eggs, larvae and pupae. Workers are wingless and sterile females. The heads and black bodies of winged males are smaller than those of red-brown females. The size of the worker ranges from 5 to 10 mm. The lifespan of fire ant workers depends on their size, minor workers may live 30 to 60 days, medium workers 60 to 90 days, major workers 90 to 180 days, and queens may live five to seven years. A complete lifecycle from egg to adult takes between 22 and 38 days (Hedges 1997). The pedicel in the RIFA consists of two segments. Workers consist of many sizes (polymorphic) between 2.4 to 6 mm (1/8 to 1/4 in) (Hedges 1998). The mandible has four distinct teeth and the antennae are 10-segmented, ending in a two-segmented club. A sting is present at the tip of the gaster. Body color is usually red to brown in color with a black gaster (Hedges 1997).

Late spring and early summer are favorable periods for mating flights. Males die after mating and the mated female locates a suitable site for a nest, sheds her wings and excavates a small chamber in the soil in which eggs are laid within 24 hours. At first, 10 to 15 eggs are laid and the female cares for them until they become adults. These first worker ants care for the eggs laid by the queen and other tasks related to the maintenance of the colony. A queen may produce between 1500 and 5000 eggs per day.

RIFA causes economic losses by feeding on agricultural crops. They are serious seed feeders and attack sunflowers, okra, cucumbers, soybeans, corn and eggplant. RIFA also damage irrigation systems and their mounds disrupt harvesting operations. They cause death of livestock such as calves, small pigs and domestic animals. Fire ants are a social menace due to their sting. In extreme rare cases fire ant stings are lethal to humans. RIFA colonies commonly infest electrical equipment such as air conditioners,

traffic signal boxes and other electrical utilities. They can also infest telephone junctions, airport landing lights, electric pumps for oil and water wells, computers and car electrical systems, the ants chew on the insulation or carry soil into these areas and cause short circuits (Vinson 1997).

Total structural and electrical damage was estimated at $11.2 million annually. The total cost due to RIFA was estimated at $ 1 billion per year in the southern United States. *Solenopsis invicta* outcompete and prey on other invertebrates, reducing abundance, biomass and diversity (Allen et al. 1995). *Solenopsis geminata* colonies, a native fire ant, cannot compete against the RIFA. During a period of 3 years in Texas, 180 *S. geminata* colonies were replaced by 1,100 RIFA mounds. This is a replacement ratio of 6:1. Bait traps indicate that ant diversity is lower in areas where RIFA colonies are established. They compete with other taxa for food and alter abundance of prey species (Porter et al. 1988).

Fire ants quickly attack anything that disturbs their nest and they can sting repeatedly. With the help of its jaws the ants grasp the skin and stings 7-8 times in a circular pattern, or releases and moves to grasp and sting again and produce a series of stings (Vinson 1997).

Damage caused to biological communities by exotic species is a serious and growing problem which has enormous ecological and economic impacts (Pimentel et al. 2000, Mooney and Cleland 2001). Invasive insects are capable of causing great damage to animal and human communities. Ecological communities have been disrupted by several species of ants, wasps and termites in many of the introduced ranges (Vinson and Greenberg 1986, Moller 1996). The RIFA which was introduced into U.S.A. from South

America in the 1930s (Lofgren 1986), was the subject of a vast literature concerning the natural history, social biology and breeding biology in the introduced range. A considerable amount of information was available on the basic population genetics of both native and introduced fire ants (Ross 1993, Ross et al. 1993 and 1997, Ross and Shoemaker 1997).

Studies of population genetic structure in invasive species provide ways to examine the roles of evolutionary forces such as selection, gene flow and drift play in processes such as local adaptation and speciation (Barton and Clark 1990, Avise 1994, Slatkin 1994, Foster et al. 1998). The fundamental goal of population genetics is to understand the relative importance of microevolutionary forces in determining the existence of genetic variation within a species. Genetically distinct populations are usually defined on the basis of statistically significant heterogeneity in allele frequencies among samples implying nonrandom mating.

The major applications of population genetics theory have been in the estimation of genetic and demographic properties of populations. Migration is the movement of genes within a population or between two populations which causes genetic admixture. Gene flow or migration creates new combination of genes or alleles in the individuals.

Genetic drift is the alteration of allele frequencies due to sampling variation from one generation to the next and is a chance process. Genetic drift leads to loss of polymorphism within subpopulations and increased genetic differentiation between subpopulations.

Gene frequencies drift in local populations, but alleles lost locally may be restored by migration from other populations. High migration homogenizes variation in allele

frequencies between two different populations; low migration allows differentiation by genetic drift. The balance between genetic drift and migration is reflected in the population genetic structure across the species.

In population genetics the concept of effective population size N_e was introduced by the American geneticist Sewall Wright, (Wright 1931, 1938). He defined it as the number of breeding individuals in an idealized population that would show the same amount of dispersion of allele frequencies under random genetic drift or the same amount of inbreeding as the population and it is a basic parameter in many models in population genetics. The effective population size is usually smaller than the absolute population size.

From population genetic data, quantities such as mutation rate, migration rate and recombination rate can be estimated, at least as products with the effective population size. The variance effective population size (N_e) is an important quantity in evolutionary biology, which helps in describing the rate at which the genetic variance changes due to genetic drift. The possibility of estimating effective population size from temporal changes in allele frequencies is known as the temporal method (Krimbas and Tsakas 1971, Begon et al. 1980, Nei and Tajima 1981, Pollak 1983, Tajima and Nei 1984). A disadvantage of the temporal method is the effect of migration is ignored which modifies the estimates of N_e.

The estimation of N_e can be affected by migration in two ways. In the short term, migration can change allele frequencies quite quickly. In the long term, constant migration and drift would cause the population to approach an equilibrium level of genetic differentiation. The rate of change of allele frequencies in a deme would slow

down the approach which is predicted by the effective size of the whole metapopulation. The effective population size of the deme would be substantially over estimated in the long term. This can be avoided by simultaneously estimating N_e and the migration rate jointly (Wang and Whitlock 2003). Another approach of estimating N_e is by assessing the change in allele frequencies measured in a population sampled at different times, assuming that the observed change in allele frequencies is caused by genetic drift (Krimbas and Tsakas 1971). This temporal method was applied using moment estimators (Nei and Tajima 1981, Waples 1989, Jorde and Ryman 1995).

The RIFA has emerged as a case for investigating the consequences of variation in patterns of gene flow and routes to possible speciation (Ross and Keller 1995). The fire ant represents an excellent model system to conduct detailed studies of genetic structure using many markers of multiple classes.

Using allozymes, genetic markers, microsatellites and other population genetic tools one can gain useful information about the populations. Microsatellites exhibit a high level of polymorphism compared to other markers and protein encoding loci and they are co-dominant, showing two bands on electrophoresis for heterozygous individuals.

Ross et al. (1999) studied the genetic structure of RIFA between Georgia and Louisiana with multiple classes of molecular markers and F_{ST} estimates with microsatellites revealed that significant site or region differentiation was observed due to isolation by distance, socially induced barriers and due to genetic drift and migration equilibrium.

Studies of population genetic variation in different locations such as Texas, Louisiana, Mississippi, Georgia, Florida and Mobile Bay, Alabama revealed that there is significant nuclear regional differentiation between the regions in study. The regional differentiation was due to isolation by distance due to spread of fire ants by long distance, human mediated dispersal, with subfounder populations in two different forms of fire ants (Shoemaker et al. 2006).

RIFA is introduced into USA in the late 1930's or early 1940's and local populations may have not had sufficient time to get into equilibrium between migration and drift and show spatial variation. RIFA queens can reproduce up to 5-7 years which may extend the time required to attain equilibrium between drift and migration. The differentiation between populations may therefore be due to a recent introduction rather than a balance between drift and migration. We hypothesize that there is substantial gene flow between these populations which decreases variation among these populations making them relatively uniform. An alternate hypothesis is that the differentiation is due to recent introduction.

With this rationale, a study was initiated to assess the following

- To assess genetic differences of RIFA among different places and to estimate effective population sizes in the presence of gene flow.
- To assess the temporal stability of population structure and genetic diversity of RIFA.

Material and Methods

Fig 2.1 Collection sites for *S. invicta* in Mississippi and Alabama.

Ants were collected from four different sites which include Yazoo City, Meridian, Crystal Springs from Mississippi and Mobile, Alabama which is thought to be the original point of entry (Fig 2.1). The distance between Mobile to Yazoo City, Meridian and Crystal Springs study site is approximately 373.36, 218.87 and 294.50 km, respectively. Within each site 12 - 15 colonies were sampled. A minimum of 0.16 km was maintained between colonies sampled. At each site approximately 4.82 – 11.26 sq. km was covered. From each colony 10-15 ants were collected in ethanol for genetic

analysis. A total of 100 to 150 worker ants were collected using an aspirator and placed in 5 ml of hexane from each colony separately for GC-MS analysis.

The species of the fire ants was identified by gas chromatography and mass spectrophotometry (GC-MS). Ants were allowed to soak in hexane for at least 2 days to transfer cuticular hydrocarbons and venom alkaloids from the ants. Hexane samples were taken to the USDA Agricultural Research Service in Stoneville, Mississippi and GC-MS analysis for species identification was performed as described in Menzel et al. (2008).

The social form of each colony was determined by polymerase chain reaction (PCR) using primers designed by Valles and Porter (2003). The genetic composition of the monogyne colony can be identified with two alleles for the queen and one allele from the male for each locus by testing up to 8 to 12 ants.

Total genomic DNA was extracted individually from 12 ants in each colony by using DNA extraction kit for arthropods (Epicenter, Madison WI). The head and thorax tissue was placed in 1.5 ml micro centrifuge tubes and placed in liquid nitrogen. The tissue was ground with a pre-chilled sterile pestle. The Epicenter protocol for animal tissues was followed after grinding the ant tissue. Total DNA was eluted in TE buffer and stored at -80^{o} C. Ants were collected in the years 2006, 2007 and 2008 from four sites described above. We used total genomic DNA as a template for assessing the genotypes of fire ants at five polymorphic microsatellite loci. Primers used in the study were described by Krieger and Keller (1997) with modifications. The modified primer pairs and the used microsatellites are given in the Table 2.1

Table 2.1 Microsatellites and their primer pairs used in the study for fragment analysis

LOCUS	CORE REPEAT	PRIMERS (5'- 3')
SOL – 11	**(TC)15**	**F: CCAGTTTATTGCACGAGATTA R: TTGCGATAGGGAAAAAGATACG**
SOL – 20	**(TC)13**	**F: GAAACGCTCCCTCTGTG R: TTGCAAGCATGAAAAATC**
SOL – 42	**(TC)26**	**F: TAAAATTGGGCACTATCAT R: ATTTCCTTCGCCATTGTC**
SOL – 49	**(TC)19**	**F: GTAATAAACAAGTCACCTAAAAT R: TTCTCAAACGGCAACAA**
SOL – 55	**(TC)15**	**F: AACGCCGTGTGCTTTTCTTCTGC R: AGTCTCGCTCGCCTCGCTTTCTC**

The forward primer of each locus was dye labeled in the commercial synthesis and was purchased from Sigma-Proligo (St. Louis, MO). The five microsatellite regions were amplified separately for each using PCR with the help of a thermal cycler (MyCycler® Biorad, CA). The reaction mixture for each loci contained 10x PCR Buffer (100 mM Tris-HCl and 500mM KCl Mg^{2+} free), 0.2mM dNTP's, 1.9 mM Mg^{2+}, 0.125 µM forward primer (for Sol 11, 20, 42 and 49) or 0.0625 µM forward primer (for Sol 55), 0.2 µM reverse primer, 0.5 units of *Taq* polymerase and 100 to 120 ng of template DNA in the total 24 µl reaction. The following cycling profile was used for four loci: Sol 11, 20, 42, and 49: initial denaturation step 94 ^{o}C (30 s), followed by 30 cycles at 94 ^{o}C (30 s), annealing temperature 48 ^{o}C (30 s) and 72 ^{o}C (30 s), and one final elongation step at 72 ^{o}C (2 min). For Sol 55 the initial denaturation step was for 2 min, 30 sec and the annealing temperature was at 60 ^{o}C.

The WellRED dyelabeled forward primers used in amplification, helped for fragment analysis using CEQ genetics analysis system (Beckman Coulter). The fragment

analysis of the PCR product was done in two multiplex reactions. In one multiplex reaction D2-black dye for Sol 11, D3- green for Sol 42 and D4-blue dye for Sol 49 were used. In the second reaction D3-green for Sol 20 and D4-blue dye for Sol 55 were used.

After PCR, for each ant, 2.0 µl of Sol 11, 1.0 µl of Sol 42 and 0.53 µl of Sol 49 PCR products were combined in 41.6 µl of formamide and 0.57 µl of size standard in one reaction. In another reaction with the same above amount of formamide, size standard, 2.0 µl of Sol 20 and 0.53 µl of Sol 55 PCR products were combined. The samples were run in CEQ genetic analysis system (Beckman Coulter) in Life Sciences and Biotechnology Institute (LSBI), Mississippi State University for fragment analysis. The microsatellite genotypes were scored using CEQ System fragment-sizing algorithm without phosphoramadite correction. Allele frequencies, overall F_{ST} values, pair wise F_{ST} values, gene flow, Ne and m were calculated using GENEPOP and MNE software.

Data Analysis

Nearly 180 mounds were collected during this study, but samples collected from colonies that were determined to be polygyne were not included in the study. A total of 131 monogyne mounds were analyzed from four sites, over three years. A minimum of 8 colonies at each site, each year were analyzed. A total of 1310 ants were analyzed with 5 microsatellites, which includes a total of 6550 PCR reactions over three years. Fragment analysis was performed in multiplex reactions with 3 loci in one reaction and two loci in one reaction, a total of 2620 reactions in all the three years. Alleles obtained from 8-10 worker individuals from each colony of the location are entered in the software. This helped in obtaining the whole colony genetic composition (two alleles for the queen and

one allele from the male) in the monogyne colony. Allele frequencies and genotype frequencies for all the loci were estimated by using the software program GENEPOP 4.0 on the web (Rousset 2008). Global tests across loci and populations were conducted for conformity to Hardy-Weinberg expectations (HWE) and the alternate hypothesis of interest for heterozygote excess or deficiency using GENEPOP (Rousset and Raymond 1995). F_{ST}, the standardized genetic variance values were estimated by a weighted analysis of variance Cockerham (1973); Weir and Cockerham (1984). F_{IS} is the inbreeding co-efficient in the allele frequencies. F_{ST} values measure the extent of population differentiation between different spatial populations in allele frequencies. F_{ST}, values were estimated in each year 2006, 2007 and 2008 for overall populations to check temporal stability in F_{ST} values (Raymond and Rousset 1995). Gene flow ($N_m = 0.25(1-F_{ST})/F_{ST}$) estimates were estimated using F_{ST} values (Slatkin and Barton 1989) in three years of research to estimate their movement. Pair wise F_{ST}'s were estimated using standard ANOVA as in Weir and Cockerham (1984) between populations in three years to measure significant differentiation in pairs of populations. Effective population sizes (N_e) and migration rate (m) were jointly estimated using MNE software using samples over space and time (Wang and Whitlock 2003). Null allele presence was detected using Micro-checker in all the populations (van Oosterhout et al. 2004). Genetic distances among all populations were calculated using tools of population genetic analysis TEPGA version 1.3 (Miller 1997) by building a UPGMA tree of Nei's unbiased distance (Nei 1978). Statistical analysis was done to analyze significant differences in the over-all F_{ST} in the three years and pair-wise F_{ST} in the three years by using Kruskall - Wallis test

(Daniel 1990) in SAS (2004) and by using repeated measures for different years, loci, over-all and pair-wise F_{ST} values.

Results

The results from GC-MS analysis for species identification indicated that all the colonies collected in the study were *S. invicta*. Allele frequencies at each marker in each year in all the four places of study are reported in the Appendix A. Over the entire study, alleles from 4 (sol 49 in 2007) to 10 (Sol 42 in 2006) were detected at the microsatellite loci. Most of the loci deviated from Hardy Weinberg equilibrium. Heterozygote excess was detected in most of loci (except Sol 11 in 2007 and Sol 20 in 2008) in all the three years. Null alleles were detected in the loci Sol 42 and Sol 49 in all the years. Overall F_{ST} values for all the four populations decreased over time from 0.0613 (2006) to 0.0372 (2008) but this decrease was not significant over time (Fig 2.2). Pairwise F_{ST} values also decreased over time but this decrease was not significant over time (Fig 2.3). The results from repeated measures also provided that there was no significant decrese over time. Genetic distances between populations were very similar across all the sampling times. Meridian population differed from all the three populations in all the three years. Overall gene flow estimates among populations over three years (2006 - 2.149, 2007 - 2.205 and 2008 - 2.472) increased over years. N_e and m were jointly estimated from Mobile to other places with minimum number of generations over three years. The resulting data for gene flow estimates indicated the presence of movement between populations (N_e = 10 and m = 0.33, N_m = 3.3 (N_e x m) for Meridian, N_e = 11 and m = 0.25, N_m = 2.75 for Yazoo City and N_e = 17 and m = 0.30, N_m = 5.1 for Crystal Springs). The overall F_{IS}, F_{ST}, F_{IT}

and pairwise F_{ST} values for the three years are presented in the Tables 2.2, 2.3, 2.4 and 2.5. The overall F_{IS} values are slightly higher in all the three years.

Discussion

In the current study, nuclear genetic structure of RIFA was assessed spatially and temporally using microsatellites. Genetic structure is one of the important aspects of population biology. RIFA were introduced into United States in the early 1940's or late 1930's. The fire ant queen reproduces for a life span up to 5 -7 years (Tschinkel 1993) and it takes some time for them to get into equilibrium between genetic drift and migration and to show spatial variation. The overall F_{IS} values are slightly higher which indicates the presence of inbreeding in these populations. The reasons for the presence of higher F_{IS} values may be due to the presence of null alleles in two of the loci and using 8 to 10 individuals from the same colony. Individuals from the same colony are siblings; hence the software provided the evidence of inbreeding. The over all F_{ST} and pair wise F_{ST} values estimated during each year decreased over time. Overall gene flow estimates among populations increased over time. N_e and m jointly estimated between Mobile and other populations over time also provided information about their movement (N_e x m = Nm). These results provide evidence of extensive movement of fire ants between these populations and low spatial variation. Earlier reports of spatial variation among fire ant populations may be due to subfounder effects (Shoemaker et al. 2006) caused due to introduction rather than due to isolation by distance or genetic drift. From earlier studies, fire ants were introduced with few founder populations, 9 -20 unrelated mated queens in

Mobile, Alabama (Ross and Shoemaker 2008), which also provides that these little number of founder populations have spread to other places with their rapid expansion.

Table 2.2 Multilocus estimates for fire ants in 2006

Locus	F_{IS}	F_{ST}	F_{IT}
Sol11	-0.0339	0.0490	0.0168
Sol42	0.4424	0.0570	0.4742
Sol49	0.1480	0.0254	0.1696
Sol20	-0.0381	0.0482	0.0120
Sol55	0.0361	0.1314	0.1628
All:	0.1124	0.0613	0.1669

Table 2.3 Multilocus estimates for fire ants in 2007

Locus	F_{IS}	F_{ST}	F_{IT}
Sol11	-0.0980	0.0515	-0.0415
Sol42	0.5348	0.0317	0.5495
Sol49	0.2102	0.1065	0.2943
Sol20	-0.0463	0.0182	-0.0273
Sol55	-0.0165	0.0491	0.0335
All:	0.1217	0.0509	0.1664

Table 2.4 Multilocus estimates for fire ants in 2008

Locus	F_{IS}	F_{ST}	F_{IT}
Sol11	-0.0703	0.0455	-0.0216
Sol42	0.4544	0.0440	0.4784
Sol49	0.1141	0.0429	0.1521
Sol20	-0.0459	0.0247	-0.0200
Sol55	-0.0118	0.0287	0.0173
All:	0.0934	0.0372	0.1272

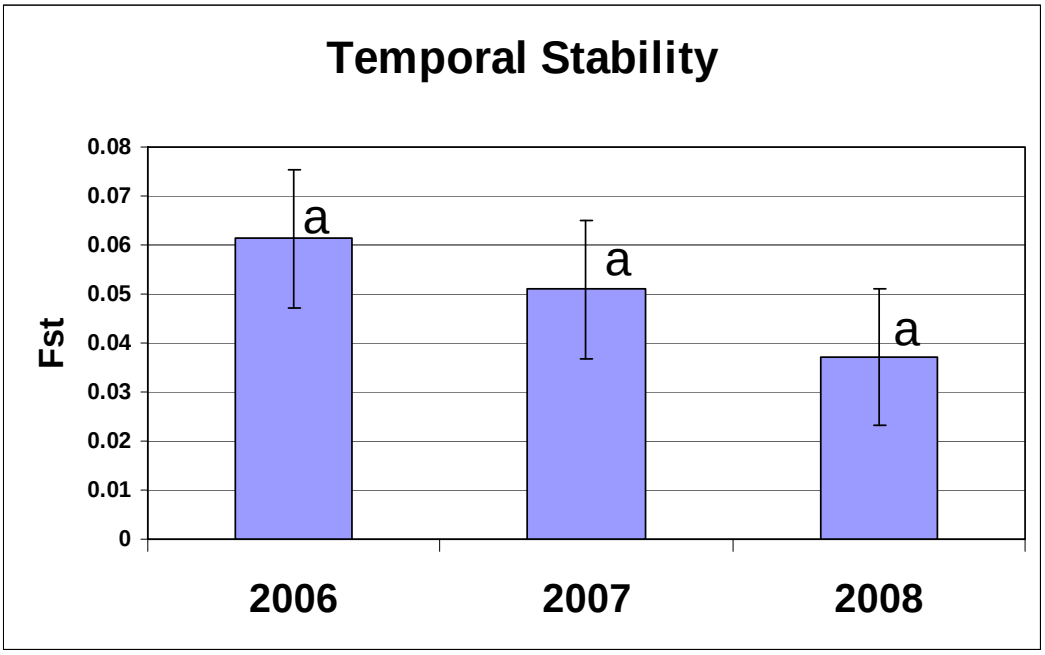

Fig 2.2 Overall F_{ST} of four populations in three different years of study

Table 2.5 Pairwise F_{ST} estimates between population pairs in different years.

Population Pairs	2006	2007	2008
Mobile and Meridian	0.0617	0.0501	0.0425
Meridian and Yazoo City	0.0759	0.0724	0.0512
Meridian and Crystal Springs	0.0717	0.0687	0.0482
Mobile and Crystal Springs	0.0529	0.0344	0.0268
Mobile and Yazoo City	0.0616	0.0453	0.0299
Yazoo City and Crystal Springs	0.0384	0.0314	0.0251

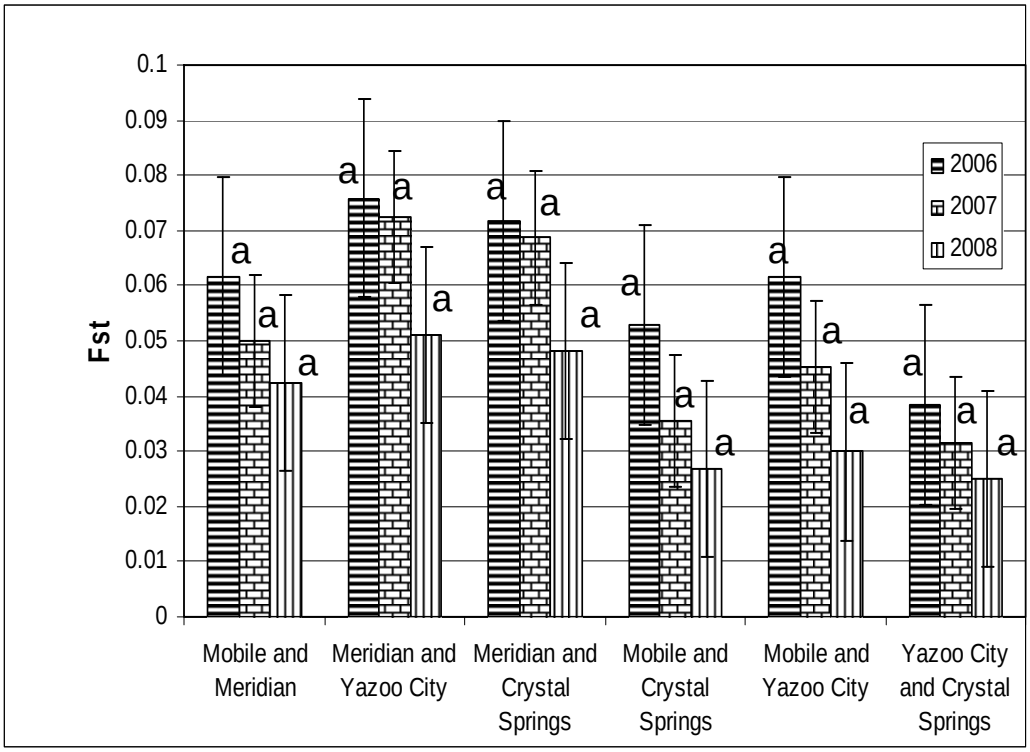

Fig 2.3 Pairwise F_{ST} estimates between population pairs in different years

Low spatial variation in all the populations studied, from the overall F_{ST} also suggests that all these populations may have been originated from a few queens of RIFA which were introduced earlier. From the decreasing trend of F_{ST} values over time and increase of gene flow estimates over time supports that gene flow is homogenizing all the four populations over time and decreasing their differentiation. The main objective of our study was to estimate gene flow between these populations. The data provided evidence that four populations are homogenizing over time and there is little to moderate genetic differentiation between these populations from the overall F_{ST} values. The N_+ values obtained are low (< 20) which is generally normal due to complementary sex determination in the haplodiplody hymenopterans (Zayed 2004). The number of sex alleles found in the introduced populations of fire ants is less when compared to the total sex alleles in the natural populations of fire ants in South America (Ross et al. 1993). This could be another possible cause for the low effective population size of the fire ants in the current study. The heterozygote excess for most of the loci also provided information about population mixing between these four populations. The nuclear genetic structure of these populations is not in equilibrium and these populations are homogenizing over time. There is no equilibrium between genetic drift and migration in the populations studied. In future studies, temporal stability of genetic structure in fire ants in other places away from the source of introduction should be estimated. This helps to estimate gene flow and identify the presence/ absence of genetic equilibrium in other fire ant populations, and in turn helps to understand the expansion and movement of fire ants and the genetic processes that occur during colonization by invasive species.

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CHAPTER III

CHARACTERISTICS OF ELEVEN POLYMORPHIC MICROSATELLITE MARKERS IN THE RED IMPORTED FIRE ANT, *SOLENOPSIS INVICTA* BUREN.

Abstract

We have isolated and characterized an additional 11 polymorphic microsatellite loci in the invasive ant *Solenopsis invicta* from a population within the United States. Primers were developed for the new microsatellite regions, and their variability was tested on 96 worker ants collected from monogyne mounds in Lauderdale County, Mississippi. The observed and effective number of alleles ranged from two to six (average 3.1818 ± S. E. 0.0486) and 1.31 to 2.64 (average 1.9684 ± S. E. 0.0455), respectively. The observed and expected heterozygosity values ranged from 0.1613 to 0.7826 (average 0.4319 ± S. E. 0.0486) and 0.1491 to 0.6242 (average 0.4630 ± S. E. 0.0486), respectively. The polymorphism information content of the microsatellites ranged from 0.1482 to 0.6208. Probability tests indicated significant deviations from the Hardy-Weinberg equilibrium at three loci. Pair-wise tests did not detect linkage disequilibrium between any pair of loci.

Introduction

Microsatellites, which are co-dominant markers, play an important role in population genetics in understanding the role of evolutionary forces such as selection, gene flow, and drift in natural populations. The red imported fire ant (RIFA), *Solenopsis invicta*, is an invasive species of significant economic and ecological importance. The RIFA has emerged as an important case study to investigate the consequences of variation due to place and time and to estimate the patterns of gene flow (Ross and Keller 1995). Unfortunately, few polymorphic nuclear markers have been available for population genetic studies. Here we report the development of 11 new polymorphic microsatellite markers for RIFA.

Material and Methods

Genomic DNA of 12 RIFA individuals from a mound collected at Crystal Springs, Copiah County, Mississippi was extracted using a DNA extraction kit for arthropods (Cartagen, San Carlos, CA). A partial genomic library of RIFA was enriched for di-, tri- and tetra– nucleotide microsatellite sequences following a previously described protocol (Hamilton et al. 1999) and using three groups of biotinylated oligonucleotides as described in Perera et al. (2007). Biotinylated oligonucleotides of group 1 ($[AC]_{13}$ and $[AGC]_6$), group 2 ($[AAC]_6$, $[AAG]_8$, $[ACT]_{12}$, $[ATC]_8$, $[AG]_{14}$), and group 3 ($[AAAC]_6$, $[AAAG]_6$, $[AAT]_6$) were hybridized to size fractionated DNA ligated with an adapter, at 58 ^{o}C, 52 ^{o}C and 48 ^{o}C, respectively. Genomic DNA fragments hybridized to biotinylated oligonucleotides were enriched by capturing with streptavidin coated magnetic particles (Promega, Madison, WI). DNA fragments enriched during the

first round were amplified using the SNX-F primer (5'-CTAAGGCCTTGCTAGCAGAAGC-3') and subjected to a second round of enrichment. The DNA fragments resulting after two rounds of enrichment were ligated into the PCR 2.1 TOPO cloning vector, and One –Shot Mach1 T1 competent cells (Invitrogen) were transformed with the ligation mix. Recombinant clones were selected on LB agar plates containing 50µg/mL kanamycin and 40µg/mL X-Gal. A total of 544 clones were sequenced. SSR Finder software (Sharapova et al. 2002) was used to design the primer pairs for amplifying SSR containing DNA sequences by following the design criteria outlined in Perera et al. (2007). A ´tail sequence´ (5´-CAGTTTTCCCAGTCACGAC-3´), which was identical to the universal fluorescent labeled primer, was added to forward primers to use a universal fluorescent labeled primer in genotyping reactions. A stabilizer sequence (5´-GTTT-3´) was added to each of the reverse primers (Taliercio et al. 2006). The sequence analysis and genotyping were performed at the USDA-ARS, Stoneville, Mississippi using ABI 3730xl instrument (Applied Biosystems). All the amplification reactions were set up with 1-2 µL of approximately 20ng/µL genomic DNA, 0.8 µL of a primer mix [0.4 p_M forward primer, 1.2 p_M reverse primer, 1.2 p_M 6-carboxyfluorescein (6- FAM)-labeled universal primer], 0.1µL of Titanium Taq polymerase (BD Biosciences), 1µL of 10m_M dNTP mix, and 0.5µL of 10x Titanium Taq polymerase buffer in a 5µL volume. Cycling conditions were: initial denaturation for 2 min at 95 ^oC, followed by 1 min at 60 ^oC, and 30 cycles of 15 sec at 95 ^oC, 15 sec at 60 ^oC, and 30 sec at 72 ^oC. The PCR products were analyzed as ten-fold dilutions on ABI 3700x1 genetic analyzer with ROX-500 size standard (Applied Biosystems) and the

resulting peaks were scored with GENEMAPPER (Applied Biosystems) software and confirmed manually.

Results and Discussion

DNA sequences of 268 unique recombinant clones yielded 92 microsatellite sequences, and all 92 primer pairs were selected for further screening. All monomorphic or stutter peak producing primer pairs were eliminated and 11 polymorphic primer pairs that consistently produced single peaks were selected for further evaluation. POPGENE version 1.3.1 (Yeh et al. 1999) was used for the statistical analyses. Probability tests for Hardy-Weinberg Equilibrium (HWE) and genotypic linkage disequilibrium (LD) with sequential Bonferroni adjustment of the P value were performed (Rice 1989). The characteristics of the 11 microsatellite loci in *S. invicta* worker ants, collected from 23 monogyne mounds in Lauderdale County, Mississippi, are given in Table 3.1 and 3.2. The observed and effective number of alleles ranged from two to six (average 3.1818 $\pm$ S. E. 0.0486) and 1.31 to 2.64 (average 1.9684 $\pm$ S. E. 0.0455), respectively. The observed and expected heterozygosity values ranged from 0.1613 to 0.7826 (average 0.4319 $\pm$ S. E. 0.0486) and 0.1491 to 0.6242 (average 0.4630 $\pm$ S. E. 0.0486), respectively. Polymorphism information content (PIC) of the markers was calculated as PIC= $1 - \Sigma P_{ij}^{\ 2}$, where P_{ij} = frequency of the j^{th} allele for i^{th} locus (Anderson et al. 1993). The PIC values ranged from 0.1482 to 0.6208. Significant deviations from HWE were observed for loci SiMS2A-01, SiMS2A-18, and SiMS2B-66. Evaluation of all loci using micro-checker software (van Oosterhout et al. 2004) did not reveal excessive null alleles at any of the above three loci. Further studies are needed to evaluate if factors such as

selection, genetic drift, and non-random mating are responsible for these deviations from HWE. Pair-wise comparisons did not reveal linkage disequilibrium between any of the loci.

In the present study, we report an additional 11 polymorphic microsatellite loci that are suitable for extensive population studies of the red imported fire ants. Selection of primer pairs with 60 ^{o}C annealing temperatures that produce single peak alleles and less then 250 bp amplicons facilitate accurate, high-throughput analysis of populations. These polymorphic markers can be used for area wise population genetic studies of fire ant populations which help to assess the equilibrium.

Table 3.1 Description of 11 microsatellite loci isolated in *S. invicta*.

Locus (Accession no.)	**Primer Sequence (5´- 3´)**	**Repeat**	**Allele size Range (bp)**
SiMS1A-43 (EU307210)	F: GCTGCTGTTAAATTCGATATTCCG R: GTCGCGTCGAACAAAGTGTTAAAT	$(AC)_8$	171-174
SiMS2A-01 (EU307211)	F: TAGCCTATAAATCAACCCGTTGCC R: GCCTTCGTACCTGATTATGCAGC	$(AG)_{11}$	178-206
SiMS2A-18 (EU307212)	F: GCTCAAGCAACGACAAAGAGAAAT R: ACTTGGTGCATCGACTGTACTGAT	$(AG)_7AC(AG)_6$	162-187
SiMS2A-50 (EU307213)	F: CTATTAAAGAGCCACTGCACCGAT R: GTTTAATTTCGTTACGCATGAGCC	$(TC)_9$	124-132
SiMS2B-65 (EU307214)	F: ATTTTATCGAACGGGAGGAAAAAG R: TGCTTTCAAATTAACCTTGCGAAT	$(AG)_4AA(AG)_8$	157-171
SiMS2B-66 (EU307215)	F: ACCGTTGAAATTGAGAAAAACCAA R: AAATGCTTAAAAGTCGAGCGACAG	$(GA)_{13}$	151-167
SiMS3A-39 (EU307216)	F: GCCTTTCAAACGCTTCGTATTACA R: TTTGCGATACAAGACCATCGTTTA	$(ATG)_4ATA(ATG)_7$	183-192
SiMS3A-47 (EU307217)	F: AGGGAAAAGGAAAGAAGAGCAAGA R: CTCTTCCTGGACTCTCCGAACTAA	$(AG)_3AA(AG)_9$	162-168
SiMS3A-96 (EU307218)	F: TCCGAACAATATCATTCCCGTATC R: GGGAAAAGATTGTTGAAGGAGAGAA	$(TCTT)_6$	118-122
SiMS3B-13 (EU307219)	F: AAATCGACAGGCGAGACATTTAAC R: GCGTACGTAACTTTGTCGAGTCCT	$(TCTT)_7$	138-142
SiMS3B-17 (EU307220)	F: AAGTCTTCCTACTTCACATGCAATCT R: TCCGAGATATTTAGGTGTACCAAGC	$(AT)_6$	154-156

Note: Accession no. denotes GenBank accession number for the sequence from which the primers were designed; “F” and “R” denote forward and reverse primer sequence respectively.

Table 3.2 Characteristics of 11 microsatellite loci isolated in *S. invicta* collected from Lauderdale County, Mississippi

Locus	**Sample Size**	**H_o**	**H_E**	**n_o**	**n_e**	**PIC**
SiMS1A-4	94	0.4149	0.4967	3	1.9765	0.588
SiMS2A-01‡	92	0.7826	0.6242	4	2.6372	0.6208
SiMS2A-18‡	94	0.4894	0.5586	4	2.2504	0.5556
SiMS2A-50	94	0.2128	0.2406	3	1.3146	0.2393
SiMS2B-65	93	0.4409	0.5912	6	2.4274	0.588
SiMS2B-66‡	94	0.5	0.5354	3	2.1392	0.5326
SiMS3A-39	94	0.4255	0.3868	3	1.6253	0.3847
SiMS3A-47	93	0.4624	0.5863	3	2.3992	0.5832
SiMS3A-96	94	0.3723	0.4862	2	1.9367	0.4836
SiMS3B-13	92	0.4891	0.4380	2	1.7720	0.4357
SiMS3B-17	93	0.1613	0.1491	2	1.1741	0.1482
Mean	93.36	0.4319	0.4630	3.1818	1.9684	0.4604
S.E		0.0486	0.0455	0.3520	0.1403	0.0452

Note: The observed number of alleles (n_o), the effective number of alleles (n_e), the observed heterozygosity (H_o), the expected heterozygosity (H_E), the polymorphism information content (PIC) value and the standard error (S.E). ‡ Loci significantly deviated from HWE

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CHAPTER IV

PHENOTYPIC AND GENOTYPIC CHARACTERS ASSOCIATED WITH POLYGYNE HYBRID IMPORTED FIRE ANT

Abstract

A study was initiated to identify the origin of polygyny in the hybrid imported fire ant (*Solenopsis invicta* x *richteri*) and to confirm the association of polygyne phenotypic and genotypic characters. *Gp-9*, a major gene in imported fire ants, is associated with the expression of social form. Identifying multiple inseminated queens in a colony and sequencing the critical region of *Gp-9* locus (1,600 base pairs) in individuals with the polygyne allele strongly indicate the association of phenotypic and genotypic characters of polygyny in a hybrid imported fire ant colony. A 100% match of $Gp\text{-}9^{B}$ and $Gp\text{-}9^{b}$ alleles and concordance in the critical amino acid substitutions of the *Gp-9* locus in the hybrid fire ant with *S. invicta* Buren provides the evidence that polygyny in the hybrid imported fire ant colony is derived from *S. invicta*.

Introduction

Imported fire ants in the genus *Solenopsis* (Hymenoptera: Formicidae) exist primarily in tropical and subtropical areas (Buren 1972). Fire ants are conspicuous

elements of local ant fauna because of the large size of their nests and the aggressive habits of the workers. In the early 20th century, fire ants that were native to South America were introduced to the United States through the port of Mobile, Alabama (Wilson 1958, Lofgren et al. 1975). Initially two forms were identified in the introduced fauna and recognized as subspecific variants (Wilson 1953, 1958). In 1972, Buren assigned species status to both forms based on morphological characters. *S. richteri* Forel, the black imported fire ant, which was introduced prior to 1920, was replaced in much of the southern United States by *S. invicta* Buren, the red imported fire ant. *S. invicta*, introduced in the 1930's, which rapidly colonized most of the southeast and south-central United States. *S. invicta* has become a devastating pest since its introduction to the USA, causing 6 billion dollars damage annually (ARS 2003). *S. richteri* is confined to a very limited area which includes Tennessee, northern Mississippi and Alabama (Oliver et al. 2009).

Two gas chromatographic studies of fire ants collected at the contact zone of *S. invicta and S. richteri* in Mississippi were conducted in the 1980's (Vander Meer et al. 1985 and Ross et al. 1987). Through the examination of the two imported fire ant species' cuticular hydrocarbons and venom alkaloids it was confirmed that hybridization had occurred. Streett et al. (2006) reported the presence of a zone of hybridization between the red and black imported fire ants in central to northern Mississippi and Alabama and southern Tennessee. Gardner et al. (2008) reported the presence of *Solenopsis invicta* x *richteri* (hybrid form) in northwestern Georgia, northern Mississippi and Alabama. They reported that hybrid fire ant colonies are better adapted to cooler and more northerly latitudes than *S. invicta.*

The first populations of *S. invicta* discovered were monogyne colonies, in which a single queen and her workers were hostile to individuals from other colonies. From the1970's to the present, polygyne colonies have been found and with increasing prevalence. In these colonies more than one queen is present (Ross and Keller 1995). The reasons for the evolution of polygyny in *S. invicta* are not clear and may be due to the greater ecological success of this social organization (Ross and Keller 1995, Chapman and Bourke 2001). This represents a major social polymorphism in fire ant populations.

The fundamental genetic difference between the two social forms of fire ants is mostly associated with variation at or near the *Gp-9* locus and number of queens. Colony organization and the number of egg laying queens are associated with the variation at the gene *Gp-9*. In monogyne colonies a single queen is present. In polygyne colonies more than one queen is present, ranging from 2 to 200 queens (Ross and Keller 1995). In the monogyne form, the queen and workers have the *Gp-9*^{BB} genotype whereas workers in the polygyne colonies have both *Gp-9*^{BB} and *Gp-9*^{Bb} genotypes. The queens of the polygyne colonies are almost always heterozygous and rarely *Gp-9*^{bb} (Keller and Parker 2002). Due to the *Gp-9* allele or a closely linked locus, a worker with the *Gp-9*^{b} allele will selectively kill the queens with the *Gp-9*^{BB} genotype immediately after the queen starts reproduction. *Gp-9*^{bb} queens and workers are generally absent in polygyne colonies due to the lethal recessive *Gp-9*^{b} (b) allele which causes the death of homozygous females soon after their eclosion from the pupal stage (Ross 1997). Most of the queens of polygyne fire ant colonies are heterozygotes. A stable polymorphism exists between the *Gp-9*^{B} (B) and b alleles. This is maintained by the selection against *Gp-9*^{BB} queens which

is compensated by the inviabilty of *Gp-9*^{bb} queens in the polygyne form (Keller and Parker 2002).

The protein product of the *Gp-9* region is believed to be a molecular component in the chemical recognition of conspecifics, enabling the workers to discriminate and recognize potential egg laying queens in a colony. Through this means, queen number and social recognition are regulated in fire ants (Krieger and Ross 2002).

Polygyny in the hybrid imported fire ant was reported based on morphological and behavioral characters by Glancey et al. (1989). Menzel et al. (2008) identified the b allele by molecular methods using primer pairs developed by Valles and Porter (2003). A study was initiated in the hybrid imported fire ant to identify the origin, and to confirm the association of phenotypic and genotypic characters of polygyny. The polygyne phenotype, by definition, is the presence of multiple inseminated queens in a colony. Genotypic characters are defined by the presence of molecular markers coding for the different amino acid replacements that cause the expression of polygyny in a colony.

Material and Methods

Hybrid imported fire ant colonies were found in Lauderdale County, Mississippi, an area where red imported fire ants predominate. Ants were collected in hexane for species confirmation and ethanol for DNA extraction. Three colonies were brought to the lab in 18.3 L buckets for identification of the number of dealates in each colony. Ants were separated from the soil by sieving in a screen mesh through running water. The dealates, which were surrounded by other worker ants due to pheromonal activity, were separated as they were believed to be mated (Glancey et al. 1975). The abdomens of the

dealated ants were dissected for assessing mated/unmated status through examination of spermatheca to confirm them as inseminated queens (Glancey et al. 1989). Total DNA was extracted individually from 12 ants in each colony by using a DNA extraction kit for arthropods (Epicenter, Madison WI). The head and thorax tissue was placed in 1.5 ml micro-centrifuge tubes and placed in liquid nitrogen. The tissue was ground with a pre-chilled sterile pestle. The Epicenter protocol for animal tissues was followed after grinding the ant tissue. Total DNA was eluted in TE buffer and stored at -80^{o} C. Total DNA was also extracted from sperm, isolated from the inseminated queens. A CEQ 8000 Genetic Analysis System (Beckman Coulter) was used for fragment analysis of five microsatellite regions, Sol 11, 20, 42, 49 and 55 (Krieger and Keller 1997, with modifications) to identify the alleles in both the dealate and the sperm sample DNA. Males encountered during our collection were dissected to observe gonadal development which also provides evidence for confirmation of social form. The males in polygyne colonies are thought to be sterile and diploid (Ross and Fletcher 1985). In sterile males, the testicular lobes are undeveloped, and the vasa deferentia are transparent and devoid of sperm (Hung et al. 1974). Fragment analysis was done for the above five microsatellite regions to identify the males' ploidy, which also aids in confirming polygyny.

The species of the fire ants was identified by gas chromatography and mass spectrophotometry (GC-MS). A total of 100 to 150 worker ants were collected using an aspirator and placed in 5 ml of hexane. Ants were soaked in hexane for at least 2 days to transfer cuticular hydrocarbons and venom alkaloids from the ants. Hexane samples were taken to the USDA Agricultural Research Service in Stoneville, Mississippi, and

GC-MS analysis for species/hybrid status was performed as described in Menzel et al. (2008).

Polymerase chain reaction was used to identify the presence or absence of B and b alleles in the hybrid imported ants using primer pairs designed by Valles and Porter (2003). A monogyne individual yields only the 517 base pairs (bp) band, and a polygyne heterozygous individual yields two bands, at 517 and 423 bp by agarose gel electrophoresis.

To reconfirm the presence of B and b alleles, a PCR/RFLP assay (Krieger and Keller 2002) was conducted. Total genomic DNA was amplified with primers *Gp-9*_169.for and *Gp-9*_490.rev and then digested with BSa AI restriction enzyme. The digested products yielded 2 bands, at 545 and 283 bp, for B allele and 3 bands, at 428, 283 and 117 bp, for b allele. The resulting fragments from a heterozygous polygyne individual appear as 545, 428, 283 and 117 bp bands on the gel. After confirmation of polygyny by identifying the B and b alleles from both the above PCR reactions, the critical region of *Gp-9* was sequenced by using two sets of primers. The first pair, *Gp-9*_169.for and *Gp-9*_490.rev covers only a part of the critical *Gp-9* region. Primers were designed using Primer 3 software (Rozen and Skaletsky 2000) in the *Gp-9* region of a polygyne individual (EU220181) to include the other coding regions of amino acid replacements which are believed to cause polygyny in *S. invicta*. A forward primer, *Gp-9*_163.for (5'CAATGCCCTTATTGCATGTAGA3') and reverse primer, *Gp-9*_1105.rev (5'CATGGGAAGACGTTATGAAAAA 3') were obtained to sequence the other region. Total genomic DNA was amplified with the new primer pairs (92 ^{o}C [2 min], 30 cycles of 92 ^{o}C [20s], 51.0 ^{o}C [30s] and 72 ^{o}C [1 min], followed by 72 ^{o}C for 2 min).

Both PCR products from the two sets of primer pairs were cloned into pGEM-T easy vector (Promega, USA), and the resulting recombinant plasmids were transferred to *Escherichia coli* Top 10 competent cells (Invitrogen, USA). The cloned products were sequenced in three individual ants for each allele. The sequence, consisting of all critical amino acid substitutions at different positions for polygyny, was approximately 1,300 bp in size (Krieger and Keller 2002). Molecular data generated in this work were compared with sequences available in the NCBI/GenBank using the BLAST (Altschul et al. 1997) on-line resources of NCBI.

Results and Discussion

The results obtained from GC-MS analysis indicated that ants of the three colonies collected from the location were hybrids. After thoroughly searching each colony separately, two dealated individuals were found in one of the three colonies. After dissecting the abdomens of the two dealated individuals, we found spermatheca with sperm and the two individuals were confirmed as inseminated queens. Microsatellite fragment analysis at five loci showed allele differences between the delate and sperm DNA, providing further evidence of insemination of both queens In another colony we found sterile males with undeveloped testicular lobes, and the vasa deferentia of these sterile males were transparent and devoid of sperm. Fragment analysis for the same microsatellite loci showed that the sterile males possessed two alleles (diploid) for two or more loci tested. The presence of diploid males is known to be an indicator of polygyny (Ross and Fletcher 1985).

At least ten individuals from each colony and a control *S. invicta* polygyne individual had both the 517 and 423 bp bands when tested with primer pairs designed by Valles and Porter (2003). This provides evidence for the presence of B and b alleles, a molecular indicator in the hybrid imported fire ant.

When the total DNA was amplified using the primers developed by Krieger and Keller (2002) and digested with *Bsa*AI restriction enzyme, all the individuals which were heterozygous yielded 545, 428, 283 and 117 bp bands by agarose gel electrophoresis. Males which are homozygous (*Gp-9* ^{bb}) yielded only the 428, 283 and 117 bp bands in one of the colonies. All the above evidence also supports the existence of B and b allele in the hybrid fire ants collected in our study.

A 1,574 bp sequence was amplified using both primer pairs in both B and b alleles in the hybrid imported fire ant. GenBank BLASTN searches of both sequence fragments revealed 100% match with the *S. invicta* sequences. When checked for different nucleotide substitutions for amino acid variations, the fragments revealed concordance with *S. invicta* B and b sequences. When the similarity with *S. richteri* B and b allele was checked, they differed by more than 1%. The sequence fragments were submitted to GenBank.

We checked different positions of nucleotides for amino acid substitution in different codons that are believed to result in the expression of polygyny. These are listed in the Table 4.1.

Table 4.1 Nucleotide substitutions between B and b alleles in hybrid fire ants.

Amino acid Position	B allele	b allele
42	AGT	GGT
95	ATG	ATA
139	GTC	ATC
151	GAA	AAA

The nucleotide substitutions were exactly the same as in the *S. invicta* found in North America (Krieger and Keller 2002), and were not similar to the *S. richteri* b allele found in South America. There is no recorded evidence of polygyny in *S. richteri* populations in North America.

Gotzek et al. (2007) reported that all the three characteristic b-like residues at codon positions 42, 95 and 139 may be jointly required for the expression of polygyny in South American fire ants. The North American polygyne *S. invicta* and hybrid fire ant possess amino acid substitutions associated with polygyny at all four positions: 42, 95, 139 and 151. The primers developed by Valles and Porter (2003) help to identify the difference at codon position 151 between North American monogyne and polygyne populations of *S. invicta*.

The main objective of the study was to confirm the association of phenotypic and genotypic characters of polygyny in the hybrid imported fire ant for a colony, and to sequence the critical region of the *Gp-9* gene in the hybrid fire ant. Earlier studies by Glancey et al. (1989), showed the presence of multiple inseminated queens as the phenotypic evidence in hybrid polygyne fire ant colonies. Menzel et al. (2008) showed

that molecular techniques can be used to identify polygyny (presence of b allele) in the hybrid fire ant. In this study we not only provide phenotypic evidence but also the sequence for the critical region of *Gp-9* for both B and b alleles, which provides strong evidence for the existence of polygyny. The identical match for those critical regions of the B and b alleles of the hybrid fire ant with *S. invicta* and the nearly 100% match of the rest of the allelic sequences confirm the origin of polygyny in the hybrid form to be from *S. invicta*. All the above evidence corroborates the transfer of the polygyne trait to the hybrid fire ant colony from *S. invicta* during hybridization.

Polygyne *S. invicta* populations exhibit a higher density than monogyne populations, which has potential for greater economic and environmental losses (Macom and Porter 1996). Likewise, polygyne hybrid fire ant populations are likely to cause greater damage and more economic losses than the hybrid monogyne form. Current reports suggest that the hybrid fire ant populations are expanding in the southeastern United States where both red and black imported fire ants are found. In a recent study, Leal and Ishida (2008) suggested that the polygyne individuals of *S. invicta* may or may not have the protein coded by b like alleles in the individuals where b allele was present. Additional studies will be necessary to examine the proteomic difference in polygyne and monogyne individuals in hybrid fire ant colonies.

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CHAPTER V

CONCLUSIONS

Five main objectives have been met in this study. The first objective was, to assess genetic differences of red imported fire ants (RIFA) among different places (Meridian, Yazoo City and Crystal Springs in Mississippi and Mobile, Alabama) and to estimate effective population sizes in Mississippi populations in the presence of gene flow with the help of microsatellites. This study indicated that there is low spatial variation among four populations and the effective population sizes are low (10- 17) in Mississippi populations. The reason for the low numbers of effective population size may be due to complementary sex determination in the haplodipoidy social hymenopterans and fewer number of sex alleles in introduced fire ant populations compared to the native fire ants in South America. RIFA populations in the study have high gene flow values which indicate that there is extensive movement among populations. Overall F_{ST} and Pairwise F_{ST} between these populations is less indicating, low spatial variation in the nuclear genetic structure. The low spatial variation also indicates that they were recently introduced into United States and it takes some time to get into equilibrium and show spatial variation. The fire ant queen reproduces on average for 5 -7 years, so it extends time to get into equilibrium between genetic drift and migration and show spatial variation.

The goal of the second objective is to assess the temporal stability of genetic structure of fire ant populations. Genetic structure is an important aspect of population biology. In this study genetic structure was assessed spatially and temporally using microsatellites. Over a period three years the overall gene flow values increased and the overall F_{ST} values decreased among the populations in the study. Pairwise F_{ST} values between populations also decreased over time. This suggests that genetic structure of fire ant populations is not in equilibrium and these populations are homogenizing over time. There is extensive movement between these populations. The earlier reports of isolation by distance may be due to founder effects as red imported fire ants were introduced in the late 1930's or early 1940's and it takes time to get into equilibrium between migration and genetic drift. In future studies, temporal stability of genetic structure in fire ants in other places away from the source of introduction should be estimated. This helps to estimate gene flow and identify the presence/ absence of genetic equilibrium in other fire ant populations.

The third objective was to develop additional polymorphic microsatellites in RIFA which helps to find more variation within and between populations. In this regard, an additional 11 polymorphic microsatellites were isolated and characterized from a population in Lauderdale County, Mississippi. The observed and effective number of alleles ranged from two to six (average 3.1818 $\pm$ S. E. 0.0486) and 1.31 to 2.64 (average 1.9684 $\pm$ S. E. 0.0455), respectively. The observed and expected heterozygosity values ranged from 0.1613 to 0.7826 (average 0.4319 $\pm$ S. E. 0.0486) and 0.1491 to 0.6242 (average 0.4630 $\pm$ S. E. 0.0486), respectively. The polymorphism information content of the microsatellites ranged from 0.1482 to 0.6208. Probability tests indicated significant

deviations from the Hardy-Weinberg equilibrium at three loci. Pair-wise tests did not detect linkage disequilibrium between any pair of loci. These new microsatellites which are polymorphic may help in further population genetic studies of fire ants and provide more information about the genetic structure of fire ant populations and can assess genetic equilibrium.

The fourth and fifth objectives were to identify the origin of polygyny in the hybrid imported fire ant (*Solenopsis invicta* x *richteri*) and to confirm the association of polygyne phenotypic and genotypic characters. Identifying multiple inseminated queens in a colony and sequencing the critical region of *Gp-9* locus (1300 base pair) in individuals with the polygyne allele strongly indicate the association of phenotypic and genotypic characters of polygyny in a hybrid imported fire ant colony. A 100% match of *Gp*-$9^{B}$and *Gp*-9^{b} alleles and concordance in the critical amino acid substitutions of the *Gp-9* locus in the hybrid fire ant with *S. invicta* Buren provides strong evidence that polygyny in the hybrid imported fire ant is derived from *S. invicta*. Polygyne *S. invicta* populations exhibit a higher density than monogyne populations, which has potential for greater economic and environmental losses. Likewise, polygyne hybrid fire ant populations are likely to cause greater damage and more economic losses than the hybrid monogyne form. Current reports suggest that hybrid fire ant populations are expanding in the southeastern United States where both red and black imported fire ants are found.

APPENDIX A

ALLELIC FREQUENCIES FOR EACH LOCUS

Table A.1 Allelic frequencies for each locus in 2006:

Population	Locus: Sol11 Alleles					
	157	159	161	165	167	169
YC	0.333	0.031	0.222	0.247	0.000	0.167
MR	0.389	0.037	0.086	0.253	0.049	0.185
MB	0.106	0.000	0.188	0.425	0.000	0.281
CS	0.275	0.025	0.175	0.469	0.006	0.050

Population	Locus: Sol42 Alleles									
	184	186	188	190	192	198	200	202	210	212
YC	0.006	0.287	0.369	0.000	0.000	0.006	0.287	0.000	0.000	0.044
MR	0.000	0.338	0.400	0.131	0.050	0.000	0.000	0.000	0.000	0.081
MB	0.000	0.069	0.419	0.181	0.000	0.006	0.269	0.000	0.000	0.056
CS	0.000	0.119	0.494	0.175	0.000	0.000	0.113	0.087	0.013	0.000

Table A.1 continued

Population	Locus: Sol49 Alleles					
	84	96	98	102	104	116
YC	0.200	0.306	0.275	0.219	0.000	0.000
MR	0.150	0.450	0.212	0.188	0.000	0.000
MB	0.050	0.600	0.200	0.150	0.000	0.000
CS	0.050	0.469	0.244	0.225	0.006	0.006

Population	Locus: Sol20 Alleles				
	126	128	130	148	154
YC	0.031	0.512	0.235	0.167	0.056
MR	0.275	0.356	0.069	0.256	0.044
MB	0.081	0.287	0.338	0.231	0.063
CS	0.094	0.494	0.138	0.163	0.113

Table A.1 continued

Population	Locus: Sol 55 Alleles							
	210	214	218	220	222	224	226	228
YC	0.000	0.000	0.704	0.037	0.080	0.099	0.080	0.000
MR	0.000	0.050	0.338	0.056	0.000	0.125	0.000	0.431
MB	0.000	0.000	0.388	0.106	0.000	0.281	0.000	0.225
CS	0.013	0.000	0.719	0.037	0.006	0.144	0.050	0.031

YC - Yazoo City; MR- Meridian; MB – Mobile; CS – Crystal Springs.

Table A.2 Allelic frequencies for each locus in 2007:

Population	Locus: Sol11 Alleles				
	157	159	161	165	169
MR	0.171	0.141	0.103	0.453	0.132
MB	0.152	0.110	0.117	0.508	0.114
YC	0.278	0.075	0.111	0.248	0.288
CS	0.309	0.000	0.230	0.442	0.018

Table A.2 continued

Population	Locus: Sol42 Alleles					
	186	188	190	200	212	214
MR	0.177	0.151	0.259	0.159	0.254	0.000
MB	0.158	0.296	0.196	0.227	0.085	0.038
YC	0.255	0.393	0.114	0.138	0.101	0.000
CS	0.138	0.193	0.201	0.299	0.169	0.000

Population	Locus: Sol49 Alleles				
	84	94	96	98	102
MR	0.123	0.000	0.264	0.577	0.036
MB	0.025	0.020	0.365	0.230	0.361
YC	0.227	0.000	0.309	0.118	0.345
CS	0.181	0.000	0.478	0.085	0.256

Table A.2 continued

Population	Locus: Sol20 Alleles					
	126	128	130	148	154	156
MR	0.243	0.396	0.144	0.149	0.068	0.000
MB	0.225	0.411	0.271	0.023	0.070	0.000
YC	0.192	0.281	0.242	0.232	0.053	0.000
CS	0.201	0.354	0.164	0.193	0.051	0.036

Population	Locus: Sol55 Alleles						
	218	220	222	224	226	228	230
MR	0.404	0.026	0.039	0.254	0.061	0.215	0.000
MB	0.406	0.211	0.047	0.055	0.039	0.242	0.000
YC	0.590	0.037	0.023	0.193	0.043	0.113	0.000
CS	0.575	0.164	0.004	0.097	0.097	0.037	0.026

YC - Yazoo City; MR- Meridian; MB – Mobile; CS – Crystal Springs.

Table A.3 Allelic frequencies for each locus in 2008:

Population	Locus: Sol11 Alleles				
	157	159	161	165	169
MR	0.260	0.087	0.021	0.580	0.052
MB	0.188	0.120	0.136	0.393	0.162
YC	0.220	0.000	0.208	0.534	0.038
CS	0.391	0.039	0.141	0.324	0.106

Population	Locus: Sol42 Alleles					
	186	188	190	200	210	212
MR	0.250	0.465	0.176	0.039	0.000	0.070
MB	0.240	0.217	0.132	0.188	0.020	0.204
YC	0.461	0.186	0.140	0.081	0.000	0.132
CS	0.338	0.201	0.092	0.165	0.000	0.204

Table A.3 continued

Population	Locus: Sol49 Alleles			
	84	96	98	102
MR	0.220	0.383	0.344	0.053
MB	0.032	0.384	0.265	0.319
YC	0.239	0.314	0.170	0.277
CS	0.215	0.393	0.133	0.259

Population	Locus: Sol20 Alleles				
	126	128	130	148	154
MR	0.238	0.402	0.066	0.217	0.077
MB	0.212	0.422	0.190	0.098	0.078
YC	0.143	0.446	0.306	0.062	0.043
CS	0.157	0.364	0.179	0.250	0.050

Table A.3 continued

Population	Locus: Sol55 Alleles						
	218	220	222	224	226	228	230
MR	0.443	0.064	0.028	0.103	0.113	0.248	0.000
MB	0.390	0.084	0.126	0.184	0.039	0.177	0.000
YC	0.576	0.084	0.034	0.141	0.000	0.164	0.000
CS	0.585	0.064	0.000	0.117	0.131	0.085	0.018

YC - Yazoo City; MR- Meridian; MB – Mobile; CS – Crystal Springs.